新規試験法評価書

眼刺激性試験代替法　SIRC-CVS:TEA法

令和 3 年 7 月

国立医薬品食品衛生研究所

新規試験法評価書

令和 3 年 7 月 30 日

No.　2021-01

眼刺激性試験代替法　SIRC-CVS:TEA法に関する評価

令和 3 年 7 月 23 日に国立医薬品食品衛生研究所にて開催された新規試験法評価会議（通称：JaCVAM 評価会議）において以下の評価がなされた。

当該試験法は化学物質の SIRC 細胞に対する細胞生存率を指標に用いて眼刺激性を評価する試験法であり、生きた動物を用いないという点で、3Rs の精神に合致している。また、本試験法は安価であり、短時間で実施でき、特殊な機材や試薬を必要とせず、必要な手技も複雑なものではない。したがって、基本的な細胞培養の技術と設備を有する施設であれば実施可能であり、技術移転性は高く、再現性も高い方法である。

しかし、当該試験法の予測性評価においては、適用除外物質の設定に科学的な根拠が乏しいことから、当該試験法は化学物質による眼刺激性をボトムアップ方式において国際連合化学品の分類および表示に関する世界調和システム（UN GHS）区分に該当しない物質を検出する方法として、行政的に用いることは適切ではないと考える。

この評価書は、眼刺激性試験資料編纂委員会によりまとめられた文書を用いて、JaCVAM 評価会議が評価および検討した結果、その行政活用上の位置付けが確認されたことから作成された。

JaCVAM 評価会議

西 川 秋 佳 （国立医薬品食品衛生研究所 安全性生物試験研究センター/済生会宇都宮病院）：座長
板 垣　　宏 （ITACS コンサルティング）
中村るりこ （独立行政法人 製品評価技術基盤機構）
西 村 次 平 （独立行政法人 医薬品医療機器総合機構）
平 林 容 子 （国立医薬品食品衛生研究所 安全性生物試験研究センター）
松 本 一 彦 （名古屋市立大学大学院）

任期：令和 2 年 4 月 1 日～令和 4 年 3 月 31 日

JaCVAM 運営委員会

平 林 容 子　（国立医薬品食品衛生研究所 安全性生物試験研究センター）：委員長
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束 野 正 明　（厚生労働省 医薬・生活衛生局 医薬品審査管理課 化学物質安全対策室）
広 瀬 明 彦　（国立医薬品食品衛生研究所 安全性生物試験研究センター 安全性予測評価部）
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横 田 雅 彦　（独立行政法人 医薬品医療機器総合機構）
足利太可雄　（国立医薬品食品衛生研究所 安全性生物試験研究センター 安全性予測評価部 第二室）：事務局
小 島　肇　（国立医薬品食品衛生研究所 安全性生物試験研究センター 安全性予測評価部 第二室）：事務局

JaCVAM statement on the SIRC-CVS: TEA method for evaluating ocular irritation

At a meeting held on July 23, 2021 at the National Institute of Health Sciences (NIHS) in Kanagawa, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

The SIRC-CVS: TEA test method assesses cytotoxicity by exposing SIRC cells to a test chemical, then staining the exposed SIRC cells with crystal violet to measure their viability. As the test method does not use living animals, it is in accordance with the spirit of 3Rs. In addition, this test method is inexpensive, can be completed easily, and does not include special components, equipment, or other scientific procedures. Therefore, no requirement of practical training is a good indication of the robustness of the test method, and it has high reproducibility. However, this method could not assess the ocular irritation potential of chemicals, which when used in a bottom-up approach to identifying chemical substances that do not require classification and labeling under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) is suitable for use in a regulatory context. The applicability domain of the test method was adjusted without providing clear mechanistic insights and justification of the proposed domain restrictions.

This statement was prepared, following the review prepared by the eye irritation test JaCVAM Editorial Committee, to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the value of the test method for regulatory acceptance.

July 30, 2021

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Akiyoshi Nishikawa (Center for Biological Safety and Research: CBSR, National Institute of Health Sciences: NIHS / Saiseikai Utsunomiya Hospital) : Chairperson
Ms. Yoko Hirabayashi (CBSR, NIHS)
Mr. Hiroshi Itagaki (ITACS Consulting)
Mr. Kazuhiko Matsumoto (Nagoya City University)
Ms. Ruriko Nakamura (National Institute of Technology and Evaluation)
Mr. Jihei Nishimura (Pharmaceuticals and Medical Devices Agency)

Term: From 1st April 2020 to 31st March 2022

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Ms. Yoko Hirabayashi (CBSR, NIHS): Chairperson
Mr. Osamu Fueki (Pharmaceuticals and Medical Devices Agency)
Mr. Yukihiro Goda (NIHS)
Mr. Akihiko Hirose (Division of Risk Assessment, CBSR, NIHS)
Mr. Koji Ishii (National Institute of Infectious Diseases)
Mr. Yasunari Kanda (Division of Pharmacology, CBSR, NIHS)
Mr. Satoshi Kitajima (Division of Toxicology, CBSR, NIHS)
Ms. Kumiko Ogawa (Division of Pathology, CBSR, NIHS)
Mr. Takayuki Okubo (Ministry of Health, Labour and Welfare)
Mr. Keiichi Sugiyama (Division of Genetics and Mutagenesis, CBSR, NIHS)
Mr. Masahiro Takahata (Ministry of Health, Labour and Welfare)
Mr. Yuhji Taquahashi (Animal Management Section of the Division of Toxicology, CBSR, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Masahiko Yokota (Pharmaceuticals and Medical Devices Agency)
Mr. Takao Ashikaga (Division of Risk Assessment, CBSR, NIHS): Secretary
Mr. Hajime Kojima (Division of Risk Assessment, CBSR, NIHS): Secretary

眼刺激性試験代替法　SIRC-CVS:TEA法

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評価会議報告書

眼刺激性試験代替法　SIRC-CVS:TEA 法

JaCVAM 評価会議

令和 3 年(2021 年)5 月 13 日

JaCVAM 評価会議

西 川 秋 佳 （国立医薬品食品衛生研究所　安全性生物試験研究センター/済生会宇都宮病院）：
座長

板 垣　　宏 （ITACS コンサルティング）*

中村るりこ （独立行政法人　製品評価技術基盤機構）

西 村 次 平 （独立行政法人　医薬品医療機器総合機構）

平 林 容 子 （国立医薬品食品衛生研究所　安全性生物試験研究センター）

松 本 一 彦 （名古屋市立大学大学院）

*：最終的な結論には関与しなかった。

任期：令和 2 年 4 月 1 日～令和 4 年 3 月 31 日

略語

CVS: Crystal Violet Staining

GHS: Globally Harmonized System of Classification and Labelling of Chemicals

JaCVAM: Japanese Center for the Validation of Alternative Methods

OECD: Organisation for Economic Co-operation and Development

SIRC: Statens Seruminstitut Rabbit Cornea

TEA: Triethanolamine

TG: Test Guideline

UN: United Nations

JaCVAM 評価会議は、眼刺激性試験資料編纂委員会により作成された「眼刺激性試験代替法　SIRC-CVS:TEA 法評価報告書」[1)]をもとに本試験法の科学的妥当性、社会的および行政的な受け入れについて検討した。

1. 試験法の定義

名称：　眼刺激性試験代替法　SIRC-CVS:TEA 法

代替する対象毒性試験：ウサギを用いた急性眼刺激性／腐食性（Acute Eye Irritation/Corrosion）を評価する Draize 法（OECD TG405）[2)]

試験法の概略：　本試験法は、ウサギ角膜由来細胞（SIRC 細胞）を用いて、クリスタルバイオレット（CVS）が細胞膜を透過して生体高分子を染色する性質を利用した細胞毒性を測定する試験法である。本試験法は、トリエタノールアミン（TEA）を比較対照物質として、ボトムアップ方式 [3)]で国際連合化学品の分類および表示に関する世界調和システム（UN GHS）区分に該当しない化学物質を検出することができる方法である SIRC-CVS:TEA 法として改定された [4)]。

試験法の科学的妥当性：

眼に異物が入った場合、眼の刺激は、神経等の特定の受容体に作用する場合を除き、一般に結膜や角膜の細胞傷害から始まる。Draize 法における眼刺激性の程度の判定は、主に角膜の初期傷害の程度に大きく影響され、それは角膜上皮細胞の壊死の程度と相関関係にある [5,6)]。本試験法は、SIRC 細胞を用いて、CVS による細胞生存率を指標として被験物質の眼刺激性を評価する試験法である。これらのことから、本試験法はウサギを用いる眼刺激性試験の代替法として科学的妥当性がある。

SIRC-CVS:TEA 法については、リードラボとは異なる 3 施設において 116 の化学物質を用いたバリデーション研究が行われた [7)]。その結果、本試験法は、施設内および施設間再現性についてバリデーション実行委員会が定めた基準を満たした。また、ボトムアップ方式で UN GHS 区分に該当しない化学物質の予測性評価において、バリデーション実行委員会は適用除外物質の設定によっては偽陰性率が低くなると結論した。しかし、JaCVAM の第三者評価 [8)]では、適用除外物質の設定により、偽陰性率は低くなるものの、除外物質の設定に科学的な根拠が乏しく、UN GHS 区分に該当しない化学物質を検出する方法として相応しくないと結論された。JaCVAM 眼刺激性試験資料編纂委員会は、これらの資料を用いて本試験法を評価しており、第三者評価 [9)]と同様の見解を示している [1)]。

2. 目的とする物質又は製品の毒性を評価する試験法としての、社会的受け入れ性および行政上の利用の可能性

社会的受け入れ性：

本試験法は化学物質の SIRC 細胞に対する細胞生存率を指標に用いて眼刺激性を評価す

る試験法であり、生きた動物を用いないという点で、3Rs の精神に合致している。また、本試験法は安価であり、短時間で実施でき、特殊な機材や試薬を必要とせず、必要な手技も複雑なものではない。したがって、基本的な細胞培養の技術と設備を有する施設であれば実施可能であり、技術移転性は高く、再現性も高い方法である。以上より、本試験法の社会的受け入れ性は高い。

行政上の利用性：

予測性評価において、適用除外物質の設定に科学的な根拠が乏しいことから、本試験法は化学物質による眼刺激性をボトムアップ方式において UN GHS 区分に該当しない物質を検出する方法として、行政的に用いることは適切ではないと考える。

引用文献

1) JaCVAM 眼刺激性代替法資料編纂委員会：評価報告書　SIRC-CVS:TEA 法（2021 年 5 月 6 日）
2) OECD (2012). Test Guideline for Testing of Chemicals (No.405): Acute Eye Irritation/ Corrosion.
3) Organisation for Economic Cooperation and Development, Paris.
4) Scott L, Eskes C, Hoffmann S, Adriaens E, Alepée N, Bufo M, Clothier R, Facchini D, Faller C, Guest R, Harbell J, Hartung T, Kamp H, Varlet B L, Meloni M, McNamee P, Osborne R, Pape W, Pfannenbecker U, Prinsen M, Seaman C, Spielmann H, Stokes W, Trouba K, Berghe CV, Goethem FV, Vassallo M, Vinardell P, and Zuang V. A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-Up and Top-Down approaches. Toxicology In Vitro 2010;24 , 1-9.
5) Hagino S, Okazaki Y, Kitagaki M and Itagaki H. Further verification of an in vitro tier system for the identification of cosmetic ingredients that are not ocular irritants. Altern Lab Anim. 2010;38, 139-152.
6) 大野泰雄. 眼刺激性試験代替法のバリデーション. 組織培養. 1996;22(6), 211-217.
7) Jester JV, Li L, Molai A and Maurer JK. Extent of initial corneal injury as a basis for alternative eye irritation tests. Toxicology in Vitro. 2001;15, 115-130.
8) Validation Study for the Statens Seruminstitut Rabbit Cornea-Crystal Violet Staining Cytotoxicity Test 1 Method with Triethanolamine (SIRC-CVS:TEA Test Method) as an Alternative to Eye Irritation Test-Study Report Version 8.9 (2019)
9) SIRC-CVS Test Method: Report of the Peer Review Panel on a JaCVAM coordinated study programme addressing the validation status of the SIRC-CVS test method for the prospective identification of eye irritating substances (2019)

評価報告書

眼刺激性試験代替法　SIRC-CVS:TEA 法

眼刺激性試験資料編纂委員会

令和 3 年(2021 年)5 月 6 日

眼刺激性試験資料編纂委員会

山 本 直 樹（委員長：藤田医科大学）
佐々木正治（アレクシオンファーマ合同会社）
竹 内 小 苗（P&G イノベーション合同会社）*
波多野浩太（ホーユー株式会社）
山 下 晴 洋（大正製薬株式会社）

*：SIRC-CVS Test Method の peer review panel

略語

CVS:	Crystal Violet Staining
DMSO:	Dimethyl sulfoxide
EIT:	Eye Irritation Test
GHS:	Globally Harmonized System of Classification and Labelling of Chemicals
IC50:	Half maximal (50%) Inhibition Concentration
JaCVAM:	Japanese Center for the Validation of Alternative Methods
OECD:	Organisation for Economic Co-operation and Development
PBS(-):	Ca^{2+}/Mg^{2+} free Phosphate-Buffered Saline
PRP:	Peer Review Panel
SIRC:	Statens Seruminstitut Rabbit Cornea
SDS:	Sodium Dodecyl Sulfate
TEA:	Triethanolamine
TG:	Test Guideline
UN:	United Nations

要旨

SIRC-CVS法は、ウサギ角膜由来細胞（SIRC細胞）を用いて、クリスタルバイオレットが細胞膜を透過して生体高分子を染色する性質を利用した細胞毒性を測定する試験法である。本試験法は、株式会社資生堂により、トリエタノールアミン（TEA）を比較対照物質として、ボトムアップ方式で国際連合化学品の分類および表示に関する世界調和システム（UN GHS）区分に該当しない化学物質を検出することができる方法であるSIRC-CVS:TEA法として改定され、JaCVAMによるバリデーション研究が実施された。本報告書では、SIRC-CVS:TEA法のバリデーション研究報告書、第三者評価報告書、関連論文などをもとに試験法の概要を説明し、JaCVAM眼刺激性試験資料編纂委員会の意見をまとめた。

SIRC-CVS:TEA 法の信頼性を確認するため、バリデーション研究が行われた。この研究において、20 物質を用いて 3 施設の施設内再現性はすべての施設で 100%、また、30 物質を用いた 3 施設での施設間再現性は 90%であり、いずれの再現性もバリデーション実行委員会の定めた 80%の基準を満たした。一方、正確性は、116 物質を用いてバリデートされ、正確度 55.2%、感度 60%、特異度 47.8%であった。この値は行政的利用を目指す試験法にとって十分なものではなく、予測性を改良するため、種々の適用除外物質の検討が開発者により提案された。しかし、長年にわたる検討を経ても、開発者と第三者評価委員会（PRP）の間で科学的に十分な説明がつく適用除外に合意ができず、予測性の適切な改善には至らなかった。

以上より、本委員会においても、PRP の見解同様、SIRC-CVS:TEA 法はボトムアップ方式で UN GHS 区分に該当しない化学物質を検出する方法として、行政的に用いることは相応しくないと結論した。

キーワード：眼刺激性試験、代替法、細胞毒性、*in vitro*、バリデーション

1. 緒言

SIRC-CVS 法は、板垣らにより開発されたウサギ角膜由来細胞（SIRC 細胞）の細胞毒性を指標として、クリスタルバイオレット染色（CVS）により細胞生存率を測定し、化学物質の眼刺激性を評価する試験法であり [1,2]、1990 年代に厚生科学研究にて化粧品原料のみを対象にバリデーション研究が行われた [3-6]。本試験法を用いて「代替法を用いて化粧品原料の眼刺激性を評価するにあたっての指針」も作成されている [7]。

細胞毒性を指標として用いた眼刺激性試験として、短時間曝露法が OECD TG491[8]に、3 次元培養角膜モデルを用いた EpiOcular™ 眼刺激性試験（EIT）、SkinEthic™ EIT、LabCyte CORNEA-MODEL24 EIT および MCTT HCE™ EIT が OECD TG492 に収載されている [9]。これらの方法は、ボトムアップ方式で国際連合化学品の分類および表示に関する世界調和システム（UN GHS）区分に該当しない化学物質を検出する方法として公定化されている。

SIRC-CVS 法も上記の細胞毒性を指標とする方法同様、ボトムアップ方式により UN GHS 区分に該当しない化学物質を検出することができる方法として、株式会社資生堂によって TEA を比較対照物質として用いた SIRC-CVS:TEA 法に改定され [10]、JaCVAM によりバリデーション研究が実施された。

本報告書では、SIRC-CVS:TEA 法のバリデーション研究報告書 [11]、第三者評価報告書（Annex 1）[12]、関連論文などをもとに試験法の概要を説明し、JaCVAM 眼刺激性試験資料編纂委員会の意見をまとめたものである。

2. 試験法の原理

CVS 法はクリスタルバイオレット（CV）が細胞膜を透過して生体高分子を染色する性質を利用した細胞毒性を測定する試験法である。CVS 法は多くの細胞に適用でき、得られる結果も比較的安定しているため、細胞毒性の簡易試験法として用いられている。細胞毒性試験は角膜の障害を予測することを想定しており、バリデーション研究報告書 [11] の introduction では“Cytotoxicity is considered a useful index of the eye irritation potency of various substances, as the corneal damage that has a greater impact on the total eye irritation is related to damage of the corneal epithelium cell[13]”、また、大野によると Draize 試験から得られる情報と代替法から得られる情報を分類し、細胞毒性は角膜上皮細胞の変性、剥離の情報と対応するとされている [7]。

3. 試験手順

SIRC-CVS:TEA 法の手順を以下に示す。

3-1. SIRC 細胞の培養、継代と細胞浮遊液の調製

1) SIRC 細胞は培養液（10% FBS : Fetal Bovine Serum を含む MEM : Minimum Essential Medium）を用い、37℃、5% CO_2 インキュベータで培養される。
2) SIRC 細胞の継代はまず培養フラスコから培養液を取り除き、さらにトリプシン阻害剤となる血清を充分取り除くため、PBS(-) 10 mL で細胞表面を 2 回洗浄する。
3) PBS(-)を除去した後、0.25% Trypsin 液（1.5-2 mL）を細胞表面の全体に行き渡るよ

う加える（2-10 秒程度）。

4) 0.25% Trypsin 液を吸い取った後、細胞を剥離させるために 37℃で 2～3 分間インキュベートし、フラスコの細胞接着面裏から軽くタップする。剥離後、適量の培養液を加えた後、十分なピペッティングにより単細胞化させ均等な細胞浮遊液を調製する。細胞数を計測し、培養液にて $6～8\times10^5$ cells/mL に調製する。1 mL の細胞（$6～8\times10^5$ cells）を 15～30 mL の培養液に加え継代する。

5) 実験開始の際には、細胞数を計測し、培養液にて 2×10^5 cells/mL に調製する。

3-2. 被験物質の適用

1) PBS(-)、陰性対照物質、並びに被験物質、陽性対照物質、比較対照物質の希釈系列（100 µL/well）を図 1 に示すように 96 穴マイクロプレート内に作製する。溶媒は培養液、10,000 µg/mL DMSO 培養液溶液または 10,000 µg/mL Ethanol 培養液溶液を用いる。陰性対照物質は、被験物質を溶解させた溶媒を用いる。陽性対照物質として SDS を用いる。比較対照物質として TEA を用いる。対照物質の溶媒には培養液を用いる。被験物質の最高適用濃度は 10,000 µg/mL とし、溶解または均一に懸濁させるために必要に応じて 5,000 µg/mL とする。それでも均一に懸濁しないものは試験に不適な物質と判断する。SDS および TEA はそれぞれ 1,000 µg/mL と 10,000 µg/mL とする。被験物質、および陽性対照物質、比較対照物質は公比 2 で 8 段階に調整する。

2) 2×10^5 cells/mL の細胞浮遊液を 0.1 mL、図 2 に示すウェルに添加する。

3) 被験物質が揮発し周囲のウェルへ影響を与える可能性を考慮し、ウェルを覆うマイクロプレートシーリングテープを貼付する。さらに、ラップフィルムを用いることができる。なお、被験物質が他のウェルに影響を与えた場合には、希釈して試験することができる。

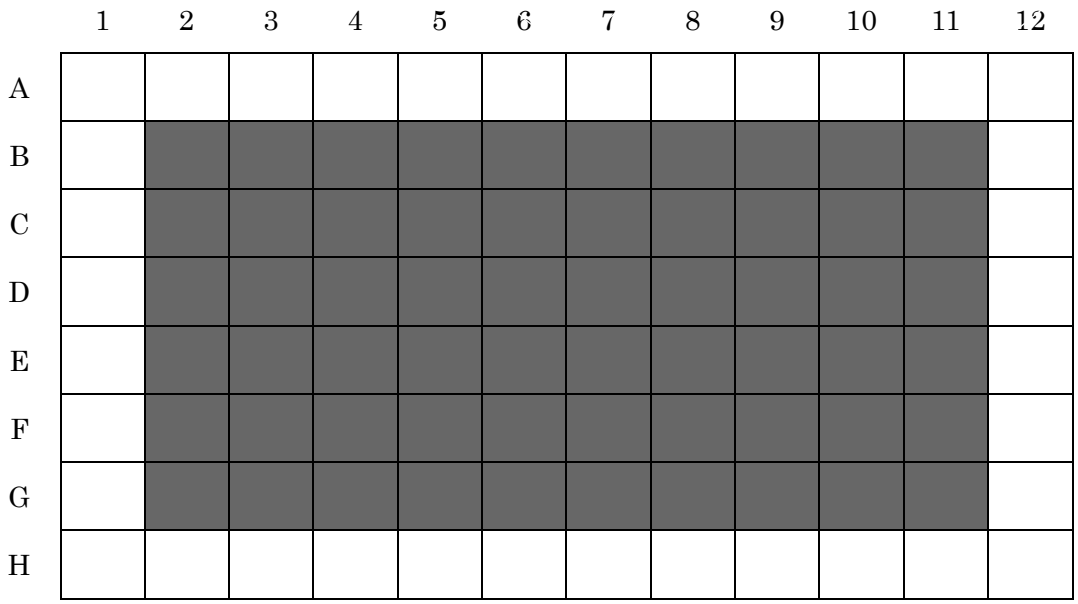
4) 添加した 96 穴マイクロプレートは細胞を培養床に均一に接着させるために、そのままクリーンベンチ内で静置（室温、20 分間）し、その後、CO_2 インキュベータ中に移す。

5) 約 72 時間、37℃、5% CO_2 条件下で培養する。

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
C	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
D	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
E	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
F	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
G	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

PBS : PBS(-)を 200 µL、NC : 培養液、10,000 µg/mL DMSO 培養液溶液または 10,000 µg/mL Ethanol 培養液溶液を 100 µL、S : 被験物質の 2 倍希釈系列（100 µL）、R : 比較対照物質の 2 倍希釈系列（100 µL）、P : 陽性物質の 2 倍希釈系列（100 µL）

図 1. 96 穴マイクロプレートのフォーマット



■ : 細胞浮遊液（100 µL）

図 2. 細胞浮遊液の添加

3-3. CVS

1) 培養期間終了後､96 穴マイクロプレートを静かに反転し被験物質を含む培養液を捨てる。
2) PBS(-)を 200 µL 添加し優しく撹拌した後、反転させ PBS(-)を捨てる。これを 2 回繰り返す。
3) 96 穴マイクロプレートの各ウェルに CV メタノール溶液を 100 µL 分注し、30 分間染色する。
4) 染色時間が終了後、96 穴マイクロプレートを静かに反転させ CV 溶液を捨て、十分に水洗する。ペーパータオル上にプレートを伏せ、水分を吸い取らせる。
5) 十分に風乾した後、マイクロプレートリーダーを用いて各ウェルの吸光度(588 nm)を測定する。

3-4. IC50 の算出

被験物質を含まない陰性対照ウェルの細胞生存率を 100%とした場合における各ウェルの細胞生存率を吸光度から算出する。細胞生存率 50%を示す被験物質濃度(IC50)の算出にあたっては、細胞生存率 50%をはさむ 2 濃度とその濃度における細胞生存率から式 LogIC50=［(50-y1)logx2-(50-y2)logx1］/(y2-y1)を用いて算出する。(※記号は、被験物質濃度 x1(低濃度側)、x2(高濃度側)におけるそれぞれの細胞生存率を y1、y2 で示す。Log は常用対数である。)

被験物質の最高濃度である 5,000 µg/mL で細胞生存率が 50%以下にならない場合は IC50>5,000 µg/mL とする。また、試験した最低濃度である 39.1 µg/mL で細胞生存率が 50%未満の場合は、IC50<39.1 µg/mL とする。

なお､表計算ソフト(Excel)において、細胞生存率を算出する段階以降で小数点以下 2 桁目を四捨五入する。

3-5. 評価

比較対照物質として TEA を用い、被験物質の眼刺激性を予測し、評価する。被験物質の IC50 が TEA の IC50 以上を陰性、TEA の IC50 未満を陽性と判定する。試験は 2 回を繰り返して行い､その結果に基づき評価する。2 回の評価結果が異なった場合には同様に 3 回目を実施し、2 回の同じ評価結果を採用し､その結果に基づき評価する。

なお、TEA は UN GHS 分類で眼刺激性に分類されない物質の中で、IC50 が高い参照物質としてバリデートされており、その結果から妥当な選択物質であると本委員会も考えている。本委員会は本物質が化学兵器の禁止及び特定物質の規制等に関する法律(平成七年四月五日法律第六十五号)[14]により第二種指定物質として指定されていることは把握しているものの、さまざまな化粧品の製造時に pH 調整剤として使用されていることから安全性上も利便上も問題ないと考える。

3-6. 品質基準

試験の精度管理を以下の 5 項目で行う。全ての項目で基準を満たすことを試験成立の要件とする。試験成立の要件を満たさない場合には該当プレートについて再試験を

行う。特に、揮発した物質の毒性により要件を満たさない場合には、被験物質の濃度を下げて試験を行う。

1) 陰性対照から得られる吸光度の絶対値は、各ウェルに播種した 2×10^4 個の細胞が 72 時間のアッセイ期間中に正常な増加を示しているか否かを表している。したがって、96 穴マイクロプレートの左右に設定した陰性対照の平均吸光度が 0.4 を上回ること。
2) 標準的なプロトコルで試験された陽性対照 SDS の IC50 は 77.7～258.7 µg/mL の範囲であること。
3) 標準的なプロトコルで試験された比較対照物質 TEA の IC50 は 1,000～2,500 µg/mL の範囲であること。
4) 体系的に試験精度を見極めるために、96 穴マイクロプレートの左右に陰性対照を設定し、両者の吸光度が同様であることを確認する。左右の陰性対照の平均吸光度が全体の平均吸光度の±15%以内（平均値±15%）に収まること。
5) 採用された 2 回の試験結果が同様であることを確認するために、2 試験間の誤差を確認することが必要である。したがって、2 試験間での陽性対照の IC50 値が±2 倍以内（高値/低値≦2）に収まること。

4. バリデーション研究

バリデーション報告書に示すようなバリデーション実行委員会（VMT）を組織し、開発者とは異なる 3 施設（日本コルマー株式会社：Lab A、株式会社ボゾリサーチセンター：Lab B、Biotoxtech Co., Ltd, Seoul, : Lab C）の協力を得て、SIRC-CVS:TEA 法のバリデーション研究が行われた [3]。これら 3 施設はバリデーション研究の開始前に開発者からビデオで試験手順の説明を受けた後、技術移転性を確認した。

4-1. 試験法の信頼性

4-1-1. 技術移転性

技術移転性については、プレバリデーション研究（Phase I）にて評価した。3 施設によるコード化していない 4 物質（Ethyl-2-methyl acetoacetate, Safflower oil, 3-Chloropropionitrile, Sodium dehydroacetate）、陽性対照物質および比較対照物質を用いた試験が行われ、プロトコルの妥当性が確認された [3]。その結果、表 1 に示すように、すべての施設で一致した結果が得られた。なお、Lab A の 1 回目の結果は、他の施設と一致しなかった。調査の結果、被験物質の希釈法が不適切であったことが判明し、再試験を行ったところ、他施設と同様の結果となった。この結果から、プロトコルに大きな改訂の必要はなく、順守すれば、適切な結果が得られることが明らかになった。

表 1．SIRC-CVS:TEA 法バリデーション研究　Phase I の結果

Chemical Code	Name of test substances	Laboratory A			Laboratory A (Retest)			Laboratory B			Laboratory C			GHS Category
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
P1-001	Ethyl-2-methyl acetoacetate	N	N	N	N	N	N	N	N	N	N	N	N	2
P1-002	Safflower oil	N	N	N	N	N	N	N	N	N	N	N	N	No
P1-003	3-Chloropropionitrile	P	P	P	P	P	P	P	P	P	P	P	P	2
P1-004	Sodium dehydroacetate	P	N	N	P	P	P	P	P	P	P	P	P	No

* N: Negative, P: Positive

4-1-2. 施設内再現性

SIRC-CVS:TEA 法の施設内再現性は、バリデーション研究 Phase II の 20 物質を用いて 3 施設で実施された。その結果、表 2 に示すように、SIRC-CVS:TEA 法の施設内再現性はすべての施設で 100%となり、バリデーション実行委員会の定めた基準（80%）を満たした。

表 2．SIRC-CVS:TEA 法バリデーション研究　Phase II の施設内再現性結果

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation	GHS Category
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3		
P2-001	Piperonylbutoxide	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-002	2,5-Dimethylhexaediol	1	N	N	N	N	N	N	N	N	N	N	1
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	1	N	N	N	N	N	N	N	N	N	N	2B
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-004	Ammonium nitrate	1	P	P	P	P	P	P	P	P	P	P	2A
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-005	Potassium tetrafluoroborate	1	N	N	N	N	N	N	N	N	N	N	No
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-006	3,4,4'-Trichlorocarbanilide	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-007	1-Bromohexane	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	1	N	N	N	N	N	N	N	N	N	N	No
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-009	Propylene glycol propyl ether	1	N	N	N	N	N	N	N	N	N	N	2A
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-010	Ethyl thioglycolate	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation	GHS Category
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3		
P2-011	Sodium oxalate	1	P	P	P	P	P	P	P	P	P	P	1
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-012	2-Phospho-L-ascorbic acid trisodium salt	1	N	N	N	N	P	N	N	N	N	N	No
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		
P2-013	1-Bromo-4-chlorobutane	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-014	Sodium hydrogensulfite	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-015	Isobutyraldehyde	1	P	P	P	P	P	P	P	P	P	P	2B
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-016	1-Naphthaleneacetic acid	1	P	P	P	P	P	P	P	P	P	P	1
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-017	Propyl 4-hydroxybenzoate	1	P	P	P	P	P	P	P	P	P	P	No
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	1	P	P	P	P	P	P	P	P	P	P	2B
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-019	Camphene	1	P	P	P	P	P	P	P	P	P	P	2B
		2	P	P	P	P	P	P	P	P	P		
		3	P	P	P	P	P	P	P	P	P		
P2-020	Cyclopentanol	1	N	N	N	N	N	N	N	N	N	N	2A
		2	N	N	N	N	N	N	N	N	N		
		3	N	N	N	N	N	N	N	N	N		

*N: Negative, P: Positive

4-1-3. 施設間再現性

Phase II の 20 物質および Phase III の 10 物質、合計 30 物質を用いて 3 施設で施設間再現性が確認された。表 3 に示すように、Phase II の 20 物質ではすべての施設で施設間再現性が一致した。さらに追加して、Phase III の 10 物質で施設間再現性を確認したところ、表 4 に示すように、3 物質で不一致があった。P3-010（n,n-Dimethylguanidine sulfate）および P3-012（Polyethylene hydrogenated castor oil (40E.O.)）はいずれの施設でも同様の濃度依存性であったが、3 施設で結果が一致しなかった。P3-003（Dipropyl disulfide）の濃度依存性は施設間で異なっていた。その原因は溶媒の選択による溶解性の差とバリデーション実行委員会は判断した。用いた溶媒が Lab A で 培養液、Lab B で Ethanol および Lab C が DMSO であったことによる。

結果として、施設間再現性は 90%となり、バリデーション実行委員会の定めた基準（80%）を満たした。なお、一致しなかった物質を予測性の評価に用いる場合には、多数決の結果を用いることがバリデーション実行委員会により決定した。

表 3．SIRC-CVS:TEA 法バリデーション研究　Phase II の施設間再現性結果

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P2-001	Piperonylbutoxide	P	P	P	1
P2-002	2,5-Dimethylhexaediol	N	N	N	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	Ammonium nitrate	P	P	P	1
P2-005	Potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-Trichlorocarbanilide	P	P	P	1
P2-007	1-Bromohexane	P	P	P	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	Propylene glycol propyl ether	N	N	N	1
P2-010	Ethyl thioglycolate	P	P	P	1
P2-011	Sodium oxalate	P	P	P	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-Bromo-4-chlorobutane	P	P	P	1
P2-014	Sodium hydrogensulfite	P	P	P	1
P2-015	Isobutyraldehyde	P	P	P	1
P2-016	1-Naphthaleneacetic acid	P	P	P	1
P2-017	Propyl 4-hydroxybenzoate	P	P	P	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	P	P	P	1
P2-019	Camphene	P	P	P	1
P2-020	Cyclopentanol	N	N	N	1

*1: N: Negative, P: Positive
*2: 1: The results from all three laboratories were concordant

表 4. SIRC-CVS:TEA 法バリデーション研究　Phase III の施設間再現性結果

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P3-003	Dipropyl disulfide	P	P	N	0
P3-005	2-(2-Ethoxyethoxy)ethanol	N	N	N	1
P3-010	n,n-Dimethylguanidine sulfate	N	P	N	0
P3-012	Polyethylene hydrogenated castor oil (40E.O.)	N	P	N	0
P3-019	Diethyl toluamide	P	P	P	1
P3-020	4-Nitrobenzoic acid	N	N	N	1
P3-024	2-Amino-3-hydroxy pyridine	P	P	P	1
P3-028	Tetraethylene glycol	P	P	P	1
P3-029	Dodecanoic acid	P	P	P	1
P3-033	gamma-Butyrolactone	N	N	N	1

*1: N: Negative, P: Positive
*2: 1: All laboratories' judge agreed, 0: Only two laboratories' judge agreed

4.2. 試験法の正確性

正確性は、表 5 に示すように、Phase II の 20 物質と Phase III の 96 物質、合計 116 物質を用いて評価された。Phase III では当初、100 物質を用いて実施され、各施設にそのうちの 40 物質が配布され、データが取得された。しかしながら、P3-023 および P3-095 に物質の重複（3,3-Dithiodipropionic acid）があり、それらの結果が一致していることから P3-023 のみの結果が解析に用いられた。また、P3-066（Calcium thioglycolate trihydrate）は沈殿が生じて IC50 を計測できなかった。この試験法の技術的な限界と考えられた。さらに、P3-067（Citric acid）および P3-068（Potassium sorbate）については、個々の動物実験結果を見つけられなかった。よって、Phase III に用いられた 100 物質のうちこれら 4 物質が正確性の解析から削除され、最終的に 96 物質が正確性の評価に加えられた。結果として、表 6 に示すように、ボトムアップ方式での正確性は正確度 55.2%、感度 60.0%、特異度 47.8%であった。

表 5．SIRC-CVS:TEA 法バリデーション研究　Phase III の全結果

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation	GHS Category
P3-001	B	2-Ethoxyethyl methacrylate	P	P	P	No
P3-002	C	iso-Octylthioglycolate	N	N	N	No
P3-003	A/B/C	Dipropyl disulfide	P/P/N	P/P/N	P	No
P3-004	C	1-Bromo-octane	P	P	P	No
P3-005	A/B/C	2-(2-Ethoxyethoxy)ethanol	N/N/N	N/N/N	N	No
P3-006	C	Dioctyl ether	P	P	P	No
P3-007	C	3-Phenoxybenzyl alcohol	P	P	P	No
P3-008	B	Glycidyl methacrylate	P	P	P	No
P3-009	C	2-Ethylhexylthioglycolate	N	N	N	No
P3-010	A/B/C	n,n-Dimethylguanidine sulfate	N/P/N	N/P/N	N	No
P3-011	C	6-Hydroxy-2,4,5-triaminopyrimidine Sulfate	P	P	P	No
P3-012	A/B/C	Polyethylene hydrogenated castor oil (40E.O.)	N/P/N	N/P/N	N	No
P3-013	C	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	N	N	N	No
P3-014	C	Cellulose, 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride	N	N	N	No
P3-015	C	3,4-Dimethoxy benzaldehyde	P	P	P	No
P3-016	C	3-Chloropropionitrile	P	P	P	2B
P3-017	C	2-Methyl-1-pentanol	N	N	N	2B
P3-018	C	Ethyl-2-methylacetoacetate	N	N	N	2B
P3-019	A/B/C	Diethyl toluamide	P/P/P	P/P/P	P	2B
P3-020	A/B/C	4-Nitrobenzoic acid	N/N/N	N/N/N	N	2B
P3-021	C	Sodium chloroacetate	P	P	P	2B
P3-022	A	2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	P	P	P	2A
P3-023	C	3,3-Dithiodipropionic acid	N	N	N	2B
P3-024	A/B/C	2-Amino-3-hydroxy pyridine	P/P/P	P/P/P	P	2A
P3-025	C	Sodium benzoate	N	N	N	2A
P3-026	C	Methylthioglycolate	P	P	P	1
P3-027	A	3-(2-Aminoethylamino)propyl] trimethoxysilane	P	P	P	1
P3-028	A/B/C	Tetraethylene glycol	P/P/P	P/P/P	P	1
P3-029	A/B/C	Dodecanoic acid	P/P/P	P/P/P	P	1
P3-030	C	1,2-Benzisothiazol-3(2H)-one	P	P	P	1
P3-031	C	2-Hydroxy-1,4-naphthoquinone	P	P	P	2B
P3-032	C	Disodium 4,4'-bis(2-sulfonatostyryl) biphenyl	P	P	P	1

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation	GHS Category
P3-033	A/B/C	gamma-Butyrolactone	N/N/N	N/N/N	N	2A
P3-034	C	1-Methylpropyl benzene	N	N	N	No
P3-035	C	4-(Methylmercapto)benzaldehyde	P	P	P	No
P3-036	C	1,9-Decaine	P	P	P	No
P3-037	C	2,4-Dimethyl-3-pentanol	N	N	N	No
P3-038	C	1-Ethyl-3-methylimidazolium ethylsulfate	N	N	N	No
P3-039	C	1,2,4-Triazole,sodium salt	P	P	P	1
P3-040	C	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl) bis[2,6-dibromophenol]	P	P	P	1
P3-041	C	Benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene) methyl-2-methy HCL	P	P	P	1
P3-042	A	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	P	P	P	No
P3-043	B	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	P	P	P	No
P3-044	C	Isopropyl acetoacetate	N	N	N	2B
P3-045	A	(3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	P	P	P	2A
P3-046	B	1-Octanol	P	P	P	2A
P3-047	B	2-Benzyloxyethanol	N	N	N	2A
P3-048	B	Butanol	N	N	N	1
P3-049	B	Isobutyl alcohol	P	P	P	1
P3-050	B	Isopropyl alcohol	N	N	N	2A
P3-051	B	Myristyl alcohol	P	P	P	2A
P3-052	B	Hexyl cinnamic aldehyde	P	P	P	2
P3-053	B	n-Butanal	P	P	P	2B
P3-054	B	Monoethanolamine	P	P	P	2B
P3-055	B	m-Phenylenediamine	P	P	P	1
P3-056	B	Ethyl acetate	N	N	N	No
P3-057	B	Isopropyl myristate	N	N	N	No
P3-058	B	Methoxyethyl acrylate	P	P	P	1
P3-059	B	Methyl acetate	N	N	N	2A
P3-060	B	Methyl cyanoacetate	N	N	N	2A
P3-061	B	Imidazole	P	P	P	1
P3-062	B	Pyridine	N	N	N	1
P3-063	B	Isopropyl bromide	N	N	N	No
P3-064	B	Cyclohexanone	N	N	N	No

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation	GHS Category
P3-065	B	2-Methylbutyric acid	N	N	N	1
P3-066	B	Calcium thioglycolate trihydrate	NA	NA	NA	1
P3-067	B	Citric acid	P	P	P	No data
P3-068	B	Potassium sorbate	N	N	N	No data
P3-069	B	Sodium salicylate	N	N	N	1
P3-070	B	Distearyldimethyl ammonium chloride	P	P	P	1
P3-071	B	n-Lauroylsarcosine sodium salt	P	P	P	2B
P3-072	B	Sodium lauryl sulfate	P	P	P	2A?
P3-073	A	Triton X-100 (5%)	P	P	P	2B
P3-074	A	2-Ethylhexyl p-dimethylamino benzoate	P	P	P	No
P3-075	A	Promethazine hydrochloride	P	P	P	1
P3-076	A	2-Ethyl-1-hexanol	P	P	P	2A
P3-077	A	3-Methoxy-1.2-propanediol	N	N	N	No
P3-078	A	Cyclohexanol	N	N	N	1
P3-079	A	Ethanol	N	N	N	2A
P3-080	A	n-Hexanol	N	N	N	2A
P3-081	A	3,3-Dimethylpentane	P	P	P	No
P3-082	A	Methyl cyclopentane	P	P	P	No
P3-083	A	Toluene	N	N	N	2B?
P3-084	A	Acetone	N	N	N	2A
P3-085	A	Gluconolactone	N	N	N	No
P3-086	A	Methyl amyl ketone (2-heptanol)	N	N	N	No
P3-087	A	Methyl ethyl ketone (2-butanone)	N	N	N	2A
P3-088	A	Methyl isobutyl ketone(4-methyl 2-pentanol)	N	N	N	No
P3-089	A	Glycerol	N	N	N	No
P3-090	A	Cetylpyridinium bromide	P	P	P	1
P3-091	C	Triton X-100	P	P	P	1
P3-092	C	Tween20	P	P	P	No
P3-093	A	Sodium hydroxide	P	P	P	1
P3-094	A	Glycolic acid	N	N	N	2B
~~P3-095~~	~~C~~	~~3,3-Dithiodipropionic acid~~	~~N~~	~~N~~	~~N~~	~~2B~~
P3-096	A	Sucrose fatty acid ester	N	N	N	2A?
P3-097	A	methyl para-Hydroxybenzoate	P	P	P	2?
P3-098	A	Silic acid	P	P	P	No

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation	GHS Category
P3-099	A	Benzyl alcohol	P	P	P	1
P3-100	A	Lactic acid	N	N	N	1

*1: N: Negative, P: Positive
*2: Eye irritation potential of common test substances were expressed as a representative of three laboratories.
*3: NA: Not applicable

表 6．SIRC-CVS:TEA 法のボトムアップ方式における正確性（TEA の有無による）

Regulatory System	Judgement by IC50 value of triethanolamine	Judgement by IC50 at 1600 µg/mL
Accuracy	55.2% (64/116)	58.9% (66/112)
Sensitivity	60.0% (42/70)	69.1% (47/68)
Specificity	47.8% (22/46)	43.2% (19/44)
False Negative Rate	40.0% (28/70)	30.9% (21/68)
False Positive Rate	52.2% (24/46)	56.8% (25/44)

5. 試験法の有用性と限界

本試験法は、眼刺激性試験代替法として原理も明確な *in vitro* の手法であり、同じ目的のために実施される動物実験と比べ、「動物の愛護及び管理に関する法律」および 3Rs の精神と合致している。また、細胞毒性を指標とした試験法であるため、操作が簡便で、標本の資料保管が可能である。実施の際に必要となる消耗品費については、細胞株は細胞バンクから購入でき、試薬・実験資材等も高価なものを用いておらず、動物実験よりも安価である。

本試験法の正確性を改良するため（すなわち、偽陰性を減らすため）、開発者はバリデーション結果に加え、株式会社資生堂の in-house のデータも合わせて解析した。まず、開発者は分子量 180 未満のアルコール、エステル、エーテル、ケトン、ヘテロ環状物質、カルボン酸の適用除外物質案を示した。PRP はこの案により、感度 90%以上（偽陰性 10%以下）と高くなるものの、科学的な合理性が乏しいと判断した。そこで、開発者は新たに、酸解離定数 pKa が 4 以下の酸，および-1.5<log D<2 の物質を除外という適用限界を提案した。しかし、この提案も PRP は感度 90%以上（偽陰性 10%以下）と高くなるが、科学的に十分な説明がつくものではないと判断し、開発者との間で明確なメカニズムに基づく正確性の高い適用除外物質案の合意には至らなかった。参考までに、予測性値の一覧を Annex2 にまとめた。株式会社資生堂の in-house のデータを含めるか否かを問わず、どの案も同程度の正確性を示している。また、Cosmetic Europe が発表した *in vivo* 結果の検証論文（Barroso ら、Archives of Toxicology, 2017, 91(2), 521-547）を基に、Barroso の

結果で解析した SIRC-CVS:TEA の結果も検討したが（Annex1）、PRP は採用しなかった。その理由は、正確性があまり変わらなかったことによる。

なお、この評価の経験から、今後新規代替法のバリデーションを実施する場合には、被験物質選択時に *in vivo* 試験結果の個別確認を事前に行うことの重要性を本委員会から提言させて頂く。

6. 本委員会の結論

SIRC-CVS:TEA 法は施設内および施設間再現性の高い方法ではあるけれども、表 6 に示すように、比較対照物質 TEA との比較による正確度は約 60%と低い。参考資料として、同表に比較対照物質を用いず、IC50 値 1,600 µg/mL を基準として正確性を求めたが、大きな改善はみられなかった。PRP の見解同様、本委員会においても適用除外物質の設定により、偽陰性率は低くなるものの、科学的な根拠が乏しく、ボトムアップ方式で UN GHS 区分に該当しない化学物質を検出する方法として行政的に用いることは相応しくないと結論した。

7. 利益相反（COI）について

特になし

8. 引用文献（最終アクセス日 2021 年 7 月 30 日）

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SIRC-CVS Test Method

Report of the Peer Review Panel

on

a JaCVAM coordinated study programme addressing the validation status of the SIRC-CVS test method for the prospective identification of eye irritating substances

Report completed by the Peer Review Panel on August 19, 2019.

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Executive Summary

The Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining Cytotoxicity Test Method with Triethanolamine (SIRC-CVS Test Method) has been proposed as an *in vitro* test method to discriminate eye irritating chemicals from non-eye irritating chemicals. It could contribute to replacing the Draize eye irritation test as being used as the first test method in a bottom-up approach. The test method main advantages are the use of a rabbit cornea cell line and that it is relatively simple to perform. Coordinated and sponsored by the Japanese Center for the Validation of Alternative Methods (JaCVAM), the SIRC-CVS Test Method was prospectively validated by a Validation Management Team (VMT).

The peer review panel (PRP) found the Validation Management Team's report presented the necessary information for an independent review. The PRP concluded that the SIRC-CVS Test Method was, with the exception of the applicability domain, sufficiently well defined, with a clear protocol and criteria for data interpretation. Both within and between laboratory reproducibility information were considered to be satisfactory. However, the predictive capacity could not be assessed, as the applicability domain of the test method was adjusted several times without providing clear mechanistic insights and justification of the proposed domain restrictions. Accordingly, the PRP concluded that, based on the provided information, the scientific validity of the SIRC-CVS Test Method could not be demonstrated for its use as a part of an integrated testing strategy for distinguishing chemicals classified from chemicals not classified according to the UN GHS classification system.

Peer Review Panel Composition

Sebastian Hoffmann (chair)	seh consulting + services, Paderborn, Germany
Chantra Eskes	Swiss 3R Competence Centre, Bern, Switzerland (until August 2018 SeCAM, Agno, Switzerland)
Pertti Hakkinen	National Institutes of Health, Bethesda, USA
Tae Cheon Jeong	Yeungnam University, Gyeongsan, South Korea
Tadashi Kosaka	Institute of Environmental Toxicology, Ibaraki, Japan
Jill Merrill	U.S. Food and Drug Administration, Silver Spring, USA
Sanae Takeuchi	P&G Innovation Godo Kaisha, Kobe, Japan

Background

The SIRC-CVS Test Method was evaluated in a prospective validation study by a Validation Management Team (VMT), which was chaired, coordinated and sponsored by the Japanese Center for the Validation of Alternative Methods (JaCVAM). The study was designed to assess the usefulness of the SIRC-CVS Test Method as an alternative to the *in vivo* Draize test method to identify ocular non-irritants in a bottom-up testing strategy approach according to the UN GHS classification scheme (Scott et al., 2010).

The PRP was assembled at the end of 2014 and met in March 2015 to discuss the outcome of the validation study with the VMT, and to review, in the absence of the VMT, the validation study of the SIRC-CVS Test Method. The peer review was conducted based on 14 evaluation criteria. As these criteria were derived from the modular approach (Hartung et al., 2014), the PRP review is structured by the seven validation modules. Upon repeated requests for clarifications by the PRP on a number of elements, but most importantly on the applicability domain, the VMT provided replies that were discussed via several teleconferences and meetings from 2015 to 2018. In March 2019, the PRP held a final telephone conference. With the provision of all of the amended, updated and additional material, including the final VMT report, this PRP report was prepared.

SIRC-CVS Test Method Definition

The PRP concluded that the SIRC-CVS Test Method has been fully described in the report of the Validation Management Team (VMT) and in the associated detailed test protocol. Protocol amendments made during and after the validation study were traceable over the various versions. The validation report adequately stated the need for the assay in the current regulatory context. Furthermore, a rationale for the test method has been given and helpfully included references to existing *in vitro* methods for eye irritation that have been validated and adopted into OECD guidelines. The PRP agreed that the mechanistic basis of the method and how it related to eye irritation was sufficiently described in the report although further details would have been appreciated.

The PRP agreed that this method is intended to contribute to the replacement of animal usage for eye irritation assessment and that, when compared to other *in vitro* cell-based methods, which aim at discriminating irritating from non-irritating chemicals, it is likely to offer time, throughput, and cost benefits. Furthermore, the PRP was of the opinion that the use of corneal cells in the SIRC-CVS Test Method can present mechanistic advantages as compared to other cell types for predicting ocular irritation.

Within Laboratory Reproducibility

Based on information provided in the VMT report, the PRP agreed that the results demonstrated a good within-laboratory reproducibility. The PRP achieved this conclusion on the basis of the data of Phase II of the validation study, in which 20 substances were tested three times in three laboratories, regardless of any applicability domain considerations. As all substances were consistently predicted in the laboratories, resulting in a within-laboratory reproducibility of 100%, the success criterion of a within-laboratory reproducibility of at least 80% set by the VMT was met.

In addition, the results provided in the validation report indicate that bovine serum and TEA from different manufacturing lots have no effect on the reproducibility.

Transferability

The PRP noted that the fact that the three participating laboratories were all naïve and that no practical training was provided is a good indication of the robustness of the test method.

Between Laboratory Reproducibility

As with within laboratory reproducibility, the PRP based its evaluation of the between-laboratory reproducibility on the information provided in the VMT report. The PRP agreed that the results demonstrated a good between-laboratory reproducibility. The PRP achieved this conclusion on the basis of the data of Phase II and of 10 substances of Phase III of the validation study, in which 30 substances were tested in three laboratories, regardless of any applicability domain considerations. As 27 substances were consistently predicted across the three laboratories, resulting in a between-laboratory reproducibility of 90%, the success criterion of a between-laboratory reproducibility of at least 80% set by the VMT was met.

Predictive Capacity

Demonstration of a test method's performance should be based on the testing of representative, preferably coded, reference chemicals. The PRP concluded that the validation study used an appropriate level of test chemical coding to ensure fully blinded evaluation. With respect to predictive capacity, the PRP confirms that a suitable balance of stronger, weaker, and non-classified (according to the GHS classes of 1, 2A, 2B and no class) test chemicals was selected. The PRP agreed that the rabbit was considered as the target species, noting that prediction of

human eye irritation is the ultimate goal and acknowledging that high quality human data are in general not available. The PRP notes that *in vivo* data have been reviewed by ICCVAM. Additionally, the majority of test chemicals were also used in other validation studies (e.g. of the RhCE and STE). Finally, the majority of tested chemicals differed from those used in initial method development.

The PRP agreed that the predictive capacity for an unrestricted applicability domain was based on a sufficiently large and representative set of test chemicals. However, the accuracy of 55% (64/116) did not meet the VMT success criteria of 80%, and the false negative rate of 40% (28/70) did not meet the success criterion of <5%.

The PRP understood that this poor predictive performance triggered the test developer to explore various physical and chemical properties to identify possible reasons for misclassification. Various restricted applicability domains, all of which resulted in the exclusion of a substantial amount of substances, but also in improved predictive performance, were presented to the PRP. However, the VMT failed to provide a clear mechanistic justification for the restrictions. Consequently, the PRP could not conclusively assess the predictive capacity of the SIRC-CVS Test Method.

Applicability Domain

Due to unsatisfactory predictive capacity with an unrestricted applicability domain, the VMT explored different potential reasons for misclassification, including chemical classes and physicochemical properties, but not *in vivo* drivers of classification as suggested by the PRP (Adriaens et al., 2014). The proposals presented to the PRP restricted the applicability, for example using a combination of molecular weight and chemical classes, the dissociation constant (pKa) and the distribution coefficient (log D). However, the VMT failed to provide a clear mechanistic justification for any of these. Consequently, the PRP concluded that the applicability domain of the SIRC-CVS Test Method was not sufficiently defined.

Performance Standards

Because the assay does not include components, equipment, or other scientific procedures that are covered by (or pending) intellectual property rights, the PRP initially agreed that performance standards are not mandatory at this stage, but could be useful if similar or modified test methods become available. However, due to the lack of a clearly justified applicability domain, performance standards are not required.

Additional Comments

The PRP concluded that the validation study management and conduct met the criteria set out in OECD GD 34 (2005). However, based on the information provided to the PRP, including a dedicated discussion at a PRP meeting, the PRP concluded also that it is unclear whether the study was conducted in accordance with the principles of GLP.

The PRP notes that during the conduct of the review, access to the full raw data files associated with SIRC-CVS Test Method validation work was provided.

Conclusions and Recommendations

The PRP concluded that, based on the provided information, the scientific validity of the SIRC-CVS Test Method could not be demonstrated for its use as a part of an integrated testing strategy for distinguishing chemicals classified from chemicals not classified according to the UN GHS classification system.

Acknowledgements

The PRP is grateful to the members of the VMT for their hard work and patience and to JaCVAM for their support in setting up and hosting meetings in Japan, as well as for the setting up of several telephone conferences.

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Annex: Short professional bios of the member of the peer review panel

Dr. Sebastian Hoffmann (PhD, ERT)
is running the independent consultancy 'seh consulting + services' in Paderborn, Germany, since 2009, specialised in validation and assessment of new approach methods (NAM) and test strategies as well as in the regulation of chemicals (REACH) and cosmetic ingredients. Being statistician by training, he received a Ph.D. from the University of Konstanz (Germany) in 2005 for his thesis 'Evidence-based *in vitro* toxicology'. He has being working for the European Centre for the Validation of Alternative Methods of the European Commission for five years, contributing inter alia to the management of various validation studies of NAM. His research activities focus on methodological challenges in the assessment of *in vitro* tests, the construction and evaluation of integrated testing strategies and in exploring the application of evidence-based approaches to toxicology. He is an appointed member of the European Commision's Scientific Committee on Occupational Exposure Limits and member of the Board of Trustees of the Evidence-based Toxicology Collaboration.

Dr. Chantra Eskes (PhD)
is director of the Swiss 3R Competence Centre (www.swiss3rcc.org). With a background in food sciences, *in vitro* neurotoxicity and topical toxicity, Chantra has over 20 years of experience in the development, optimization, validation, peer-review and regulatory acceptance of alternative methods to animal toxicity testing conducted in Europe and abroad. Her activities contributed to the formal validation and international acceptance of a number of test methods, and to the development of a number of international test guidelines and guidance documents. Chantra currently acts as the chairwoman of the EURL-ECVAM Scientific Advisory Committee (ESAC), and as a Swiss Nominated Expert for the Organisation for Economic Co-operation and Development (OECD). In the past she has been the president of the European Society of Toxicology In Vitro and Executive Secretary of the Animal Cell Technology Industrial Platform on the production of biopharmaceuticals (ACTIP).

Dr. Pertti Hakkinen (PhD, S-FRA)
is the Senior Toxicologist and the Toxicology and Environmental Health Science Advisor at the (U.S.) National Institutes of Health's National Library of Medicine. His job includes providing leadership in the development of new resources in exposure science, toxicology, risk assessment, and risk communication, and enhancements to existing resources in these fields. In addition, he is the project leader for the Chemical Hazards Emergency Medical Management (CHEMM) tool and the recently updated and enhanced ToxTutor educational resource. Further, he is an Adjunct Associate Professor in Preventive Medicine & Biostatistics in the F. Edward Hébert School of Medicine at the Uniformed Services University of the Health Sciences (USU) in Bethesda, and a co-leader of the Environmental Health Sciences graduate level course offered by the Foundation for Advanced Education in the Sciences at the NIH (FAES@NIH). Dr. Hakkinen earned a B.A. in Biochemistry and Molecular Biology from the University of California, Santa Barbara, and received a Ph.D. in Comparative Pharmacology and Toxicology from the University of California,

San Francisco. He is a member of the Society of Toxicology, a Councilor of the International Society of Exposure Science, and a Fellow of the Society for Risk Analysis. Recent publications include a) Exploring Global Exposure Factors: Resources for Use in Consumer Exposure Assessments and b) New Studies About Everyday Types of Chemical Exposures: What Readers Should Consider. He is a co-editor of an upcoming (mid-2019) book on the Practice of Consumer Exposure Assessment.

Prof. Tae Cheon Jeong (PhD)
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Dr. Jill C. Merrill (PhD, DABT)
is a senior reviewer for the Division of Dermatology and Dental Products, Center for Drug Evaluation and Research (CDER), USFDA. She received her doctorate in toxicology from Texas A&M University and was a postdoctoral fellow at the University of Texas Southwestern Medical Center and at Monsanto Co. In 1990 she joined the Gillette Medical Evaluation Laboratories as a product safety toxicologist. Under Gillette's no animal testing policy she coordinated the company's first use of the BCOP assay to further the development of innovative products otherwise sidelined by ocular irritation concerns. Subsequently she served as Study Director (2000-2003) at the Institute for In Vitro Sciences, Inc., specializing in alternative ocular and dermal irritation assays conducted under contract for pharmaceutical and personal care companies. Since 2003 she has evaluated the significance of results from both nonclinical and alternative toxicology studies conducted in support of dermal drug products prior to first-in-human studies. Dr. Merrill proactively supports CDER's use of alternative test methods.

Sanae Takeuchi (MSc)
joined Procter & Gamble Far East Inc. (current: P&G Innovation Godo Kaisha) in 1992 after getting her master's degree of science in biology from Ochanomizu University in Tokyo. After working in Clinical Development department for years, she has been working for Central Product Safety function in Global Product Stewardship, since 2001, supporting Beauty Care sector.
From 2008, she has been working with JaCVAM (Japanese Center for the Validation of Alternative Methods) as a member of Editorial Committee of alternatives for ocular irritation testing. She is a member of International Committee and Council for The Japanese Society for Alternatives to Animal Experiments, and also a member of the Japan SOT.

SIRC-CVS:TEA の予測性一覧

表 1. Overall analysis by the judgment based on IC_{50} value of Triethanolamine (TEA) in UN GHS classification system in a bottom-up approach in the validation study

Regulatory System	Analysis
Accuracy	55.2% (64/116)
Sensitivity	60.0% (42/70)
Specificity	47.8% (22/46)
False Negative Rate	40.0% (28/70)
False Positive Rate	52.2% (24/46)

表 2. Analysis after cut Molecular weight <180 for alcohol, ester, ether , ketone heterocyclic compound and carboxylic acid , and purity ≧80% (GHS, Bottom-up, TEA) in the validation study.

Regulatory System	Analysis in applicability domain
Accuracy	64.9% (37/57)
Sensitivity	92.3% (24/26)
Specificity	41.9% (13/31)
False Negative Rate	7.6% (2/26)
False Positive Rate	58.1% (18/31)

表 3. Analysis after cut LogP (-1.5<log D<2) and pKa 4 or less (GHS, Bottom-up, TEA) in the validation study(see appendix 15).

Regulatory System	Analysis in applicability domain
Accuracy	57.7% (30/52)
Sensitivity	93.8% (15/16)
Specificity	41.7% (15/36)
False Negative Rate	6.3% (1/16)
False Positive Rate	58.3% (21/36)

表 4. Overall analysis by the judgment based on IC_{50} value of Triethanolamine (TEA) in UN GHS classification system in a bottom-up approach in the Shiseido in house 46 data (see appendix 16) and validated data

Regulatory System	Analysis
Accuracy	57.4% (93/162)
Sensitivity	64.8% (59/91)
Specificity	47.9% (34/71)
False Negative Rate	35.2% (32/91)
False Positive Rate	52.1% (37/71)

表 5. Analysis after cut Molecular weight <180 for alcohol, ester, ether , ketone heterocyclic compound and carboxylic acid , and purity ≧80% (GHS, Bottom-up, TEA) in the Shiseido in house 22 data (see appendix 11) and validated data

Regulatory System	Analysis in applicability domain
Accuracy	64.6% (51/79)
Sensitivity	94.6% (35/37)
Specificity	38.1% (16/42)
False Negative Rate	5.4% (2/37)
False Positive Rate	61.9% (26/42)

表 6. Analysis after cut LogP (-1.5<log D<2) and pKa 4 or less (GHS, Bottom-up, TEA) in the validation study(see appendix 15) (GHS, Bottom-up, TEA) in the Shiseido in house 31 data (see appendix 16) and validated data

Regulatory System	Analysis in applicability domain
Accuracy	65.1% (41/63)
Sensitivity	96.3% (26/27)
Specificity	42.9% (24/56)
False Negative Rate	3.7% (1/27)
False Positive Rate	57.1% (32/56)

表 7. Overall analysis by the judgment based on IC_{50} value of Triethanolamine (TEA) in UN GHS classification system in a bottom-up approach in the validated data based on Barroso' data (see appendix 14)

Regulatory System	Analysis
Accuracy	50.0% (49/98)
Sensitivity	52.9% (27/51)
Specificity	46.8% (22/47)
False Negative Rate	47.1% (24/51)
False Positive Rate	53.2% (25/47)

表 8. Analysis after cut Molecular weight <180 for alcohol, ester, ether , ketone heterocyclic compound and carboxylic acid , and purity ≧80% (GHS, Bottom-up, TEA) in the validated data based on Barroso' data (see appendix 14)

Regulatory System	Analysis in applicability domain
Accuracy	56.5% (26/46)
Sensitivity	87.5% (14/16)
Specificity	40.0% (12/30)
False Negative Rate	12.5% (2/16)
False Positive Rate	60.0% (18/30)

Validation Study for the Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining Cytotoxicity Test Method with Triethanolamine (SIRC-CVS:TEA Test Method)

as an Alternative to Eye Irritation Test Draft）

Study Report

Version 9.0

February 20, 2019

SIRC-CVS:TEA Validation Management Team (VMT)

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Abbreviations

ATCC	American Type Culture Collection
BCOP	Bovine corneal opacity and permeability
CVS	Cell-Crystal Violet Staining
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
IC50	50% of Inhibitory concentration
GHS	Globally Harmonized Systems of Classification and Labeling
GLP	Good Laboratory Practice
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	Isolated Chicken Eye
JaCVAM	Japanese Centre for the Validation of Alternative Methods
MEM	Eagle's Minimal Essential Medium
MAS	Maximal Average Draize Total Score
MHW	Ministry of Health and Welfare
MW	molecular weight
NI	Non-irritant
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co-operation and Development
SIRC	Statens Seruminstitut Rabbit Cornea
SOP	Standard Operating Procedure
TEA	Triethanolamine
TG	Test guideline
UN	United Nations
VMT	Validation Management Team

1 Abstract

The Statens Seruminstitut Rabbit Cornea-Cell-Crystal Violet Staining (SIRC-CVS) test method was developed as a simplified alternative to the Draize rabbit eye test for use in screening test chemicals used as ingredients in cosmetics and quasi-drugs for ocular irritation (Itagaki, 1991). The SIRC-CVS test method was validated in the 1990s under the Ministry of Health and Welfare (MHW) Project on alternatives to the Draize test (Itagaki, 1995; Ohno, 1999; Ohno, 2004; Tani, 1999), and a modified version of the SIRC-CVS test method that uses triethanlamine (TEA) as a relative control has been developed by Hagino (See Appendix 1). This validation study was implemented at three participating laboratories in accordance with the spirit of GLP to validate the SIRC-CVS:TEA test method for intra- and inter-laboratory reproducibility as well as usefulness in distinguishing non-irritants from irritants in a bottom up approach (Scot, 2010).

The SIRC-CVS:TEA test method assesses cytotoxicity by exposing SIRC cells to a test chemical for 72 hours, then staining the exposed SIRC cells with crystal violet in order to measure their viability. These results are then used to calculate an IC_{50} value for the test chemical, and if this value is smaller than the IC_{50} value of triethanlamine, the test chemical is predicted to be an irritant. The test chemicals were selected to provide a balanced representation of United Nations (UN) Globally Harmonized Systems of Classification and Labeling (GHS) categories and were coded prior to distribution to the participating laboratories.

In Phase I of the validation study, VMT assessed transferability of the test method using four test chemicals. In Phase II, we assessed intra-laboratory reproducibility using twenty test chemicals. During Phases II and III, we assessed inter-laboratory reproducibility using thirty common test chemicals at the three participating laboratories. Also during Phases II and III, we assessed predictive capacity using 117 test chemicals.

These results demonstrated that the test method:

1. Was easily transferable to technically proficient laboratory technicians,
2. Has excellent intra-laboratory reproducibility (100%, 20/20) and inter-laboratory reproducibility (90%, 27/30),
3. Has a low predictive capacity for distinguishing non-irritants from irritants per UN GHS categories in a bottom-up approach,

Even after considerable review of the test data, the VMT was unable to identify a scientifically valid applicability domain that would provide a high predictive capacity. We therefore concluded that the SIRC-CVS:TEA test method has excellent intra- and inter-laboratory reliability, but were unable to reach a consensus as to whether or not this test method was useful as an alternative to the Draize test for distinguishing ocular non-irritants from irritants.

2 Introduction

Assessing the ocular toxicity of test chemicals used as cosmetic ingredients is an essential part of product development. The Draize eye irritation test has been commonly used for more than 50 years to assess rabbit eyes for in vivo ocular damage caused by exposure to test chemicals (Draize, 1959). At present,

however, modern views of animal welfare and regulation of the drug industry have made in vitro test methods to replace the Draize test highly desirable. In fact, a variety of in vitro eye irritation test methods have been developed and validated. In September 2009, the bovine corneal opacity and permeability (BCOP) test and the isolated chicken eye (ICE) test were adopted as Test Guidelines 437 and 438, respectively, by the Organization for Economic Cooperation and Development (OECD) for assessing test chemicals for severe eye irritation potential. Both of these test guidelines were later revised and adopted by the OECD in July 2013 for assessing non-irritants and severe eye irritants. Also, the fluorescein leakage test was adopted in October 2012 as Test Guideline 460 for assessing severe eye irritation potency. And, in July 2015, the OECD adopted two test methods for assessing non-irritants using corneal cells: the Short Time Exposure (STE) In Vitro Test Method as Test Guideline 491 and the Reconstructed human Cornea-like Epithelium (RhCE) test method as Test Guideline 492.

The SIRC-CVS test was designed as a cytotoxicity test using an established SIRC cell line derived from the corneas of rabbit eyes. Corneal cells are considered suitable for use in in vitro alternatives to in vivo eye irritation tests, although Ohno et al. (1999) reported that the differences between cell types and endpoints found in previous Japanese validation studies were small. SIRC cells are easily cultured and are used in cytotoxicity tests such as the STE test method (TG 491).

The SIRC-CVS method had previously been considered for use as an alternative to the Draize test. Itagaki et al. (1991) assessed the eye irritation potential of twelve surfactants using the SIRC-CVS test method and reported in vitro results that correlated well with in vivo results, thereby suggesting the SIRC-CVS test method is useful for assessing the eye irritation potency of various substances. Cytotoxicity is considered a useful index of the eye irritation potency of various substances, as the corneal damage that has a greater impact on the total eye irritation is related to damage of the corneal epithelium cell (Jester 2001). Cytotoxicity tests are reported to be useful for identifying ocular non-irritants that have almost no effect on the cornea. An analysis of in vivo data from previous studies (Ohno et al., 1999) showed that maximal eye irritation generally occurs within 72 hours of ocular instillation, which is the rationale for the 72-hour exposure time. Also, as a practical matter, volatile substances generally have a shorter application time than non-volatile substances, since the former are eliminated from the eye fairly rapidly by volatilization. Therefore, a 72-hour period of exposure for SIRC-CVS test method is safer and easier to schedule.

The SIRC-CVS:TEA test assesses cytotoxicity by exposing SIRC cells to a test chemical, then staining the exposed SIRC cells with crystal violet in order to measure their viability. The crystal violet penetrates via a cell membrane treated with methanol and stains biological macromolecules. The crystal violet staining method is suitable for a variety of cultured cells and produces highly consistent results (Saotome, 1989). Not only is the SIRC-CVS:TEA test procedure simple and easy to perform, but the tested microplate can be stored and use to verify test results at any time. In this respect, it is unique among cytotoxicity tests and also is less expensive than 3D culture models or isolated tissue. This staining method has no interference by reduction action of test substances such as interaction with 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

On the other hand, a disadvantage of this method is that test chemicals must be dissolved or uniformly suspended in a liquid medium. As the SIRC-CVS test method can detect only cytotoxicity, it cannot detect loss of transepithelial impermeability due to damaged tight junctions and desmosomal junctions, as the

Fluorescein leakage test (TG460) can. The SIRC-CVS test method detects cytotoxicity and therefore cannot predict the reversibility of eye irritation.

A three-phase validation study of the SIRC-CVS test method was planned and performed with the support of the MHW research project, entitled Studies on the Test Methods to Evaluate the Safety of New Ingredients of Cosmetics, which was carried out by six independent laboratories from 1991 to 1999 (Ohno et al., 1999). During the first phase of the study, assessment of nine surfactants and saline indicated good intra- and inter-laboratory reproducibility as well as good correlation between in vitro and in vivo tests results (Itagaki et al., 1995). Also, during a review of the data from all three phases of the study, a strong correlation ($r = -0.805$) between in vitro (cell viability measured as IC_{50}) and in vivo (MAS) was found for twenty-nine chemical substances (Tani et al., 1999). After the validation study, the SIRC-CVS test method was modified for use in distinguishing ocular non-irritants from those which are irritants, and polyoxyethylene sorbitan monolaurate (20E.O.) was chosen as a non-irritant reference substance at a concentration of 10% (Ohno, 2004). The use of a relative control is useful in obtaining consistent results (Ohno, 1999, 2004). This is because, even though the slightest variance in serum lot or other aspects of the culture medium can affect the absolute value of IC_{50}, the use of a relative control ensures that the relative ranking of the test chemicals remains consistent. This is one conclusion drawn from the previous MHW research project.

Data from the Japanese validation as reported by Tani et al. (1999) and the study reported by Hagino et al. (Appendix 1) were reanalyzed using a cut-off value for triethanolamine (TEA) as a reference in evaluating undiluted test chemicals. A Japanese Centre for the Validation of Alternative Methods (JaCVAM) peer review of the SIRC-CVS test method based on this data, which was obtained between 2009 and 2011, concluded that this test method was useful in identifying non-irritants, but that a validation using the modified SIRC-CVS:TEA test protocol was necessary.

The purpose of this study is to validate intra- and inter-laboratory reproducibility as well as the predictive capacity of the SIRC-CVS:TEA cytotoxicity test method. As a specific goal, this validation study was designed to clarify whether or not the SIRC-CVS:TEA cytotoxicity test method is a useful alternative to the Draize test method in a bottom-up approach for distinguishing chemical substances and which are ocular non-irritants from those which are irritants under the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS). To this end, we planned a validation of the proposed SIRC-CVS:TEA cytotoxicity test protocol to be performed in three phases and using a sufficient number of coded test chemicals for three laboratories to assess eye irritation potency.

3 Methods

3.1 Study Plan

3.1.1 Purpose

This validation study was designed in three phases to assess the transferability, intra- and inter-laboratory reproducibility, and predictive capacity of a proposed SIRC-CVS:TEA test protocol. More specifically, it was designed to demonstrate that the proposed SIRC-CVS:TEA cytotoxicity test method is a useful in vitro alternative to the in vivo Draize test method for identifying non-irritants under the GHS. These study plans were organized and approved by the members of the Validation Management Team (VMT) and the participating laboratories.

3.1.2 Organization

The validation study was organized as shown in Fig. 1 to assure scientific pertinence and smooth implementation.

The SIRC-CVS:TEA VMT comprises a chairperson, members of the chemical management group, the data analysis group, the record management group, and a representative of test development lead laboratory. Support to participating laboratories was provided by the lead laboratory. A representative of ICCVAM acted as a liaison to the VMT and the representatives of the participating laboratories were observers. The VMT prepared, reviewed, and finalized all draft study plans and protocols. In addition, the VMT management of the validation study included following its progress, assuring the quality of its records, contacting and coordinating between participants, and handling other administrative duties as necessary. Table 1 shows the organization of the VMT.

3.1.2.1 Chairperson

A chairperson elected by vote of the VMT members was responsible for preparing draft study plans, the study protocol, and the test chemical list as well as for convening ad hoc VMT meetings for review and finalization of such documentation. The chairperson was also responsible for other administrative duties related to the validation study.

3.1.2.2 Chemical management group

The chemical management group comprised two members selected from the VMT and was responsible for preparing list of test chemicals as well as conferring with the chairperson to finalize the test chemicals used in the validation study. It also prepared and distributed lists of non-coded or coded test chemicals by chemical distributors.

3.1.2 3 Data analysis group

The data analysis group comprised one member selected from the VMT and was responsible for providing objective analysis of data obtained in this validation study from a third-party standpoint as well as for statistical processing of data.

3.1.2.4 Record management group

The record management group comprised the lead laboratory plus one member selected from the VMT and was responsible for preparing the protocol, test chemical preparation sheets, blank data sheets, and other necessary materials as well as for distributing these materials to the participating laboratories. It also collected the completed forms and data sheets, reviewed the records for errors and omissions, and requested correction as necessary.

3.1.2.5 Research laboratories

The following three laboratories participated in the assessment of test chemicals using the SIRC-CVS:TEA test method.

Lab A: Nihon Kolmar Co., Ltd, Osaka, Japan

Lab B: Bozo Research Center Inc., Tokyo, Japan

Lab C: Biotoxtech Co., Ltd, Seoul, Korea

One study director from each participating laboratory was also an observer to the VMT and was responsible for carrying out testing according to the study protocol as well as for filling out and submitting all necessary records and forms upon completion of testing.

3.1.3 Study design

Validation of the SIRC-CVS:TEA test method was carried out in three phases, as detailed in Appendix 2.

3.1.3.1 Training of participating personnel

A technical transfer workshop focusing on the principles of and protocol for the SIRC-CVS test method was held on Thursday, Nov. 11, 2011, with personnel from all three laboratories in attendance. Instructors from the lead laboratory explained the test method by video presentation. DVD was provided to all three laboratories after the workshop. Although these laboratories were all naïve of the SIRC-CVS, they were experienced in culturing cells. No practical training was provided.

3.1.3.2 Phase I

Phase I was designed to assess transferability using four non-coded test chemicals per Study Plan version 1.1. Each test chemical was predicted to be either positive or negative based on obtaining consistent results in a set comprising three separate runs.

The terms set and run are used per the following definitions:

Run: A run consists of one test chemical tested concurrently with a negative, a relative and a positive control. A run is considered qualified if it meets test acceptance criteria, as defined in the corresponding test protocol. Data from non-qualified runs are not included in sets.

Set: A test sequence containing at least two qualified runs.

3.1.3.3 Phase II

Phase II was designed to assess intra- and inter-laboratory reproducibility using twenty coded substances per Study Plan IIA version 1.51, and Study Plan IIB version 1.53, but was split into two parts: Phases IIA and IIB.

Phase IIA was designed to assess the intra- and inter-laboratory reproducibility of five test chemicals, after which Phase IIB was designed to validate an additional fifteen test chemicals. Each test chemical was predicted to be either positive or negative based on three runs per set for each of three sets.

3.1.3.4 Phase III

Phase III was designed to assess the inter-laboratory reproducibility and predictive capacity of the SIRC-CVS:TEA test method for one hundred coded test chemicals. Each laboratory tested one common set of ten test chemicals and one unique set of 30 test chemicals, as shown in Table 2, per Study Plan version 1.56. Each test chemical was predicted to be either positive or negative based on two runs. When the results of the first and second runs were consistent, a prediction was made without performing a third run. If the results of the two runs are different, a third run is performed and the data of the two runs with the same result are adopted for the prediction.

3.1.3.5 Test chemicals

The test chemicals were selected to ensure that a variety of substances were represented, including various eye-irritant levels per GHS categories, physical states, chemical classes, and eye lesions produced. Preference was given to substances for which high-quality in vivo data, especially data including results

from individual animals, was available, such as substances listed in ICCVAM or EURL ECVAM Eye Irritation Validation Studies. All selected test chemicals are available commercially.

A total of more than one hundred test chemicals were used in this validation study. These substances were selected by the chemical management group and approved by the VMT. All test chemicals used in Phases II and III were coded, and their names were revealed only after completion of the study. During Phase III, each of the three laboratories tested a total of forty test chemicals, ten of which were tested in common by all three laboratories, as shown in Table 3.

3.1.3.6 Study duration

Testing was performed from September 2011 until September 2013

Phase I , from September 2011 to March 2012 (protocol ver. 1.71E)

Phase IIA , from March 2012 to September 2012 (protocol ver. 2.12E)

Phase IIB , September 2012 to March 2013 (protocol ver. 2.12E)

Phase III, March 2013 to September 2013 (protocol ver.2.13E)

3.1.4 Success criteria

Success criteria for intra- and inter-laboratory reproducibility was 80%, for accuracy was 80%, and for false negatives was less than 5%, as determined by the VMT prior to testing. Other acceptance criteria for the test protocol are described in section 3.2.9 Quality Control. The data file used at the participating laboratories was developed by the data analysis group, and entering data from test results automatically calculates values for IC_{50} using a dose-response plot in combination with several other quality control criteria, as described in protocol Ver. 3.8 (Appendix 3).

3.2 Summary of protocol

An overview of the SIRC-CVS test method is shown in Fig. 2. The procedures are described in greater detail below.

3.2.1 Cells

The Statens Seruminstitut rabbit corneal cell used in this test is derived from rabbit corneas and obtained from the American Type Culture Collection (ATCC No. CCL-60). The cells can be frozen and stored in liquid nitrogen. Prior to performing the test, the cells should be check to ensure the absence of mycoplasma using a test such as the Venor GeM Mycoplasma Detection Kit (Minerva Biolabs GmbH, 11-1025). The cells are to undergo no more than 35 passages from their purchased stock. (e.g., if the cell culture starts at passage number 435 and is passaged every four days, it should be disposed of after passage number 470.) Quality control is to be performed as described in section 4.7 of the test protocol. The SIRC cells are cultured in MEM supplemented with 10% FBS and 1% P/S/F at 37°C in a humidified incubator at 5% CO_2 in air. The concentrations of the antibiotics are 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 250 ng/mL of Amphotericin B.

3.2.2 Determining solubility or suspensibility of test chemicals in the Medium

First, determine whether the test chemical can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 µg/mL (1% w/v). Use a vortex mixer, water bath, or sonicator as necessary. If the test chemical cannot be dissolved or uniformly suspended in the Medium, the next step is to determine whether the test chemical is more easily dissolved in DMSO or ethanol. Next, dissolve or uniformly

suspend the test substance in the more suitable solvent at a concentration of 10,000 µg/mL and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 µg/mL. If not, dissolve or uniformly suspend the test substance in the more suitable solvent at a concentration of 5,000 µg/mL (0.5% w/v) and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 µg/mL. If not, the test substance is considered to be outside the applicability domain of the test. These judgments can all be performed by visually confirming the absence or presence of precipitate in the solution.

3.2.3 Preparing test chemicals

Determine an appropriate concentration for each test chemical per the procedure described in section 3.2.2. When the maximal concentration of a stock test chemical dilution series is 10,000 µg/mL, once the test chemical dilution series in the microplate is mixed with the Medium containing the SIRC cells, the final maximal concentration is halved to 5,000 µg/mL (0.5% w/v). When either DMSO or ethanol is used as a solvent, the final maximal concentration is 5,000 µg/mL (0.5% w/v).

When the maximal concentration of a stock test chemical dilution series is 5,000 µg/mL, the final maximal concentration in the microplate is 2,500 µg/mL (0.25 w/v%) for the test chemical dilution series and 5,000 µg/mL (0.5% w/v) for the solvents. If precipitation is observed in a well at any time after mixing the test chemical solution and the cells, especially after the 72-hr incubation period, the test data must be rejected.

3.2.4 Preparing a cell suspension

1. Remove the Medium from the culture flask, then rinse the SIRC cells twice with 10 mL of modified PBS to remove the serum, which is a trypsin inhibiter.
2. Remove the modified PBS, then add and ensure that all the cells in the culture flask are exposed to 1.5 to 2.0 mL of 0.25% trypsin solution.
3. Remove the 0.25% trypsin solution, then incubate the cells as is for two or three minutes at 37°C.
4. Detach the cells from the inside surface of the flask by tapping.
5. Collect the cells in an appropriate volume of MEM (10% FBS) with a pipette.
6. Count the cells and prepare a cell suspension at a density of 2×10^5 cells/mL.

3.2.5 Application of the test chemical

1. Prepare 100 µL of modified PBS and the negative control as well as 100 µL of the serial dilutions of the test chemical, positive control, and relative control in a 96 well microplate, as shown in Fig. 4.1.
2. Add 100 µL of the 2 × 105 cells/mL cell suspension to the wells, as shown in Fig. 4.2.
3. Seal the microplate to prevent contamination from volatile test chemicals. Wrapping film may be used for this purpose. The six measurements described in steps (1)–(6) of protocol section 4.6 Quality Control are to be used to verify that there is no contamination of other wells by volatile test chemicals. The criterion for toxic effect is the same as that for quality control. If contamination is found, the test is to be redone at a lower concentration.
4. After mixing the test chemical and the cell suspension, allow to stand for 20 minutes on a clean bench. Once the cells adhere to the bottom of the wells, the microplate is moved to the incubator.

5. Incubate for about 72 hours at 37°C and 5% CO2 in air.

3.2.6 Crystal violet staining

1. After incubation, remove the Medium containing the test chemicals by gently but quickly turning the microplate upside down.
2. Add 200 µL of modified PBS and shake gently to rinse the cells, then remove the modified PBS by gently turning the microplate upside down. Perform this procedure twice.
3. Add 100 µL of crystal violet methanol solution to each well and allow to stand for 30 minutes.
4. After the staining, remove the crystal violet methanol solution by gently but quickly turning the microplate upside down. Wash the cells thoroughly with tap water and blotted away any residual water with a paper towel.
5. After drying, measure the optical absorbance at 588 nm with an automatic microplate reader. Any nearby wavelength for which equivalency can be demonstrated is suitable for measurements.

3.2.7 Calculating IC_{50}

Absorbance in the negative control wells, which contain no test chemical, minus the absorbance of the blank is considered to be 100%, and the percentage of absorbance for the mean of two wells is calculated on this basis. Cell viability is a percentage calculated by dividing the mean absorbance of two wells at the same concentration minus the absorbance of a blank well by the mean absorbance of all negative control wells minus the absorbance of a blank well.

IC50 is the concentration at which the growth of cells was inhibited to 50% of the control and calculated as follows using two concentrations around the predicted concentration of 50% cell viability.

$$\text{Log IC50} = [(50 - y1)\log x2 - (50 - y2)\log x1]/(y2 - y1),$$

where x1 is low concentration, x2 is high concentration, y1 is cell viability at low concentration, y2 is cell viability at high concentration, and log means the common logarithm.

If cell viability is greater than 50% at maximal concentration of 5,000 µg/mL, the result for that test chemical is IC_{50} > 5,000 µg/mL. Also, if the cell viability is less than 50% at a minimal concentration of 39.1 µg/mL, the result for that test chemical is IC_{50} < 39.1 µg/mL. IC_{50} at other maximal and minimal concentrations of test chemicals are expressed in the same manner.

If multiple concentrations of a test chemical yield a 50% cell viability, use the lowest value of IC_{50}.

In the Excel spreadsheet (Appendix 4), cell viability is rounded to the nearest tenth.

3.2.8 Quality control

Quality control of the SIRC cytotoxicity test is performed by taking six measurements, as shown in Appendix 5 Rational for Quality Control Ranges, which must satisfy the following criteria. Failure to satisfy the criteria means that the test substance must be retested. In particular, if a volatile test chemical fails to satisfy the criteria, it must be retested at a lower concentration.

The Excel spreadsheet automatically displays the results of the measurements when data is input. Any test that does not satisfy the quality control criteria must be redone.

1. The absolute OD obtained from the negative control is an index of the normal proliferation of SIRC cells seeded at a concentration of 2×10^4 cells/well and incubated for 72 hours. The mean OD of the negative control (right and left wells) must be greater than 0.4 for the test data to be considered valid.

2. Sodium dodecyl sulfate (SDS) is used as a positive control. The IC_{50} of SDS should be between 77.7 and 258.7 μg/mL when tested using the standard protocol. This criterion must be satisfied for the test data to be considered valid.

3. Triethanolamine is used as a relative control. The IC_{50} of triethanolamine should be between 1,000 and 2,500 μg/mL when tested using the standard protocol. This criterion must be satisfied for the test data to be considered valid.

4. Any discrepancy between the two dilution series of the test chemical is to be reviewed. The IC_{50} of both the first series and the second series must be within 20% of the mean IC_{50} of the two dilution series together. This criterion must be satisfied for the test data to be considered valid. The minimum value for IC50 is 39.1 μg/mL and the maximum value is 5000 μg/mL. IC_{50} at other maximal and minimal concentrations of test chemicals are expressed in the same manner. These values of IC_{50} are only used for quality control calculations.

5. The difference between left and right wells of the negative control should be reviewed to confirm systematic quality. The mean OD of the left side and the mean OD of the right side should be within 15% of the mean OD of both sides combined. This criterion must be satisfied for the test data to be considered valid.

6. The two test results adopted for making a prediction must be checked for equality. The higher of the two IC50 values of the two positive controls (SDS) must be no more than twice as large as the lower of the two values. (The higher value ÷ the lower value $\leqq$ 2)

During the validation study, all data was checked against these criteria using the format shown in Appendix 4.

3.2.9 Evaluation

Eye irritation potency of the test chemical is predicted using triethanolamine as a relative control. Triethanolamine is classified No Category under GHS, and using this as a reference, a test chemical is identified as negative (No Category) when the IC50 is higher than or equal to that of triethanolamine and is identified as positive (Category 1 or 2) when the IC50 is lower than that of triethanolamine. The test is performed twice. If the results of the two test runs are different, a third run is performed and the data of the two runs with the same result are used to make the prediction. If discrepancies between the three runs must be reviewed, the test is repeated three times.

3.3 Test chemicals

3.3.1 Selection of test chemicals for the Phases I, II, and III

3.3.1.1 Test chemicals for Phase I

Transferability of the SIRC-CVS:TEA test was confirmed at the three participating laboratories using sodium dodecyl sulfate as a positive control, TEA as a relative control, and four un-coded test chemicals. The four un-coded substances were ethyl-2-methyl acetoacetate (water soluble), safflower oil (oil soluble),

3-chloropropionitrile (highly volatile and cytotoxic), and sodium dehydroacetate (cytotoxic), as shown in Table 4. One run was performed for each test chemical and the results from the three participating laboratories were then compared with data from the lead laboratory.

3.3.1.2 Test chemicals for Phase II

For Phase II, the chemical management group and the VMT selected 20 substances which had previously been assessed using the Draize eye test and classified under GHS, as shown in Table 5. The test chemicals were coded prior to distribution to the three participating laboratories, as shown in Appendix 6.

3.3.1.3 Test chemicals for Phase III

For the Phase III, the chemical management group and VMT selected 100 substances, as shown in Table 6. Each of the three participating laboratories were allocated a set of 10 common test chemicals and a set of 30 unique test chemicals, as shown in Table 2. One of these, 3,3-dithiodipropionic acid, was duplicated in distribution, so one entry was eliminated from the list. The test chemicals were coded prior to distribution to the three participating laboratories, as shown in Appendix 6.

3.3.2 Test chemicals selected for the validation study

The participating laboratories participated in VMT meetings as observers but did not take part in discussions related to selection of test chemicals. The 120 test chemicals listed in Tables 2-2 and 2-3 of Appendix 6 were selected for use in this validation study. As mentioned above, a duplication of 3,3-dithiodipropionic acid was excluded from the results. Furthermore, citric acid (P3-067) and potassium sorbate (P3-068) we also excluded from the results, since they lacked individual animal data from a clear source. Thus, a total of 117 test chemicals with individual animal data were used to evaluate intra- and inter-laboratory reproducibility. The physical state, chemical class, and classification per both GHS and EPA for each of the 117 test chemicals is shown in Table 4 of Appendix 6.

The VMT considers the selected test chemicals to cover a wide variety of physiochemical properties as well as the full range of ocular irritation potency represented in GHS categories. Selection was made from a broad range of chemical classes, and existing data was obtained for many different substances, including cosmetic ingredients.

Ultimately, the final analysis was based on 116 test chemicals, since P3-066 (calcium thioglycolate trihydrate) was excluded due to an inability to form a uniform suspension, as shown in Fig. 6.

3.3.3 Purchase, coding, and distribution of test chemicals

All of the test chemicals used in Phases I, II, and III were obtained from commercial sources, as shown in Table 4 of Appendix 6. Test chemicals used in the Phases II and III were coded and distributed to the participating laboratories by JaCVAM.

3.4 Quality assurance

The participating laboratories conducted all tests in accordance with the spirit of Good Laboratory Practice (GLP, OECD 1999) and submitted the test results to the VMT, which documented and discussed the test results. Preparation of test chemicals was recorded using a format developed for this validation by the lead laboratory. Researchers in participating laboratories recorded information such as the code name of each test chemical, solvent name, and date of the preparation, solubility or suspensibility, and concentration of the sample solution. These records were sent from the participating laboratories to JaCVAM, where their validity and accuracy were checked. These records are maintained by JaCVAM.

3.5 Record collection and analysis

Data collection and analysis were performed in close collaboration with biostatisticians. The data sheets used by the participating laboratories were developed by the lead laboratory and modified for use in this validation by the data analysis group to calculate the value of IC_{50} using a dose-response plot and quality control criteria. The data was decoded and analyzed statistically. The data management procedures and the statistical tools were approved by the chairperson and the data analysis group. Any deviations found in the analysis were documented and their impact on study results discussed by the VMT. The eye irritation potency of the test chemicals was evaluated using TEA as a relative control in accordance with the test protocol. Test results were evaluated against with GHS classification based on an analysis of specific IC_{50} criteria.

Predictive capacity of the SIRC-CVS:TEA test method was evaluated using data from Phases II and III, starting with an analysis to assess predictive capacity using TEA IC_{50} as a reference to determine GHS classification in a bottom-up approach.

4. Results

4.1 Data quality

All data sheets were analyzed by biostatisticians is shown in Appendix 7. Error found during quality checks are shown in Tables 7.1 and 7.2. The Quality Assurance group reviewed the records to assure that all tests were performed in the spirit of GLP.

4.1.1 Phase I

Phase I was designed to assess transferability and intra-laboratory reproducibility of the SIRC-CVS:TEA test method. The four non-coded substances selected for the Phase I were ethyl-2-methyl acetoacetate (water soluble), safflower oil (oil soluble), 3-chloropropionitrile (highly volatile and cytotoxic), and sodium dehydroacetate (cytotoxic). JaCVAM provided test chemicals to the three participating laboratories. Import/export restrictions prevented JaCVAM from supplying either TEA or bovine fetal serum to Biotoxtech Co., Ltd (Lab C), so these two substances were obtained from a local supplier in Korea. Since it was not possible for all three participating laboratories to use reagents from a single manufacturing lot, the VMT decided to assess only transferability during Phase I.

Testing during Phase I comprised three runs of four test chemicals, however there was a lack of awareness on the part of all three participating laboratories as to the need to perform testing in the spirit of GLP. Lab A submitted all data sheets and records for Phase I. Lab B submitted all records but only a portion of the data sheets. Therefore, they did not provide enough data to meet quality control criteria. Lab C submitted all data sheets but none of the records. Thus, after Phase I, quality criteria for the negative, positive, and reference controls was developed.

The means and standard deviations of IC_{50} for the relative and positive control at all three participating laboratories are shown in Table 8.1. The mean and standard deviation of IC_{50} for the relative control was 1898.1 ±350.3 at Lab A, 1529.3 ±132.7 at Lab B, and 1382.8 ±33.3 at Lab C. The mean and standard deviation of IC_{50} for the positive control was 170.9 ±7.4 at Lab A, 87.0 ±1.7 at Lab B, and 82.0 ±3.6 at Lab C.

Discrepancies in the test results led the VMT to direct Lab A to repeat the tests for all four test chemicals in Tables 9.1. The classification of sodium dehydroacetate at Lab A differed from that at the other two

621 labs as well as from that at the lead lab. Investigation revealed that the cause was likely improper dilution
622 of the test chemical, which prompted Lab A to offer to redo all Phase I testing, and the VMT accepted this
623 offer. The results of the retest were not only more consistent, they also matched the classifications obtained
624 by Lab B, Lab C, and the lead lab.

625 As a result of retesting, the standard deviations was between 33.3 and 132.7 for the relative controls and
626 between 1.5 and 3.6 for the positive control. The coefficient of variation was between 2.4% and 8.7% and
627 between 1.8% and 4.3% , indicating a small variation.

628 ### 4.1.2 Phase II

629 Phase II was divided into two parts and carried out using twenty coded test chemicals: five test chemicals
630 in Phase IIA and fifteen in Phase IIB. After obtaining permission to ship TEA to Korea from the Chemical
631 Weapon and Drug Materials Control Policy Office of the Japanese Ministry of Economy, Trade and
632 Industry, JaCVAM procured and shipped twenty coded test chemicals as well as TEA to all three
633 participating laboratories. Bovine fetal serum from a single lot was procured from Gibco International Co.
634 Ltd in the USA, which shipped directly to Lab C in Korea and to JaCVAM in Japan. JaCVAM then shipped
635 to Bozo Research Center and Nihon Kolmar in Japan.

636 JaCVAM received a report on Jan. 10, 2012, from Lab C, stating that test chemical P2-007 (1-
637 Bromohexane) had leaked from its container, so a new shipment was sent. There were no other problems
638 found with the containers.

639 Also, JaCVAM received a report that the test chemical supervisor at both Lab B and Lab C had
640 inadvertently opened the MSDS. This report included a signed affidavit that the content was kept secret
641 from the test technicians. JaCVAM instructed all three participating laboratories not to open the MSDS
642 during Phase III or later testing.

643 Phase II comprised three runs per set for each of three sets of test chemicals. Two of the participating
644 laboratories were able to perform the SIRC-CVS:TEA test in conformance with the six quality control
645 criteria stipulated in section 3.2.9. Lab A, however, had a total of 6 deviations from the criteria, as shown
646 in Table 7.1 and 7.2. All deviations were retested and the data were accepted for Phase II.

647 Lab A submitted all data sheets and records for Phase II. Lab B submitted all data sheets and records for
648 the testing of the test chemicals but failed to submit data sheets for preliminary set up, such as establishing
649 solvents and concentrations. Lab C submitted all data sheets and records for the testing of the test
650 chemicals but failed to submit any data sheet or records for preliminary set up. Unfortunately,
651 miscommunication between the VMT and the participating laboratories resulted in both Lab B and Lab C
652 failing to submit all necessary records for Phase II testing.

653 The means of IC_{50} for the relative control were between 1232 μg/mL and 1605 μg/mL, while those for the
654 positive control were between 85 μg/mL and 92 μg/mL, as shown in Table 8.2. These variations were
655 small.

656 The following issues were found during Phase II testing, and minor revisions were made to the protocol
657 to resolve them.

658 1. Some volatile test chemicals were found to have affected the negative control. The VMT also
659 thinks that the quality of the plate seal was also affected.

P2-010: ethyl thioglycolate, P2-013: 1-bromo-4-chlorobutane, P2-014: sodium hydrogensulfite, P2-015: isobutyraldehyde

2. Considerable variation was found in the values of IC_{50} for solid test chemicals and suspensions that required ultrasonic processing
P2-006: 3,4,4'-trichlorocarbanilide, P2-008: 4,4'-methylenbis (2,6-di-tert-buthylphenol), P2-013: 1-bromo-4-chlorobutane, P2-16: 1-naphthalenacetic acid, P2-017: propyl 4-hydroxybenzoate, P2-018: ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate, P2-019: camphene

3. Labs found that, in cases where the test chemical solution adheres to the bottom of the well, absorbance after crystal-violet staining tended to yield higher measured values. (V graph) Thus, a lower concentration was used in the test for the following chemicals.
P2-006: 3,4,4'-trichlorocarbanilide, P2-007: 1-bromohexane

4. Records of observation are particularly important to confirm solubility, suspensibility, precipitation, and other characteristics of the test chemicals during testing. The VMT agreed to add instructions for recording observations to section 3.7.2 Preparing test chemicals of the protocol and to add a column for recording those comments to the records.

5. Data for wells that were found to include precipitation after exposure of the cells to the test chemical, particularly after the 72-hour incubation period, were not used in Phase II or later because they were not uniformly suspended.

4.1.3 Phase III

Phase III was designed to validate inter-laboratory reproducibility and predictive capacity of the SIRC-CVS:TEA test method using one hundred coded test chemicals. JaCVAM provided each participating laboratory with forty coded test chemicals, comprising one set of ten common test chemicals and one set of thirty unique test chemicals. During Phase III, JaCVAM received complaints from the study directors at two of the three participating laboratories regarding eight test chemicals, all of which were liquid and highly volatile compounds. A significant quantity of these test chemicals was lost during storage and transportation, because the bottles were not sealed properly prior to distribution. JaCVAM received notification on May 7, 2013, from Lab A and on May 10, 2013, from Lab C, stating that some test chemicals delivered for use in Phase III had evaporated. Four test chemicals each at these two laboratories were replaced with new shipments, which were also found to exhibit evidence of evaporation. JaCVAM obtained reagent bottles, which are significantly evaporation resistant, and redistributed the following test chemicals.

Subject test chemicals (none of which were common to both laboratories)

Lab A:
P3-082 (Methyl cyclopentane), P3-083 (Toluene), P3-084 (Acetone), and P3-087 (Methyl ethyl ketone)

Lab B:
P3-053 (n-Butanal), P3-056 (Ethyl acetate), P3-063 (Isopropyl bromide), and P3-094 (Methyl ethyl ketone)

Upon receipt of these complaints, JaCVAM redistributed these substances in properly sealed bottles, and testing at the two laboratories was performed with no difficulty.

Phase III was designed so that a third run was needed only when the results of the first two runs were not concordant. Lab C, however, followed the procedure used in Phase IIB and conducted three runs for all forty test chemicals. Due to this mistaken procedure, our analysis of data from Lab C ignores the third run when the results of the first and second runs are concordant.

All three participating laboratories performed the SIRC-CVS:TEA test in conformance with the six quality control criteria stipulated in section 3.2.9 and as shown in Table 7.2. The VMT confirmed the data sheets and record sheets for the Phase III in the spirit of GLP. The mean values for IC_{50} were between 1119.6 µg/mL and 1358.7 µg/mL for the relative control and between 89.2 µg/mL and 123.2 µg/mL for the positive control at all three participating laboratories, as shown in Table 8.3. The coefficient of variation was between 5.5% and 14.0% for the relative control and 2.3% and 10.0% for the positive control. Thus, just as in Phases I and II, variation for both the relative and positive controls was small.

The following issues were found during Phase III, and the VMT agreed to the deletion of some data and to analyze their deviations.

Absorbance values for test chemical P3-030 (1,2-benzisothiazol-3(2H)-one) at concentrations of up to 19.5 µg/mL were assumed to be 0, irrespective of the presence of precipitation after the 72-hour incubation period at Lab C. This precipitation has no effect to the IC_{50} value.

Absorbance values for test chemical P3-042 (1-(9H-Carbazol-4-yloxy)-3-{[2-(2-methoxyphenoxy)ethyl]amino}-2-propanol) at concentrations of 5000, 2500, 1250, and 625 µg/mL were deleted due to the presence of precipitation after the 72-hour incubation period at Lab A. This deletion has no effect to the IC_{50} value.

Absorbance values for test chemical P3-075 (promethazine hydrochloride) at concentrations of 5000, 2500, 1250, and 625 µg/mL were deleted due to the presence of precipitation after the 72-hour incubation period at Lab A. This deletion has no effect to the IC_{50} value.

Absorbance values for test chemical P3-090 (cetylpyridinium bromide) at concentrations of 5000, 2500, and 1250 µg/mL were deleted due to the presence of precipitation after the 72-hour incubation period at Lab A. This deletion has no effect to the IC_{50} value.

One of the two data sets for P3-95 (3,3-dithiodipropionic acid) was excluded from analysis of predictive capacity, due to duplication. Although no precipitation was found when P3-95 was tested at Lab A, Lab C reported the presence of precipitation in medium. The VMT requested that Lab A retest P3-95, however, and no precipitation was observed. The IC_{50} values were similar at the two labs irrespective of the presence of precipitation after the 72-hour incubation period reported at Lab C. Therefore, the VMT decided to include the data for P3-23 from Lab C in the analysis.

At Lab B, no value of IC_{50} for P3-066 (calcium thioglycolate trihydrate) could be calculated due to precipitation. This data was excluded from analysis.

Lab C performed three runs per set during testing, but since all three runs showed similar results, only data from the first two runs were included in analysis.

Rather than using the Phase III record sheet version 2.2, which includes a column for recording solubility of the test chemical during the 72-hour exposure period, Lab C used the Phase II record sheet version 2.1,

738 which did not contain such a column. The VMT decided to accept the submitted Phase II record sheet
739 version 2.1 for analysis.

740 The results at Lab B for the common test chemical P3-028 (tetraethylene glycol), which is soluble in the
741 Medium, were cytotoxic for all concentrations at Lab B. The fact that no other laboratory found
742 cytotoxicity for all concentrations suggests the possibility that the microplates were not properly sealed.
743 The VMT accepted this data as valid.

744 Since there might be discrepancies in solubility of test chemicals introduced by ultrasonic processing or
745 other factors, we recognize that careful judgment based on visual observation is required.

746 **4.2 Transferability**

747 Throughout the validation, most results for the relative control and the positive control were accepted with
748 only small variations, as shown in Tables 8.1, 8.2, and 8.3. (Provided that the data from retests during
749 Phase I are adopted). Most instances of problematic data came from volatile test chemicals (See Table
750 7.2.). Therefore, the VMT considers this test method to be highly transferable.

751 On the other hand, the data from Phase I shown in Table 9.1 and Fig. 5 indicates that, although Labs B
752 and C obtained very consistent results for each individual test chemical, Lab A exhibited considerable
753 variability. As shown in Table 9.2 and Table 9.3, all three laboratories classified ethyl-2-methyl
754 acetoacetate and Safflower oil as non-irritants as well as 3-chloropropionitrile as an irritant. The lead
755 laboratory also obtained similar results for these substances. The classification of sodium dehydroacetate
756 at Lab A differed from that at the other two labs and the lead lab. The results of the retest, on the other
757 hand, were more consistent and also matched the classifications obtained by Lab B, Lab C, and the lead
758 lab. After retesting at Lab A, all three participating laboratories classified sodium dehydroacetate as an
759 irritant. Moreover, variation of the reference controls during the retest was much lower than in the first
760 test, as shown in Table 9.1. The VMT therefore considered transferability of the SIRC-CVS:TEA test
761 method to be validated. The protocol was revised several times during the validation study to compensate
762 for test chemicals that induced precipitation in medium, volatile substances, or inhibition of absorbance
763 measurement due to color or precipitation.

764 **4.3 Intra- and inter-laboratory reproducibility**

765 4.3.1 Intra-laboratory reproducibility

766 In Phase II, a common set of twenty coded test chemicals was tested by the three participating laboratories.
767 Data from Phase II is shown in Tables 10.1 to 10.4.

768 The dose response curves for P2-001 (piperonylbutoxide), P2-007 (1-bromohexane), and P2-013 (1-
769 bromo-4-chlorobutane) were U shaped, indicating that cytotoxicity was recovered at high doses), as shown
770 in Fig.6 (for example, P2-001 (piperonylbutoxide)). There is no clear indication that this was a result of
771 using DMSO as a solvent. Nor were there any problems to using the IC_{50} at the lowest dose as indicated
772 by the Excel sheet.

773 As shown in Table 8.2, variation for the twenty test chemicals, relative control, and positive control was
774 low at each laboratory. Prediction of eye irritation potency by evaluation described in 3.2.9 was congruent
775 for all three sets at all three participating laboratories, as shown in Tables 10.5 to 10.8, and the results
776 satisfied the 80% acceptance criteria. The VMT therefore considered intra-laboratory reproducibility for
777 Phase II to be validated.

4.3.2 Inter-laboratory reproducibility

In Phase II, a common set of twenty test chemicals, and Phase III, a common set of ten test chemicals were tested by all three participating laboratories to validate inter-laboratory reproducibility. The test results by evaluation described in 3.2.9 for these thirty test chemicals were highly consistent at all three laboratories. The data from Phase the II is shown in Table 10.1, and data from Phase III is shown in Tables 11.1 to 11.3.

Predictions for eye irritation potency of the twenty test chemicals from Phase II were completely concordant (20/20) at all three participating laboratories, as shown in Table 12, indicating excellent inter-laboratory reproducibility. Concordance on prediction of eye irritation potency in Phase III, however, was 7/10, as shown in Tables 13 and 14.

Of the ten common test chemicals used at all three participating laboratories during Phase III, the results for P3-010 (n,n-dimethylguanidine sulfate) and P3-012 (polyethylene hydrogenated castor oil (40E.O.)) were not concordant at two laboratories, showing a dose response curve similar to that of the TEA reference control. The dose response curve for P3-003 (dipropyl disulfide) varied between laboratories, but the VMT confirmed that this was not due to differences in solubility. The solvents used were 10% FBS-MEM at Lab A, ethanol at Lab B, and dimethylsulfoxide at Lab C, as shown in Fig. 7. The VMT is aware that the choice of solvents can cause differences in solubility. As shown in Table 14, predictions based on the criteria are determined by a simple majority.

Overall inter-laboratory reproducibility, however, was 27/30 or 90%, indicating a high degree of inter-laboratory reproducibility and satisfying the acceptance criteria of 80%. The solvents used in this validation study were 10% FBS-medium, DMSO, and ethanol, but there were no effects on inter-laboratory reproducibility that could be ascribed to the solvents.

4.4 Predictive capacity

As shown in Tables 12 and 14, the results from the testing of twenty test chemicals in Phase II and ninety-six test chemicals in Phase III or a total of 116 test chemicals were compared to determine a correlation between in vitro and in vivo data and evaluate the predictive capacity of the SIRC-CVS:TEA test method from a variety of perspectives. Test results of the SIRC-CVS:TEA for three sets from Phase II and a part of Phase III were summarized by the median judgment for the evaluation.

As shown in Fig.9, 3,3-dithiodipropionic acid was inadvertently duplicated as both P3-23 and P3-95, but only the data from P3-23 was included in the analysis.

IC_{50} for P3-066 (calcium thioglycolate trihydrate) could not be calculated due to the presence of precipitation, as shown in Fig. 8. Additionally, data for P3-067 (citric acid) and P3-068 (potassium sorbate) was excluded from the analysis of predictive capacity, because they lacked clear sources of individual animal data. Therefore, data from a total of 116 test chemicals was analyzed to determine a correlation between in vitro and in vivo data and evaluate predictive capacity from a variety of perspectives.

The SIRC-CVS:TEA test method was developed primarily to identify ocular non-irritants in a bottom-up approach. Analysis in a top-down approach for identifying GHS Category 1 eye irritants was also performed as a part of this validation study in order to compare the results from a bottom-up approach to those from a top-down approach, as shown in Tables 15 and 16. In a bottom-up approach, the SIRC-CVS:TEA test method demonstrated an accuracy of 55% (64/116), a sensitivity of 60% (42/70), and a specificity of 48% (22/46), and in a top-down approach, demonstrated an accuracy of 53% (62/116), a

sensitivity of 71% (20/28), and a specificity of 48% (42/88). Thus, the results were similar in either approach.

Since these results were not particularly satisfactory, further analysis was performed to determine if predictive capacity could be improved by defining the applicability domain.

4.5 Applicability domain

Further analysis was conducted to reduce false negatives by delimiting the applicability domain to certain chemical classes and properties of interest. Chemical classes with at least six representative substances were examined: alcohols, carboxylic acids, esters, ethers, halogen compounds, heterocyclic compounds, hydrocarbons, ketones, organic salts, phenols, surfactants, and thiol compounds as shown in Appendix 8. Physical chemical properties of interest were molecular weight, physical state, purity, water solubility, distribution coefficient (log D), pKa, and vapor pressure. Criteria and rationale for selection of these properties of interest are shown in Table 17. These records were summarized in Table 18.

4.5.1 Chemical class

Table 19 shows these results of an analysis of chemical class based on Appendix 8. Chemical classes employed as applicability domains for the analysis are shown in the table:

Surfactants had 0% (0/5) false negatives and an accuracy of 86% (6/7). Similarly, halogen compounds had 0% (0/5) false negatives and an accuracy of 64% (7/11). Unfortunately, a sample size of just five chemicals for these two classes is not large. In contrast, ketones, alcohols, and carboxylic acids all showed a high rate of false negatives. Thus predictive capacity for surfactants was high.

4.5.2 Properties of interest

Tables 20.1 through 20.7 show an analysis of predictive capacity based on physicochemical properties of the test chemicals. The following properties of interest were identified: phase, molecular weight, purity, water solubility, Log D, vapor pressure and pKa. Our preliminary analysis showed a high rate of false negatives, 41% (28/70), and a low accuracy of just 55% (64/116), as shown in Table 15. Further analysis, however, showed that false negatives could be reduced to less than 5% (1/22) and accuracy increased to 72% (31/43) by excluding test chemicals with a molecular weight of less than 180, as shown in Table 20.2. Further analysis showed 6% (1/16) false negatives with an accuracy of 71% (23/32) could be achieved by excluding test chemicals with a molecular weight of less than 180 and purity of at least 80%, as shown in Table 20.3. Thus, the VMT's decided that, in order to maintain a balanced selection of test chemicals in the analysis, mixtures and solutions of less than 80% purity were excluded.

As can be seen in Table 20.2 and 20.3, molecular weight was the only property of interest to demonstrate improvement in false negatives and accuracy. The VMT analyzed a Shiseido proposal (Appendix 14) to use a combination of chemical category and molecular weight. It is difficult to evaluate the eye irritancy of test chemicals that have a molecular weight of less than 180 and are alcohols (The number of hydroxyl group≤2), esters, ethers, ketones, heterocyclic compounds, or carboxylic acids including salt. Incidentally, TEA that has three hydroxyl groups which is not excluded from applicability domain, though the molecular weight is less than 180. The VMT reviewed this analysis in the light of a pre-validation proposal from Shiseido, and excluded the test chemicals shown in Tables 21.1 to 21.6. The result was 8% (2/26) false negatives, 58% (18/31) false positives, and 65% (37/57) accuracy, as shown in Table 22. The two false negatives were GHS category 2B substances: P3-083 (toluene) and P3-023 (3,3-dithiodipropionic

acid). Although this false negative rate did not meet the 5% target and the false positive rate was greater than 50%, the VMT considered this to be the most suitable applicability domain.

5. Discussion

5.1 Considerations for the validation study

In an earlier study performed in Japan (Ohno, 1999), the reproducibility and the predictive capacity of the SIRC-CVS test method was validated on the basis of assessing eye irritation potency for solutions or suspensions at a 10% concentration. In the present study, the SIRC-CVS:TEA test method was validated on the basis of assessing undiluted substances using TEA as a relative control. TEA was selected by Shiseido as a suitable control substance after a reanalysis of previous studies in which GHS No Category non-irritants were distinguished from Category 1 and 2 irritants. As shown in Appendix 10, TEA from different manufacturing lots provides consistent results. Also, differences in manufacturers or production lots of serum and SDS do not have any significant effect on test results. See Appendix 10 “Examination of difference by lot of triethanolamine and serum".

In the validation study, the test chemicals were selected from chemicals for which individual Draize scores could be confirmed, and so that chemicals from Category 1, 2, and No Category were represented appropriately. The VMT determined that a minimum sample size of 20 test chemicals was necessary to evaluate intra-laboratory reproducibility, which was evaluated in Phase II using data from three sets per test chemical at the three participating laboratories. The results for all three sets for each test chemical at each laboratory were concordant for all substances, thus intra-laboratory reproducibility for the test chemicals was 100% (20/20), which satisfied the criteria of 80%.

In order to confirm inter-laboratory reproducibility, 10 more test chemicals were added for Phase III. Inter-laboratory reproducibility was evaluated using data from the twenty Phase II test chemicals and ten Phase III test chemicals. Three of the thirty test chemicals had non-concordant results. Of these three, n,n-dimethylguanidine sulfate and polyethylene hydrogenated castor oil (40E.O.) have an IC_{50} relatively close to that of TEA. The other, dipropyl disulfide was difficult to suspend uniformly and all three participating labs used a different solvent. However, all three have in vivo data supporting a classification of No Category under UN GHS. Thus, inter-laboratory reproducibility was 90% (27/30), which satisfied the criteria of 80%. Tani et al reported that the use of different solvents at different laboratories did not affect the reproducibility, but there are exceptions to this trend.

In response to a comment about the effects of different solvents, the VMT analyzed average ±standard deviations of the O.D. for each solvent. The negative control was 0.64 ±0.08 in the Medium (n = 52) and 0.66 ±0.08 in medium containing DMSO (n = 28), as calculated from Phase III data obtained at Lab A, and 0.97 ±0.09 in the Medium (n = 76) and 0.93 ±0.10 in medium containing ethanol (n = 4), as calculated from Phase III data obtained at Lab B. Neither Lab A nor Lab C used ethanol as a solvent, nor did Lab B use DMSO as solvent, as shown in Appendix 11 “Effect of solvents in the validation study.” Actually, an investigation of the effects of different solvents was part of the previous Japanese validation study of the SIRC-CVS test. Also, the fact that the three participating laboratories were all naïve and that no practical training was given is another good indication of the robustness of the test method.

The test data record sheets were all checked by the record management group. The results indicate that the SIRC-CVS:TEA test method demonstrates good intra- and inter-laboratory reproducibility for identifying test chemicals that are not ocular irritants.

The database at the lead laboratory was not considered extensive enough to evaluate predictive capacity, and the VMT decided that data from at least 100 test substances would be needed. The 116 test chemicals selected for the analysis of predictive capacity comprised 28 from GHS Category 1, 42 from Category 2, and 46 from No Category. The VMT decided to validate the SIRC-CVS:TEA test method using as many test chemicals as possible and did not initially take into consideration as criteria for selection of test chemicals a proposal from Shiseido that the exclusion of alcohols, esters, ethers, or similar chemical classes would improve predictive capacity. Prediction of UN GHS classification by comparing the IC_{50} of the test chemicals with that of TEA as a preliminary step in a bottom-up approach yielded an accuracy of 55% (64/116), a sensitivity of 60% (42/70), and a specificity of 48% (22/46), as shown in Table 15. If a cut-off value of 1600 μg/mL is adopted instead of using TEA as a relative control, these values become 59% (66/112), 69% (48/68), and 43% (19/44), respectively, as shown in Table 16. In either case, the results are similar. Prediction of EPA classification by comparing the IC_{50} of the test chemicals with that of TEA yielded an accuracy of 54% (62/115), a sensitivity of 57% (50/88), and a specificity of 44% (12/27), as shown in Appendix 6. Thus, predictive capacity was similar for both UN GHS and EPA classification. These results show that the predictive capacity of the SIRC-CVS:TEA test method was not sufficient to permit its use as a preliminary step in a bottom-up approach. Nor was the predictive capacity good enough for use in a top-down approach, which yielded an accuracy of 53% (62/116), a sensitivity of 71% (20/28), and a specificity of 47% (8/28), as shown in Table 15. The VMT therefore concluded that revision of the applicability domain would be necessary for further improvement of predictive capacity.

5.2 Original applicability domain

The original applicability domain for the SIRC-CVS:TEA test initially included test chemicals that could not be tested properly due to precipitation in the medium, high volatility, or interference due to color. S3-066 (calcium thioglycolate) was excluded from this validation due to precipitation. Volatile chemicals tended to produce more variable results. Although some colored test chemicals could be tested successfully, the VMT feels that they could induce color interference. Although certain chemicals that have a negative effect on cell attachment may produce false positives, VMT feel this, in effect, serves as a margin of safety.

Chemical class, physical state, molecular weight, purity, water solubility, distribution coefficient (log D), vapor pressure, and pKa were all studied as potential means of improving of predictive capacity. In this validation, chemical classes were defined by existence of functional group, as detailed in Appendix 8. Only surfactants were classified on the basis of function in accordance with the actual condition. Information on surfactants was obtained from the International Cosmetic Ingredient Dictionary (CTFA, 2006). The examination of finding applicability domain was performed in consideration of decreasing false negative first and increasing accuracy second with end user in mind. Effective elements for decreasing false negatives were molecular weight and exclusion based on chemical classes such as alcohols (The number of hydroxyl group≤2), esters, ethers, ketones, heterocyclic compounds, and carboxylic acid. False positive rate did not have marked improvement for the selection of the applicability domain in consideration of decreasing false negative.

The SIRC-CVS:TEA test was not suitable for test chemicals such as some organic solvents with a molecular weight of less than 180. Because the diluted concentration of test chemicals used in the SIRC-

CVS:TEA test was not sufficient to detect cell-membrane disrupting effects of some organic solvents. It is reported that some organic solvents cause no destruction of cells at low concentration such as 0.5% or less (Ohsumi et al, 1993). On the other hand, relatively strong cell-membrane disruptions caused by surface action of test chemicals with a molecular weight of 180 or higher can be detected with the SIRC-CVS test. Needless to say, toxicity is modified by the functional groups and other factors.

Therefore, the applicability domain was defined as follows: The SIRC-CVS:TEA test is suitable for distinguishing ocular non-irritants from ocular irritants for test chemicals that are uniformly soluble in the medium, have a purity of 80% or higher, and are not alcohols, esters, ethers, ketones, heterocyclic compounds, and carboxylic acid (containing salt) with a molecular weight of less than 180. Incidentally, TEA that belongs to the alkanolamine chemical class which is not excluded from applicability domain, though the molecular weight is less than 180.

Reanalysis of validation test results suggested that the SIRC-CVS:TEA test was suitable for the identification of chemicals that were not ocular irritants when alcohols, esters, ethers, ketones, or other test chemicals with a molecular weight of less than 180 were excluded, as shown in appendix 8. In this validation, heterocyclic compounds and carboxylic acid compounds with a molecular weight of less than 180 were shown to be likely to cause false negatives. Excluding alcohols, esters, ethers, ketones, heterocyclic compounds, carboxylic acid compounds and similar chemical classes with a molecular weight of less than 180 improved the accuracy to 65% (37/57) and the false negative rate to 8% (2/26), which suggests that the predictive capacity of the SIRC-CVS:TEA test can be improved by delimiting the applicability domain. Toluene was one of the two false negatives and was > Category 2B per TSCA in vivo data, but was classified No Category, meaning “negative,” per ECETOC in vivo data.

The applicability domain was also reviewed using Shiseido’s in-house data in an attempt to find more test chemicals, as detailed in Appendix 8. Predictive capacity based on data from 57 test substances in this validation study and data from Shiseido on an additional 22 test chemicals yielded an accuracy of 65% (51/79), a sensitivity of 95% (35/37), and a specificity of 38% (16/42). Thus it is suggested that the SIRC-CVS:TEA test method is suitable for distinguishing ocular non-irritants and irritants, if the applicability domain is well defined.

Predictive capacity was further analyzed using data on 79 substances that conform to the applicability domain from this validation study and from Shiseido in-house data. Although false positives were unavoidable, the false negative rate was less than 10%. Thus, the VMT concluded that the SIRC-CVS:TEA test was a useful alternative to animal testing for distinguishing ocular non-irritants and irritants with a carefully defined applicability domain based on Appendix 12 “Analysis of predictive capacity by the data from this validation study and the additional data from Shiseido.”

5.3 Reanalysis of the original applicability domain

The original applicability domain for the SIRC-CVS:TEA test method was determined during the design of the validation study by analysis with a combination of chemical category and molecular weight. Additionally, upon completion of Phases I–III, we attempted to determine a more definite physicochemical basis for defining the applicability domain. We were unable, however, to overcome technical limitations affecting the results for test chemicals with poor solubility, high volatility, or color. As detailed in Appendix 16, we attempted to reduce false negatives by excluding (1) acids with an acid dissociation constant pKa of 4 or less or organic salts consisting of a weak acid and a strong base and (2) chemicals with a distribution coefficient (log P) of greater than -1.5 and less than 2. In this analysis, predictive

capacity was calculated relative to Draize eye test reference data by Barroso et al, though the influence on the results was not significant (Appendix 15 and 16). Additional data from Shiseido were also used to analyze the predictive capacity as shown in Appendix 16. The SIRC-CVS:TEA test method finally demonstrated an accuracy of 62% (49/79), a sensitivity of 100% (25/25), and a specificity of 44% (24/54) with a false negative rate of 0% (0/25). Reanalysis of the test results using these criteria shows that the SIRC-CVS:TEA test is capable of distinguishing ocular non-irritants from irritants per UN GHS categories once test chemicals that are strong acids or alkalis, are amphiphilic substances with high cell membrane accessibility, or are cytotoxic have been excluded from the applicability domain. Even after considerable review of the test data, however, the VMT was unable to reach a consensus regarding a scientifically valid approach to achieving the requisite level of sensitivity and was unable to identify a scientifically valid applicability domain that would provide a high predictive capacity.

6 Conclusion

This validation study of the SIRC-CVS:TEA test method was performed using a wide variety of 120 test chemicals. It was implemented at three participating laboratories in the spirit of GLP to validate intra- and inter-laboratory reproducibility as well as usefulness for distinguishing between non-irritants and irritants in a bottom up approach.

The results showed 100% (20/20) intra-laboratory reproducibility at all three laboratories and an excellent 90% (27/30) inter-laboratory reproducibility. Unfortunately, predictive capacity for distinguishing non-irritants from irritants per UN GHS categories in a bottom-up approach was not as favorable without restricting the applicability domain.

Even after considerable review of the test data, the VMT was unable to identify a scientifically valid applicability domain that would provide a high predictive capacity. We therefore concluded that the SIRC-CVS:TEA test method has excellent intra- and inter-laboratory reliability, but were unable to reach a consensus as to whether or not this test method was useful as an alternative to the Draize test for distinguishing ocular non-irritants from irritants.

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Tables for SIRC-CVS:TEA validation version 9.2

Table 1. Members of SIRC-CVS:TEA Validation Management Team (VMT)

Name	Organization	Duties
Momoko Sunouchi	NIHS Japan	Chairperson Record management
Hajime Kojima	JaCVAM, NIHS Japan	JaCVAM Chemical Management Quality Assurance Record management
Warren Casey	ICCVAM, NIH USA	NICEATM Chemical Management
Takashi Omori	Doshisha University, Japan	Data Analysis
Kohji Yamakage	Food and Drug Safety Center, Hatano Research Institute, Japan	Chemical Management
Shigenobu Hagino	Shiseido Research Center, Japan	Lead laboratory

Table 2. Distribution of 100 test substances used in Phase III study

Test substances	Laboratory A	Laboratory B	Laboratory C
10 common test substances	☑	☑	☑
30 unique test substances	☑		
30 unique test substances		☑	
30 unique test substances			☑

Table 3. Breakdown of substances used in the SIRC-CVS:TEA validation study

Phase	No. of test substances	No. of sets	No. of runs per set	Area of Validation	
I	4 non-coded	1	3	Transferability	
IIA	5 coded	3	3	Intra- and inter-laboratory reproducibility	Predictive capacity
IIB	15 coded	3	3		
III	A total of 100 coded test substances: 40 at each laboratory, including 10 common and 30 unique substances.	1	2 or 3	Inter-laboratory reproducibility	

Table 4. Substances for Phase I study and data by lead laboratory

No.	Substance	CAS	Supplier	Physical state	*In vitro* Judgment
Positive	Sodium dodecyl sulfate	151-21-3	Wako Pure Chemical	Solid	Positive
Reference	Triethanolamine (TEA)	102-71-6	Wako Pure Chemical	Liquid	-
P1-001	Ethyl-2-methyl acetoacetate	609-14-3	Wako Pure Chemical	Liquid	Negative
P1-002	Safflower oil	8001-23-8	Wako Pure Chemical	Liquid	Negative
P1-003	3-Chloropropionitrile	542-76-7	Wako Pure Chemical	Liquid	Positive
P1-004	Sodium dehydroacetate	4418-26-2	Wako Pure Chemical	Solid	Positive

Table 5. Twenty substances for Phase II study

No.	Chemical Name	CAS	Supplier	Physical state	GHS
P2-001	Piperonylbutoxide	51- 03- 6	Sigma-Aldrich	Liquid	No
P2-002	2,5-Dimethylhexanediol	110-03-2	Sigma-Aldrich	Solid	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	Sigma-Aldrich	Liquid	2B
P2-004	Ammonium nitrate	6484-52-2	Sigma-Aldrich	Solid	2A
P2-005	Potassium tetrafluoroborate	14075-53-7	Sigma-Aldrich	Solid	No
P2-006	3,4,4'-Trichlorocarbanilide	101-20-2	Sigma-Aldrich	Solid	No
P2-007	1-Bromohexane	111-25-1	Sigma-Aldrich	Liquid	No
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	Sigma-Aldrich	Solid	No
P2-009	Propylene glycol propyl ether	1569-01-3	Sigma-Aldrich	Liquid	2A
P2-010	Ethyl thioglycolate	623-51-8	Sigma-Aldrich	Liquid	No
P2-011	Sodium oxalate	62-76-0	Sigma-Aldrich	Solid	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	66170-10-3	Sigma	Solid	No
P2-013	1-Bromo-4-chlorobutane	6940-78-9	Sigma-Aldrich	Liquid	No
P2-014	Sodium hydrogensulfite	7631-90-5	Sigma-Aldrich	Solid	No
P2-015	Isobutyraldehyde	78-84-2	Sigma-Aldrich	Liquid	2B
P2-016	1-Naphthaleneacetic acid	86-87-3	Wako Pure Chemical	Solid	1
P2-017	Propyl 4-hydroxybenzoate	94-13-3	Sigma-Aldrich	Solid	No
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	Sigma-Aldrich	Solid	2B
P2-019	Camphene	79-92-5	Sigma-Aldrich	Solid	2B
P2-020	Cyclopentanol	96-41-3	Sigma- Aldrich	Liquid	2A

Table 6. The 100 substances for the Phase III study

No.	Chemical Name	CAS	Supplier	Physical state	GHS
P3-001	2-Ethoxyethyl methacrylate	2370-63-0	Sigma-Aldrich	Liquid	No
P3-002	iso-Octylthioglycolate	25103-09-7	Wako Pure Chemical	Liquid	No
P3-003	Dipropyl disulfide	629-19-6	Sigma-Aldrich	Liquid	No
P3-004	1-Bromo-octane	111-83-1	Sigma-Aldrich	Liquid	No
P3-005	2-(2-Ethoxyethoxy)ethanol	111-90-0	Sigma-Aldrich	Liquid	No
P3-006	Dioctyl ether	629-82-3	Sigma-Aldrich	Liquid	No
P3-007	3-Phenoxybenzyl alcohol	13826-35-2	Sigma-Aldrich	Liquid	No
P3-008	glycidyl methacrylate	106-91-2	Sigma-Aldrich	Liquid	No
P3-009	2-Ethylhexylthioglycolate	7659-86-1	Sigma-Aldrich	Liquid	No
P3-010	n,n-Dimethylguanidine sulfate	598-65-2	Sigma-Aldrich	Solid	No
P3-011	6-Hydroxy-2,4,5-triaminopyrimidine sulfate	1603-02-7	Wako Pure Chemical	Solid	No
P3-012	Polyethylene hydrogenated castor oil (40E.O.)	61788-85-0	Sigma-Aldrich	Solid	No
P3-013	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	103597-45-1	Sigma-Aldrich	Solid	No
P3-014	Cellulose,2-(2-hydroxy-3-(trimethyl ammonio)propoxy) ethyl ether chloride	68610-92-4	Sigma-Aldrich	Solid	No
P3-015	3,4-Dimethoxy benzaldehyde	120-14-9	Sigma-Aldrich	Solid	No
P3-016	3-Chloropropionitrile	542-76-7	Wako Pure Chemical	Liquid	2B
P3-017	2-Methyl-1-pentanol	105-30-6	Sigma-Aldrich	Liquid	2B
P3-018	Ethyl-2-methylacetoacetate	609-14-3	Sigma	Liquid	2B
P3-019	Diethyl toluamide	134-62-3	Sigma-Aldrich	Liquid	2B
P3-020	4-Nitrobenzoic acid	62-23-7	Sigma-Aldrich	Solid	2B
P3-021	Sodium chloroacetate	3926-62-3	Sigma-Aldrich	Solid	2B
P3-022	2,4,11,13-Tetraazatetra (Chlorohexidine glucocinate)	18472-51-0	Wako Pure Chemical	Liquid	2A
P3-023	3,3-Dithiodipropionic acid	1119-62-6	Wako Pure Chemical	Solid	2B
P3-024	2-Amino-3-hydroxy pyridine	16867-03-1	Sigma-Aldrich	Solid	2A
P3-025	Sodium benzoate	532-32-1	Sigma-Aldrich	Solid	2A
P3-026	Methylthioglycolate	2365-48-2	Sigma-Aldrich	Liquid	1
P3-027	[3-(2-Aminoethylamino)propyl] Trimethoxysilane	1760-24-3	Chemo's	Liquid	1
P3-028	Tetraethylene glycol	17831-71-9	Sigma-Aldrich	Liquid	1
P3-029	Dodecanoic acid	143-07-7	Sigma-Aldrich	Solid	1
P3-030	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Wako Pure Chemical	Solid	1
P3-031	2-Hydroxy-1,4-naphthoquinone	83-72-7	Sigma-Aldrich	Solid	2B
P3-032	Disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	27344-41-8	Wako Pure Chemical	Solid	1
P3-033	Gamma-Butyrolactone	96-48-0	Sigma-Aldrich	Liquid	2A
P3-034	1-Methylpropyl benzene	135-98-8	Wako Pure Chemical	Liquid	No
P3-035	4-(Methylmercapto)benzaldehyde	3446-89-7	Sigma-Aldrich	Liquid	No
P3-036	1,9-Decaine	1647-16-1	Sigma-Aldrich	Liquid	No

No.	Chemical Name	CAS	Supplier	Physical state	GHS
P3-037	2,4-Dimethyl-3-pentanol	3970-62-5	Sigma-Aldrich	Liquid	No
P3-038	1-Ethyl-3-methylimidazolium ethylsulfate	342573-75-5	Alfa Aesar	Liquid	No
P3-039	1,2,4-Triazole,sodium salt	41253-21-8	Sigma-Aldrich	Solid	1
P3-040	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol]	4430-25-5	Sigma-Aldrich	Solid	1
P3-041	Benzenamine,4,4'-(4-amino-3-methyl phenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCL	3248-91-7	Sigma-Aldrich	Solid	1
P3-042	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	72956-09-3	LKT.Labs, Inc	Solid	No
P3-043	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	33089-61-1	LKT.Labs, Inc	Solid	No
P3-044	Isopropyl acetoacetate	542-08-5	Wako Pure Chemical	Liquid	2B
P3-045	(3R,4R)-4-acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	76855-69-1	Sigma-Aldrich	Solid	2A
P3-046	1-Octanol	111-87-5	Wako Pure Chemical	Liquid	2A
P3-047	2-benzyloxyethanol	622-08-2	Wako Pure Chemical	Liquid	2A
P3-048	Butanol	71-36-3	Wako Pure Chemical	Liquid	1
P3-049	Isobutyl alcohol	78-83-1	Sigma-Aldrich	Liquid	1
P3-050	Isopropyl alcohol	67-63-0	Wako Pure Chemical	Liquid	2A
P3-051	myristyl alcohol	112-72-1	Wako Pure Chemical	Solid	2A
P3-052	Hexyl cinnamon aldehyde	101-86-0	Wako Pure Chemical	Liquid	No
P3-053	n-Butanal	123-72-8	Wako Pure Chemical	Liquid	2B
P3-054	Monoethanolamine	141-43-5	Sigma-Aldrich	Liquid	2B
P3-055	m-Phenylenediamine	108-45-2	TCI	Solid	1
P3-056	Ethyl acetate	141-78-6	Sigma-Aldrich	Liquid	No
P3-057	Isopropyl myristate	110-27-0	Wako Pure Chemical	Liquid	No
P3-058	Methoxyethyl acrylate	3121-61-7	Wako Pure Chemical	Liquid	1
P3-059	Methyl acetate	79-20-9	Sigma-Aldrich	Liquid	2A
P3-060	Methyl cyanoacetate	105-34-0	Sigma-Aldrich	Liquid	2A
P3-061	Imidazole	288-32-4	Sigma-Aldrich	Solid	1
P3-062	Pyridine	110-86-1	Sigma-Aldrich	Liquid	1
P3-063	Isopropyl bromide	75-26-3	Wako Pure Chemical	Liquid	No
P3-064	Cyclohexanone	108-94-1	Sigma-Aldrich	Liquid	No
P3-065	2-Methylbutyric acid	116-53-0	Sigma-Aldrich	Liquid	1
P3-066	Calcium thioglycolate trihydrate	5793-98-6	TCI	Solid	1
P3-067	Citric acid	77-92-9	Sigma-Aldrich	Solid	No
P3-068	Potassium sorbate	24634-61-5	Sigma-Aldrich	Solid	No
P3-069	Sodium salicylate	54-21-7	Wako Pure Chemical	Solid	1

No.	Chemical Name	CAS	Supplier	Physical state	GHS
P3-070	Distearyldimethylammonium chloride	107-64-2	TCI	Solid	1
P3-071	n-Lauroylsarcosine sodium salt	137-16-6	Wako Pure Chemical	Solid	2B
P3-072	Sodium lauryl sulfate	151-21-3	Wako Pure Chemical	Solid	2A?
P3-073	Triton X-100 (5%)	9002-93-1	Sigma-Aldrich	Liquid	2B
P3-074	2-Ethylhexyl p-dimethylaminobenzoate	21245-02-3	Wako Pure Chemical	Liquid	No
P3-075	Promethazine hydrochloride	58-33-3	Sigma-Aldrich	Solid	1
P3-076	2-Ethyl-1-hexanol	104-76-7	Wako Pure Chemical	Liquid	2A
P3-077	3-Methoxy-1.2-propanediol	623-39-2	TCI	Liquid	No
P3-078	Cyclohexanol	108-93-0	Sigma-Aldrich	Liquid	1
P3-079	Ethanol	64-17-5	Wako Pure Chemical	Liquid	2A
P3-080	n-Hexanol	111-27-3	Sigma-Aldrich	Liquid	2A
P3-081	3,3-Dimethylpentane	562-49-2	Sigma-Aldrich	Liquid	No
P3-082	Methyl cyclopentane	96-37-7	TCI	Liquid	No
P3-083	Toluene	108-88-3	Wako Pure Chemical	Liquid	2B?
P3-084	Acetone	67-64-1	Sigma-Aldrich	Liquid	2A
P3-085	Gluconolactone	90-80-2	Wako Pure Chemical	Solid	No
P3-086	Methyl amyl ketone (2-heptanol)	110-43-0	Wako Pure Chemical	Liquid	No
P3-087	Methyl ethyl ketone (2-butanone)	78-93-3	TCI	Liquid	2A
P3-088	Methyl isobutyl ketone(4-methyl 2-pentanol)	108-10-1	Sigma-Aldrich	Liquid	No
P3-089	Glycerol	56-81-5	Wako Pure Chemical	Liquid	No
P3-090	Cetylpyridinium bromide	140-72-7	Sigma-Aldrich	Solid	1
P3-091	Triton X-100	9002-93-1	Sigma-Aldrich	Liquid	1
P3-092	Tween20	9005-64-5	Sigma-Aldrich	Liquid	No
P3-093	Sodium hydroxide	1310-73-2	Wako Pure Chemical	Solid	1
P3-094	Glycolic acid	79-14-1	Sigma-Aldrich	Solid	2B
P3-095	See P3-023				
P3-096	Sucrose fatty acid ester	Non	TCI	Solid	2A?
P3-097	Methyl para-Hydroxybenzoate	99-76-3	Wako Pure Chemical	Solid	2?
P3-098	Silicic acid	7699-41-4	Wako Pure Chemical	Solid	No
P3-099	Benzyl alcohol	100-51-6	Sigma-Aldrich	Liquid	1
P3-100	Lactic acid	50-21-5	Wako Pure Chemical	Liquid	1

1) Phase III Test Substance No. 067, and 068 were excluded from the analysis due to a lack of in vivo data.
2) Phase III Test Substance, 3,3-dithiodipropionic acid was excluded from the analysis due to duplication.

Table 7.1. Error on the quality control check in phase II and Phase III of SIRC-CVS:TEA validation study

QC check			Laboratory A		Laboratory B		Laboratory C	
Item		Criterion	Phase II	Phase III	Phase II	Phase III	Phase II	Phase III
(1)	The mean OD of the negative control (the right and left wells) for normal proliferation of SIRC cells	> 0. 4	1/186	0/80	0/180	0/80	0/180	0/120
(2)	The IC50 of SDS	77.7 - 258.7 μg/mL	0/186	0/80	0/180	0/80	0/180	0/120
(3)	The IC50 range of triethanolamine as a relative control	1,000-2,500 μg/mL	3/186	0/80	0/180	0/80	0/180	0/120
(4)	The mean IC50 of substance in two series	within ± 20% of the mean IC50	2/186	0/80	0/180	0/80	0/180	0/120
(5)	The mean ODs of left and right wells of the negative control	within ±15% of the mean OD of negative control wells	2/186	0/80	0/180	0/80	0/180	0/120
(6)	The IC50 values of two tests of positive control	lower or equal to twice	0/186	0/80	0/180	0/80	0/180	0/120

Table 7.2. Error of quality control criteria in the all phases validation study

Phase	Lab.	Code No.	Test substance	Error run	Aberration
IIA	A	P2-001	Piperonylbutoxide	Run 3	QC(3)、(4)
	A	P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	Run 3	QC(3)
	A	P2-004	Ammonium nitrate	Run 3	QC(4)
IIB	A	P2-010	Ethyl thioglycolate	Run 1	QC(1)、(5)
	A	P2-013	1-Bromo-4-chlorobutane	Run 2	QC(5)
	A	P2-015	Isobutyraldehyde	Run 1	QC(3)

Table 8.1. Means and standard deviations of IC_{50}s for the relative controls and positive controls in Phase I of the SIRC-CVS:TEA

	Laboratory A		Laboratory A (Retest)		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	4	4	4	4	4	4	4	4
Mean	1898.1	170.9	1280.8	84.6	1529.3	87.0	1382.8	82.0
SD	350.3	7.4	61.3	1.5	132.7	1.7	33.3	3.5

*N: Number of relative controls and positive controls

*IC_{50} in μg/mL.

Table 8.2. Means and standard deviations of IC_{50}s for relative controls and positive controls in the SIRC-CVS:TEA validation Phase II study

	Laboratory A		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	60	60	60	60	60	60
Mean	1355.5	85.0	1232.1	90.8	1605.1	92.0
SD	106.7	2.7	84.2	2.7	154.6	4.6

* N: Numbers of each test substances, relative controls and positive controls

* IC_{50} in μg/mL

Table 8.3. Mean and standard deviation of IC_{50}s for relative controls and positive controls in the SIRC-CVS:TEA validation Phase III study

	Laboratory A		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	40	40	39	39	39	39
Mean	1119.6	89.7	1317.3	89.2	1358.7	123.2
SD	61.6	2.1	134.3	3.0	189.6	12.3

* N: Numbers of each test substances, relative controls and positive controls

* IC50 was expressed as μg/mL.

Table 9.1. The IC_{50}s for test substances, relative controls and positive controls in the SIRC-CVS:TEA validation Phase I study

No.	Name of test substance		Laboratory A			Laboratory A (Retest)			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
P1-001	Ethyl-2-methyl acetoacetate	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	>5000	1677.7	172.1	3296.5	1234.5	83.2	3642.0	1551.6	87.2	>5000	1349.5	82.6
		SD	-	133.1	10.3	292.3	306.2	3.3	142.1	376.1	4.2	-	62.4	1.4
P1-002	Safflower oil	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	>5000	1613.4	170.3	>5000	1265.0	86.6	>5000	1579.8	84.7	>5000	1365.5	80.2
		SD	-	426.3	6.1	-	175.8	4.0	-	31.8	4.8	-	23.3	0.1
P1-003	3-Chloro-propionitrile	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	60.6	2386.1	179.7	45.6	1370.8	84.4	38.9	1339.4	88.6	48.5	1390.3	86.7
		SD	10.1	966.0	6.0	6.3	176.5	8.3	6.9	285.3	1.3	1.1	51.8	7.4
P1-004	Sodium dehydroacetate	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	2024.3	1915.3	161.6	854.3	1252.8	84.1	720.8	1646.5	87.5	1026.4	1425.8	78.5
		SD	485.7	314.5	38.5	100.8	188.8	3.5	235.3	75.7	2.8	46.2	33.4	0.4

* N: Number of runs

Table 9.2. Eye irritation potential of test substances in the SIRC-CVS:TEA validation Phase I study

Chemical No.	Name of test substances	Laboratory A			Laboratory A (Retest)			Laboratory B			Laboratory C		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
P1-001	Ethyl-2-methyl acetoacetate	N	N	N	N	N	N	N	N	N	N	N	N
P1-002	Safflower oil	N	N	N	N	N	N	N	N	N	N	N	N
P1-003	3-Chloropropio nitrile	P	P	P	P	P	P	P	P	P	P	P	P
P1-004	Sodium dehydroacetate	P	N	N	P	P	P	P	P	P	P	P	P

* N: Negative, P: Positive

Table 9.3. Transferability of the SIRC-CVS:TEA method using the Phase I study

Chemical No.	Name of test substances	Laboratory A (Retest)	Laboratory B	Laboratory C	Transferability
P1-001	Ethyl-2-methyl acetoacetate	N	N	N	Good
P1-002	Safflower oil	N	N	N	Good
P1-003	3-Chloropropionitrile	P	P	P	Good
P1-004	Sodium dehydroacetate	P	P	P	Good

* N: Negative, P: Positive,

Table 10.1. The IC_{50} for test substances, relative controls and positive controls in the SIRC-CVS: TEA validation Phase II study Set1

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase II A	P2-001	1	98.4	1676.6	82.1	117.8	1153.5	89.9	224.6	1774.8	92.8
		2	114.4	1461.0	88.7	551.6	1575.0	91.8	276.9	1411.5	83.2
		3	210.2	1298.7	86.7	194.8	1159.3	84.6	393.4	1350.2	77.7
		Mean	141.0	1478.8	85.8	288.1	1295.9	88.8	298.3	1512.2	84.6
	P2-002	1	>5000	1762.6	80.9	>5000	1401.0	92.3	>5000	1725.7	96.1
		2	>5000	1590.1	88.4	>5000	1558.4	92.9	>5000	1721.0	93.4
		3	>5000	1454.3	84.3	>5000	1247.7	84.6	>5000	1818.3	91.5
		Mean	>5000	1602.3	84.5	>5000	1402.4	89.9	>5000	1755.0	93.7
	P2-003	1	4068.4	1484.8	87.7	2685.7	1279.2	91.8	4673.4	1780.3	90.7
		2	4020.6	1355.0	86.4	3395.0	1596.1	94.0	>5000	1696.0	88.9
		3	4301.1	1711.3	84.5	3485.9	1086.4	86.9	>5000	1950.3	92.3
		Mean	4130.0	1517.0	86.2	3188.9	1320.6	90.9	>4673	1808.9	90.6
	P2-004	1	1666.9	1708.9	88.0	1117.5	1259.8	95.3	1508.6	1556.4	81.9
		2	1332.2	1741.3	87.5	1131.5	1701.7	94.9	1414.5	1433.1	79.6
		3	1027.7	1104.5	92.6	1193.5	1280.4	89.4	1305.7	1585.8	79.5
		Mean	1342.3	1518.2	89.4	1147.5	1414.0	93.2	1409.6	1525.1	80.3
	P2-005	1	1734.8	1394.3	81.9	2090.6	1261.3	90.9	>5000	1638.7	98.9
		2	1741.5	1503.8	86.8	1712.1	1556.7	94.2	>5000	1895.5	95.8
		3	1898.5	1189.3	85.2	2046.1	1003.5	80.2	>5000	1977.1	92.9
		Mean	1791.6	1362.5	84.6	1949.6	1273.8	88.4	>5000	1837.1	95.9
Phase IIB	P2-006	1	<39.1	1443.2	79.2	<39.1	1274.7	86.2	<39.1	1611.0	85.3
		2	<39.1	1163.9	96.6	<39.1	1314.7	89.1	<39.1	1907.3	94.9
		3	<39.1	1063.1	81.2	<39.1	1382.0	82.7	<39.1	1786.5	94.3
		Mean	<39.1	1223.4	85.7	<39.1	1323.8	86.0	<39.1	1768.3	91.5
	P2-007	1	245.6	1774.5	95.5	111.3	1215.0	81.8	349.6	1694.1	88.8
		2	117.1	1174.0	82.2	78.0	1075.1	85.5	349.0	1542.1	91.0
		3	435.9	1410.1	78.1	108.6	1391.7	81.5	858.8	1605.5	92.2
		Mean	266.2	1452.9	85.3	99.3	1227.3	82.9	519.1	1613.9	90.7

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-008	1	>5000	1736.6	86.6	>5000	1025.1	87.3	>5000	1694.1	88.8
		2	>5000	1010.7	86.3	>5000	1220.3	84.3	>5000	1542.1	91.0
		3	>5000	1504.2	91.5	2345.6	1418.5	88.0	>5000	1781.9	87.1
		Mean	>5000	1417.2	88.1	>2345.6	1221.3	86.5	>5000	1672.7	89.0
	P2-009	1	4865.3	1603.3	86.0	3783.7	1359.6	94.0	>5000	1538.2	85.1
		2	>5000	1021.3	88.9	3203.2	1286.0	93.0	>5000	1152.2	93.3
		3	>5000	1412.3	85.6	3698.9	1036.0	82.6	>5000	1882.4	102.6
		Mean	>4865	1345.6	86.8	3561.9	1227.2	89.9	>5000	1524.3	93.7
	P2-010	1	<39.1	1572.0	87.4	<39.1	1093.4	95.1	<39.1	1351.9	83.4
		2	<39.1	1680.5	93.2	<39.1	1306.1	90.8	<39.1	1437.8	104.2
		3	<39.1	1604.1	87.6	51.4	1261.6	81.8	<39.1	1475.2	102.5
		Mean	<39.1	1618.9	89.4	<51.4	1220.4	89.2	<39.1	1421.6	96.7
	P2-011	1	132.4	1695.0	88.9	93.5	1179.6	88.3	192.2	1526.2	81.3
		2	142.7	1060.6	85.0	113.2	1202.0	90.6	270.1	1866.0	94.0
		3	443.2	1527.4	83.2	122.8	1098.7	90.1	218.7	1874.4	104.4
		Mean	239.4	1427.7	85.7	109.8	1160.1	89.7	227.0	1755.5	93.2
	P2-012	1	3787.4	1646.4	86.3	3670.0	1074.9	83.0	4362.3	1269.3	138.8
		2	3636.4	1255.2	87.8	3397.9	1317.7	88.2	4207.0	1797.5	90.6
		3	3302.8	1216.3	78.7	3779.3	1173.0	90.6	4589.4	1891.7	93.5
		Mean	3575.5	1372.6	84.3	3615.7	1188.5	87.3	4386.2	1652.8	107.6
	P2-013	1	420.0	1603.1	91.1	540.8	1269.2	92.2	278.1	1509.3	96.3
		2	489.2	1507.1	89.2	441.8	1026.4	84.3	396.1	1563.0	96.4
		3	395.3	1302.4	81.9	213.8	1297.5	89.4	384.2	1937.9	94.7
		Mean	434.8	1470.9	87.4	398.8	1197.7	88.6	352.8	1670.1	95.8
	P2-014	1	52.7	1625.8	83.2	45.1	1122.6	91.2	52.3	1303.3	98.6
		2	66.0	1192.3	88.7	44.4	1007.4	89.9	65.6	1831.3	91.4
		3	157.1	1486.5	82.2	<39.1	1275.9	91.7	47.7	1785.0	83.3
		Mean	91.9	1434.9	84.7	<45.1	1135.3	90.9	55.2	1639.9	91.1

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-015	1	462.9	1440.9	80.1	492.8	1133.7	81.2	1135.5	1429.9	88.1
		2	730.8	1612.3	86.6	373.2	1152.7	84.5	1216.0	1601.7	89.6
		3	798.3	1367.5	77.7	490.8	1141.9	81.7	1514.6	1628.8	92.2
		Mean	664.0	1473.6	81.5	452.3	1142.8	82.5	1288.7	1553.5	90.0
	P2-016	1	989.2	1614.7	88.4	556.4	1106.8	90.5	1492.5	1610.8	92.1
		2	518.1	1194.0	78.7	588.6	1388.9	90.1	1256.4	1535.7	101.0
		3	882.7	1091.6	80.5	709.1	1114.9	86.3	1510.9	1860.6	93.3
		Mean	796.7	1300.1	82.5	618.0	1203.5	89.0	1419.9	1669.0	95.5
	P2-017	1	63.2	1637.6	87.8	104.4	1291.5	93.7	50.9	1405.3	99.9
		2	52.0	1232.0	84.7	50.3	1342.9	84.0	45.9	1920.2	95.6
		3	57.8	1025.0	88.0	50.9	1211.8	92.9	51.5	1773.0	95.5
		Mean	57.7	1298.2	86.8	68.5	1282.1	90.2	49.4	1699.5	97.0
	P2-018	1	<39.1	1532.3	87.9	<39.1	1085.7	94.3	<39.1	1621.5	97.3
		2	46.4	1128.0	88.0	<39.1	1316.2	93.1	<39.1	1785.2	89.3
		3	<39.1	1018.3	82.5	<39.1	1515.6	93.9	<39.1	1411.5	97.0
		Mean	<46.4	1226.2	86.1	<39.1	1305.8	93.8	<39.1	1606.1	94.5
	P2-019	1	262.9	1490.2	85.1	420.0	1560.1	93.3	1264.3	1425.7	97.8
		2	382.6	1109.9	88.1	405.9	1552.9	91.1	1594.7	1805.2	95.9
		3	432.6	1217.2	81.1	332.0	1048.3	85.2	1556.3	1806.8	100.9
		Mean	359.4	1272.4	84.8	386.0	1387.1	89.9	1471.8	1679.2	98.2
	P2-020	1	2977.0	1468.2	80.6	1565.3	1320.1	87.0	3851.9	1553.4	104.1
		2	3520.5	1076.4	88.7	1927.8	1571.3	97.2	3827.3	1858.3	89.2
		3	2724.5	1153.5	91.9	1695.6	1287.2	79.8	4360.4	1753.0	90.5
		Mean	3074.0	1232.7	87.1	1729.6	1392.9	88.0	4013.2	1721.6	94.6

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

*: Each IC50 for test substances, relative controls and positive controls was expressed as an average every set

Table 10.2. The IC_{50} for test substances, relative controls and positive controls in the SIRC-CVS: TEA validation Phase II study Set2

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase II A	P2-001	1	107.2	1078.8	88.8	231.9	1282.0	95.8	280.6	1430.9	89.5
		2	77.6	1006.3	85.7	364.0	1388.1	92.1	226.1	1570.4	89.1
		3	30.4	1621.7	90.3	184.6	1311.0	93.4	293.0	1359.4	86.6
		Mean	71.7	1235.6	88.3	260.2	1327.0	93.8	266.6	1453.6	88.4
	P2-002	1	>5000	1541.3	94.3	>5000	1497.4	95.7	>5000	1054.3	95.3
		2	>5000	1067.9	86.2	3989.1	1130.8	100.0	>5000	1263.3	93.2
		3	>5000	1235.4	87.4	>5000	1364.3	93.0	>5000	1278.5	88.9
		Mean	>5000	1281.5	89.3	>3989	1330.8	96.2	>5000	1198.7	92.5
	P2-003	1	3704.2	1704.7	89.1	3660.7	1450.8	93.5	>5000	1405.1	94.5
		2	3680.7	1040.1	85.7	3229.8	1046.0	95.0	>5000	1303.2	90.4
		3	4312.2	1611.6	91.4	4073.8	1576.7	91.0	>5000	1337.3	84.3
		Mean	3899.0	1452.1	88.7	3654.8	1357.8	93.2	>5000	1348.5	89.7
	P2-004	1	978.5	1616.2	90.4	646.8	1048.7	89.2	1251.2	1564.9	96.3
		2	1014.9	1054.6	90.1	542.6	1119.0	97.3	1305.7	1512.8	85.9
		3	783.1	1386.5	91.7	1146.0	1385.7	91.1	1096.9	1521.0	92.3
		Mean	925.5	1352.4	90.7	778.5	1184.5	92.5	1217.9	1532.9	91.5
	P2-005	1	1687.7	1635.4	87.7	3630.9	1449.2	92.3	>5000	1566.5	90.9
		2	2002.2	1029.0	87.5	3630.7	1344.8	86.5	>5000	1590.9	94.3
		3	1659.6	1200.5	89.4	3630.7	1344.8	86.5	>5000	1439.0	87.6
		Mean	1783.2	1288.3	88.2	3630.8	1379.6	88.4	>5000	1532.1	90.9
Phase IIB	P2-006	1	<39.1	1163.5	83.2	<39.1	1030.9	88.9	<39.1	1597.1	112.1
		2	<39.1	1042.8	78.1	<39.1	1202.7	95.0	<39.1	1847.1	93.5
		3	<39.1	1797.2	89.5	<39.1	1133.7	94.5	<39.1	1633.1	90.7
		Mean	<39.1	1334.5	83.6	<39.1	1122.4	92.8	<39.1	1692.4	98.8
	P2-007	1	293.4	1181.3	86.7	119.5	1126.0	82.6	450.5	1857.6	90.5
		2	703.9	1177.8	88.2	101.3	1331.3	92.5	326.4	1806.5	89.5
		3	522.2	1578.9	83.8	110.1	1186.1	90.0	488.1	1490.2	97.6
		Mean	506.5	1312.7	86.2	110.3	1214.5	88.4	421.7	1718.1	92.5

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-008	1	4131.4	1426.7	80.7	>5000	1303.5	93.9	>5000	1844.3	92.8
		2	2599.3	1007.8	77.7	>5000	1217.3	90.3	>5000	1627.4	94.7
		3	>5000	1635.5	82.9	>5000	1154.1	85.7	>5000	1675.5	93.1
		Mean	>2599	1356.7	80.4	>5000	1225.0	90.0	>5000	1715.7	93.5
	P2-009	1	3048.1	1083.8	80.9	3312.4	1312.3	86.9	>5000	1355.0	94.5
		2	4218.4	1004.7	83.2	3658.1	1325.8	95.6	>5000	1820.2	95.9
		3	>5000	1559.0	86.8	3614.0	1107.8	85.4	>5000	1869.2	95.5
		Mean	>3048	1215.8	83.6	3528.2	1248.6	89.3	>5000	1681.5	95.3
	P2-010	1	<39.1	1183.4	85.3	<39.1	1339.9	94.0	<39.1	1489.2	98.9
		2	<39.1	1301.0	84.1	<39.1	1134.4	91.1	<39.1	1652.4	93.7
		3	<39.1	1517.4	86.5	<39.1	1239.5	87.3	<39.1	1715.5	86.1
		Mean	<39.1	1333.9	85.3	<39.1	1237.9	90.8	<39.1	1619.0	92.9
	P2-011	1	138.3	1327.5	85.6	117.3	1103.1	92.2	224.8	1489.2	98.9
		2	115.5	1034.0	80.9	125.2	1108.7	92.8	269.0	1776.1	96.5
		3	117.3	1533.3	84.1	122.0	1073.1	89.2	237.2	1364.9	96.8
		Mean	123.7	1298.3	83.5	121.5	1095.0	91.4	243.7	1543.4	97.4
	P2-012	1	3464.6	1191.6	84.8	3821.5	1225.9	93.9	4338.6	1801.4	98.9
		2	3265.8	1025.2	80.9	3727.8	1099.7	89.1	4057.2	1811.8	93.1
		3	4160.9	1590.1	81.5	3615.6	1443.2	91.2	4343.8	1603.4	95.1
		Mean	3630.4	1269.0	82.4	3721.6	1256.3	91.4	4246.5	1738.9	95.7
	P2-013	1	1111.0	1308.9	88.4	529.6	1347.5	91.2	331.0	1795.8	89.8
		2	1113.8	1269.1	82.7	518.4	1513.5	97.1	321.1	1538.6	91.7
		3	942.0	1411.1	85.6	584.4	1158.2	88.8	243.4	1467.4	103.6
		Mean	1055.6	1329.7	85.6	544.1	1339.7	92.4	298.5	1600.6	95.0
	P2-014	1	65.2	1214.3	88.0	103.8	1283.2	91.4	57.2	1802.5	84.9
		2	80.1	1010.3	85.5	45.1	1067.6	92.2	45.0	1770.8	95.2
		3	102.1	1517.5	81.2	45.4	1395.5	89.4	108.7	1476.1	91.4
		Mean	82.5	1247.4	84.9	64.8	1248.8	91.0	70.3	1683.1	90.5

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-015	1	995.9	1004.5	85.0	471.4	1339.2	97.0	1112.8	1384.5	93.4
		2	1023.6	1029.9	81.3	300.7	1088.6	89.0	1047.5	1459.0	83.5
		3	1437.0	1483.9	85.3	412.9	1182.8	95.5	1003.1	1642.1	106.2
		Mean	1152.2	1172.8	83.9	395.0	1203.5	93.8	1054.5	1495.2	94.4
	P2-016	1	535.6	1570.7	90.1	476.6	1189.4	88.9	1359.6	1827.8	96.7
		2	897.1	1044.5	88.8	585.9	1127.6	88.0	1200.1	1825.0	101.9
		3	712.7	1478.7	82.5	835.4	1188.7	86.5	1013.5	1898.8	92.2
		Mean	715.1	1364.6	87.1	632.6	1168.6	87.8	1191.1	1850.5	96.9
	P2-017	1	104.4	1423.8	85.2	46.0	1290.7	92.3	116.3	1307.0	98.1
		2	72.4	1043.3	84.8	42.9	1056.2	91.4	83.3	1815.7	96.9
		3	101.4	1530.2	81.6	43.2	1184.4	92.6	70.9	1338.6	87.4
		Mean	92.7	1332.4	83.9	44.0	1177.1	92.1	90.2	1487.1	94.1
	P2-018	1	49.8	1226.4	78.5	<39.1	1091.8	88.8	<39.1	1633.0	95.2
		2	79.5	1166.2	80.1	<39.1	1217.9	87.6	<39.1	1603.1	100.6
		3	80.5	1723.8	89.8	<39.1	1127.6	88.0	<39.1	1698.7	82.7
		Mean	69.9	1372.1	82.8	<39.1	1145.8	88.1	<39.1	1644.9	92.8
	P2-019	1	389.8	1169.8	87.4	116.3	1333.6	86.8	1424.8	1766.1	91.4
		2	426.7	1040.5	80.2	53.4	1232.0	89.7	1277.8	1682.0	92.0
		3	884.6	1693.7	89.8	114.6	1370.3	89.7	1101.8	1581.5	84.5
		Mean	567.0	1301.3	85.8	94.8	1312.0	88.7	1268.1	1676.5	89.3
	P2-020	1	2761.1	1300.9	88.0	2545.6	1365.8	94.5	3759.4	1768.1	100.5
		2	3333.3	1061.7	81.6	2011.1	1192.2	93.2	3491.2	1846.7	91.7
		3	1805.7	1631.8	90.1	2005.1	1126.0	83.9	3528.4	1939.5	95.3
		Mean	2633.4	1331.5	86.6	2187.3	1228.0	90.5	3593.0	1851.4	95.8

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

*: Each IC50 for test substances, relative controls and positive controls was expressed as an average every set

Table 10.3. The IC_{50} for test substances, relative controls and positive controls in the SIRC-CVS: TEA validation Phase II study Set3

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase II A	P2-001	1	45.6	1802.0	89.4	225.4	1228.9	91.9	281.0	1373.3	86.8
		2	49.7	1524.8	86.3	474.5	1267.0	91.5	348.4	1051.3	80.7
		3	210.1	1440.4	88.0	656.9	1038.0	92.1	219.2	1488.0	87.0
		Mean	101.8	1589.1	87.9	452.3	1178.0	91.8	282.9	1304.2	84.8
	P2-002	1	>5000	1702.5	86.7	>5000	1231.5	91.7	>5000	1439.3	92.6
		2	>5000	1380.5	86.4	>5000	1314.4	93.0	>5000	1594.3	84.1
		3	>5000	1069.8	91.5	>5000	1276.2	92.2	>5000	1542.3	91.7
		Mean	>5000	1384.3	88.2	>5000	1274.0	92.3	>5000	1525.3	89.5
	P2-003	1	3925.5	1715.7	87.9	3292.2	1218.0	92.9	>5000	1182.2	85.9
		2	4177.1	1511.3	90.8	3012.1	1345.4	94.6	>5000	1481.0	88.0
		3	3692.5	1313.2	87.3	2770.8	1307.4	89.6	>5000	1353.4	85.9
		Mean	3931.7	1513.4	88.7	3025.0	1290.3	92.4	>5000	1338.9	86.6
	P2-004	1	1544.5	1750.1	88.3	917.1	1286.4	91.2	1201.0	1595.8	88.3
		2	1185.5	1468.9	87.0	1285.8	1431.1	91.8	1043.7	1522.1	84.0
		3	725.7	1101.4	84.0	981.5	1170.0	89.4	1054.1	1406.5	87.4
		Mean	1151.9	1440.1	86.4	1061.5	1295.8	90.8	1099.6	1508.1	86.6
	P2-005	1	1869.8	1607.7	87.4	3634.5	1260.4	86.1	4952.0	1071.9	93.3
		2	1823.8	1337.4	79.6	3506.9	1232.7	92.5	4971.1	1317.7	83.0
		3	1912.2	1080.5	87.5	>5000	1276.2	92.2	>5000	1404.2	97.7
		Mean	1868.6	1341.9	84.8	>3507	1256.4	90.3	>4952	1264.6	91.3
Phase IIB	P2-006	1	<39.1	1215.5	82.4	<39.1	1275.7	95.3	<39.1	1697.1	87.0
		2	<39.1	1411.5	81.7	<39.1	1338.2	96.1	<39.1	1577.2	84.4
		3	<39.1	1037.0	82.3	<39.1	1155.2	93.1	<39.1	1858.2	90.7
		Mean	<39.1	1221.3	82.1	<39.1	1256.4	94.8	<39.1	1710.8	87.4
	P2-007	1	1473.5	1512.2	78.0	260.1	1139.8	92.4	417.7	1074.2	86.7
		2	213.8	1541.6	78.0	493.7	1304.7	93.4	303.4	1538.6	88.8
		3	1031.7	1066.5	78.9	471.1	1293.2	94.4	543.4	1683.2	80.1
		Mean	906.3	1373.4	78.3	408.3	1245.9	93.4	421.5	1432.0	85.2

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-008	1	2964.6	1255.0	77.7	>5000	1042.0	89.9	>5000	1125.2	82.0
		2	4353.7	1439.0	81.5	>5000	1113.3	92.3	>5000	1694.3	83.6
		3	3693.7	1023.5	84.3	>5000	1158.0	90.2	>5000	1713.0	90.2
		Mean	3670.7	1239.2	81.2	>5000	1104.4	90.8	>5000	1510.8	85.3
	P2-009	1	>5000	1496.4	82.8	3871.5	1151.4	93.5	>5000	1806.1	86.3
		2	>5000	1594.9	85.4	3446.8	1024.9	93.8	>5000	1367.3	96.0
		3	4537.5	1281.1	83.5	3667.1	1322.4	90.9	>5000	1895.4	96.9
		Mean	>4538	1457.5	83.9	3661.8	1166.2	92.7	>5000	1689.6	93.1
	P2-010	1	<39.1	1540.9	84.0	118.9	1067.7	92.6	<39.1	1777.8	88.9
		2	<39.1	1295.2	94.3	176.1	1118.8	93.3	<39.1	1579.1	87.4
		3	<39.1	1386.7	79.4	119.9	1123.3	92.5	<39.1	1718.6	97.2
		Mean	<39.1	1407.6	85.9	138.3	1103.3	92.8	<39.1	1691.8	91.2
	P2-011	1	145.2	1501.3	88.9	125.5	1211.8	96.1	143.9	1034.8	86.1
		2	116.4	1393.5	82.9	98.6	1257.1	90.8	178.1	1385.0	80.7
		3	128.9	1072.0	86.3	120.9	1198.6	94.9	207.1	1927.9	93.4
		Mean	130.2	1322.3	86.0	115.0	1222.5	93.9	176.4	1449.2	86.7
	P2-012	1	2889.7	1435.1	82.9	4212.3	1063.8	95.8	4402.0	1142.0	83.0
		2	4256.1	1434.2	84.8	4209.6	1024.2	90.6	4443.7	1429.3	85.9
		3	1751.9	1026.6	79.6	4355.5	1059.8	96.1	4922.0	1793.7	92.7
		Mean	2965.9	1298.6	82.4	4259.1	1049.3	94.2	4589.2	1455.0	87.2
	P2-013	1	1201.6	1320.9	80.3	306.0	1041.1	92.1	228.5	1024.4	92.6
		2	430.7	1010.5	84.6	563.6	1019.6	89.8	199.4	1314.5	84.6
		3	479.1	1049.6	82.1	139.2	1211.5	93.3	105.7	1641.1	92.2
		Mean	703.8	1127.0	82.3	336.3	1090.7	91.7	177.9	1326.7	89.8
	P2-014	1	103.6	1453.0	81.2	<39.1	1238.1	92.2	106.4	1686.7	100.0
		2	127.6	1603.0	81.5	44.4	1251.3	91.1	103.4	1855.9	82.4
		3	114.4	1358.0	79.6	40.9	1082.2	90.3	92.2	1866.5	90.8
		Mean	115.2	1471.3	80.8	<44.4	1190.5	91.2	100.7	1803.0	91.1

Chemical code		Run	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-015	1	1099.1	1314.1	79.5	422.3	1099.2	94.0	1470.3	1769.5	84.1
		2	1256.0	1271.2	84.2	240.9	1340.9	93.3	1006.2	1654.2	85.0
		3	72.6	1112.2	83.1	188.6	1102.7	95.4	1363.3	1838.4	93.5
		Mean	809.2	1232.5	82.3	283.9	1180.9	94.2	1279.9	1754.0	87.5
	P2-016	1	608.3	1494.6	83.3	710.0	1028.3	91.5	1098.8	1770.5	96.0
		2	759.6	1583.6	85.5	666.6	1305.6	93.6	1426.1	1817.5	88.9
		3	448.8	1079.7	78.2	512.5	1115.0	93.4	1408.7	1973.1	93.0
		Mean	605.6	1386.0	82.3	629.7	1149.6	92.8	1311.2	1853.7	92.6
	P2-017	1	54.5	1265.8	83.9	45.1	1438.8	93.0	<39.1	1035.0	94.0
		2	58.7	1472.3	83.9	42.8	1025.3	89.0	<39.1	1943.7	88.2
		3	86.4	1044.4	81.9	43.1	1143.2	92.5	<39.1	1790.5	90.6
		Mean	66.5	1260.8	83.2	43.7	1202.4	91.5	<39.1	1589.7	90.9
	P2-018	1	65.0	1506.6	85.9	<39.1	1154.1	95.1	<39.1	1078.8	111.2
		2	40.5	1627.7	88.2	<39.1	1192.5	92.2	<39.1	1803.6	103.6
		3	<39.1	1115.8	80.8	<39.1	1106.2	94.4	<39.1	1549.1	87.9
		Mean	<65.0	1416.7	85.0	<39.1	1150.9	93.9	<39.1	1477.2	100.9
	P2-019	1	397.1	1120.4	78.2	818.7	1071.7	93.1	1104.8	1654.5	93.9
		2	399.7	1564.6	78.3	223.9	1224.1	88.9	1207.1	1779.8	86.6
		3	397.1	1079.5	82.4	212.8	1298.5	97.9	1314.7	1726.8	91.0
		Mean	398.0	1254.8	79.6	418.5	1198.1	93.3	1208.9	1720.4	90.5
	P2-020	1	2858.3	1458.8	80.7	1820.8	1200.6	93.4	3774.7	1839.0	9.9
		2	3453.8	1570.7	82.6	2723.1	1236.5	91.3	3658.6	1589.1	92.8
		3	2696.2	1063.1	79.3	1784.2	1153.6	91.4	3081.5	1820.7	99.2
		Mean	3002.8	1364.2	80.9	2109.4	1196.9	92.0	3504.9	1749.6	67.3

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

*: Each IC50 for test substances, relative controls and positive controls was expressed as an average every set

Table 10.4. The IC_{50} for test substances, relative controls and positive controls in the SIRC-CVS: TEA validation Phase II study

Chemical code		Set	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase II A	P2-001	1	141.0	1478.8	85.8	288.1	1295.9	88.8	298.3	1512.2	84.6
		2	71.7	1235.6	88.3	260.2	1327.0	93.8	266.6	1453.6	88.4
		3	101.8	1589.1	87.9	452.3	1178.0	91.8	282.9	1304.2	84.8
	P2-002	1	>5000	1602.3	84.5	>5000	1402.4	89.9	>5000	1755.0	93.7
		2	>5000	1281.5	89.3	>3989.1	1330.8	96.2	>5000	1198.7	92.5
		3	>5000	1384.3	88.2	>5000	1274.0	92.3	>5000	1525.3	89.5
	P2-003	1	4130.0	1517.0	86.2	3188.9	1320.6	90.9	>4673	1808.9	90.6
		2	3899.0	1452.1	88.7	3654.8	1357.8	93.2	>5000	1348.5	89.7
		3	3931.7	1513.4	88.7	3025.0	1290.3	92.4	>5000	1338.9	86.6
	P2-004	1	1342.3	1518.2	89.4	1147.5	1414.0	93.2	1409.6	1525.1	80.3
		2	925.5	1352.4	90.7	778.5	1184.5	92.5	1217.9	1532.9	91.5
		3	1151.9	1440.1	86.4	1061.5	1295.8	90.8	1099.6	1508.1	86.6
	P2-005	1	1791.6	1362.5	84.6	1949.6	1273.8	88.4	>5000	1837.1	95.9
		2	1783.2	1288.3	88.2	3630.8	1379.6	88.4	>5000	1532.1	90.9
		3	1868.6	1341.9	85.5	>3506.9	1256.4	90.3	>4952	1264.6	91.3
Phase IIB	P2-006	1	<39.1	1223.4	85.7	<39.1	1323.8	86.0	<39.1	1768.3	91.5
		2	<39.1	1334.5	83.6	<39.1	1122.4	92.8	<39.1	1692.4	98.8
		3	<39.1	1221.3	82.1	<39.1	1256.4	94.8	<39.1	1710.8	87.4
	P2-007	1	266.2	1452.9	85.3	99.3	1227.3	82.9	519.1	1613.9	90.7
		2	506.5	1312.7	86.2	110.3	1214.5	88.4	421.7	1718.1	92.5
		3	906.3	1373.4	78.3	408.3	1242.6	93.4	421.5	1432.0	85.2
	P2-008	1	>5000	1417.2	88.1	>2346	1221.3	86.5	>5000	1672.7	89.0
		2	>2599	1356.7	80.4	>5000	1225.0	90.0	>5000	1715.7	93.5
		3	3670.7	1239.2	81.2	>5000	1104.4	90.8	>5000	1510.8	85.3
	P2-009	1	>4865	1345.6	86.8	3561.9	1227.5	89.9	>5000	1524.3	97.0
		2	>3048	1215.8	83.6	3528.2	1248.6	89.3	>5000	1681.5	95.3
		3	>4538	1457.5	83.9	3661.8	1166.2	92.7	>5000	1689.6	93.1

Chemical code		Set	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase IIB	P2-010	1	<39.1	1618.9	89.4	<51.4	1220.4	89.2	<39.1	1421.6	96.7
		2	<39.1	1333.9	85.3	<39.1	1237.9	90.8	<39.1	1619.0	92.9
		3	<39.1	1407.6	85.9	138.3	1103.3	92.8	<39.1	1691.8	91.2
	P2-011	1	239.4	1427.7	85.7	109.8	1160.1	89.7	227.0	1755.5	93.2
		2	123.7	1298.3	83.5	121.5	1095.0	91.4	243.7	1543.4	97.4
		3	130.2	1322.3	86.0	115.0	1222.5	93.9	176.4	1449.2	86.7
	P2-012	1	3575.5	1372.6	84.3	3615.7	1188.5	87.3	4386.2	1652.8	107.6
		2	3630.4	1269.0	82.4	3721.6	1256.3	91.4	4246.5	1738.9	95.7
		3	2965.9	1298.6	82.4	4259.1	1049.3	94.2	4589.2	1455.0	87.2
	P2-013	1	434.8	1470.9	87.4	398.8	1197.7	88.6	352.8	1670.1	95.8
		2	1055.6	1329.7	85.6	544.1	1339.7	92.4	298.5	1600.6	95.0
		3	703.8	1127.0	82.3	336.3	1090.7	91.7	177.9	1326.7	89.8
	P2-014	1	91.9	1434.9	84.7	<45.1	1135.3	90.9	55.2	1639.9	91.1
		2	82.5	1247.4	84.9	64.8	1248.8	91.0	70.3	1683.1	90.5
		3	115.2	1471.3	80.8	<44.4	1190.5	91.2	100.7	1803.0	91.1
	P2-015	1	664.0	1473.6	81.5	452.3	1142.8	82.5	1288.7	1553.5	90.0
		2	1152.2	1172.8	83.9	395.0	1203.5	93.8	1054.5	1495.2	94.4
		3	809.2	1232.5	82.3	283.9	1180.9	94.2	1279.9	1754.0	87.5
	P2-016	1	796.7	1300.1	82.5	618.0	1203.5	89.0	1419.9	1669.0	95.5
		2	715.1	1364.6	87.1	632.6	1168.6	87.8	1191.1	1850.5	96.9
		3	605.6	1386.0	82.3	629.7	1149.6	92.8	1311.2	1853.7	92.6
	P2-017	1	57.7	1298.2	86.8	68.5	1282.1	90.2	49.4	1699.5	97.0
		2	92.7	1332.4	83.9	44.0	1177.1	92.1	90.2	1487.1	94.1
		3	66.5	1260.8	83.2	43.7	1202.4	91.5	<39.1	1589.7	90.9
	P2-018	1	<46.4	1226.2	86.1	<39.1	1305.8	93.8	<39.1	1606.1	94.5
		2	69.9	1372.1	82.8	<39.1	1145.8	88.1	<39.1	1644.9	92.8
		3	<65.0	1416.7	85.0	<39.1	1150.9	93.9	<39.1	1477.2	100.9
	P2-019	1	359.4	1272.4	84.8	386.0	1387.1	89.9	1471.8	1679.2	98.20
		2	567.0	1301.3	85.8	94.8	1312.0	88.7	1268.1	1676.5	89.3
		3	398.0	1254.8	79.6	418.5	1198.1	93.3	1208.9	1720.4	90.5

Chemical code		Set	Laboratory A			Laboratory B			Laboratory C		
			IC50 μg/mL			IC50 μg/mL			IC50 μg/mL		
			Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
Phase II B	P2-020	1	3074.0	1232.7	87.1	1729.6	1392.9	88.0	4013.2	1721.6	94.6
		2	2633.5	1331.5	86.6	2187.3	1228.0	90.5	3593.0	1851.4	95.8
		3	3002.8	1364.2	80.9	2109.4	1196.9	92.0	3504.9	1749.6	67.3

*: Each IC50 for test substances, relative controls and positive controls was expressed as an average every run

Table 10.5. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using the Phase II study in laboratory A

Chemical code	Name of test substance	Laboratory A			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	Piperonylbutoxide	P	P	P	1
P2-002	2,5-Dimethylhexanediol	N	N	N	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	Ammonium nitrate	P	P	P	1
P2-005	Potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-Trichlorocarbanilide	P	P	P	1
P2-007	1-Bromohexane	P	P	P	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	Propylene glycol propyl ether	N	N	N	1
P2-010	Ethyl thioglycolate	P	P	P	1
P2-011	Sodium oxalate	P	P	P	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-Bromo-4-chlorobutane	P	P	P	1
P2-014	Sodium hydrogensulfite	P	P	P	1
P2-015	Isobutyraldehyde	P	P	P	1
P2-016	1-Naphthaleneacetic acid	P	P	P	1
P2-017	Propyl 4-hydroxybenzoate	P	P	P	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	P	P	P	1
P2-019	Camphene	P	P	P	1
P2-020	Cyclopentanol	N	N	N	1

*N: Negative, P: Positive, 1: Concordant results

Table 10.6. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using the Phase II study in laboratory B

Chemical code	Name of test substance	LaboratoryB			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	Piperonylbutoxide	P	P	P	1
P2-002	2,5-Dimethylhexanediol	N	N	N	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	Ammonium nitrate	P	P	P	1
P2-005	Potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-Trichlorocarbanilide	P	P	P	1
P2-007	1-Bromohexane	P	P	P	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	Propylene glycol propyl ether	N	N	N	1
P2-010	Ethyl thioglycolate	P	P	P	1
P2-011	Sodium oxalate	P	P	P	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-Bromo-4-chlorobutane	P	P	P	1
P2-014	Sodium hydrogensulfite	P	P	P	1
P2-015	Isobutyraldehyde	P	P	P	1
P2-016	1-Naphthaleneacetic acid	P	P	P	1
P2-017	Propyl 4-hydroxybenzoate	P	P	P	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	P	P	P	1
P2-019	Camphene	P	P	P	1
P2-020	Cyclopentanol	N	N	N	1

*N: Negative, P: Positive, 1: Concordant results

Table 10.7. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using the Phase II study in laboratory C

Chemical code	Name of test substance	Laboratory C			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	Piperonylbutoxide	P	P	P	1
P2-002	2,5-Dimethylhexanediol	N	N	N	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	Ammonium nitrate	P	P	P	1
P2-005	Potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-Trichlorocarbanilide	P	P	P	1
P2-007	1-Bromohexane	P	P	P	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	Propylene glycol propyl ether	N	N	N	1
P2-010	Ethyl thioglycolate	P	P	P	1
P2-011	Sodium oxalate	P	P	P	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-Bromo-4-chlorobutane	P	P	P	1
P2-014	Sodium hydrogensulfite	P	P	P	1
P2-015	Isobutyraldehyde	P	P	P	1
P2-016	1-Naphthaleneacetic acid	P	P	P	1
P2-017	Propyl 4-hydroxybenzoate	P	P	P	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	P	P	P	1
P2-019	Camphene	P	P	P	1
P2-020	Cyclopentanol	N	N	N	1

*N: Negative, P: Positive, 1: Concordant results

Table 10.8. Eye irritation potential of test substances in the SIRC-CVS:TEA validation Phase II study

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
P2-001	Piperonylbutoxide	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-002	2,5-Dimethylhexanediol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-004	Ammonium nitrate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-005	Potassium tetrafluoroborate	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-006	3,4,4'-Trichlorocarbanilide	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-007	1-Bromohexane	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-009	Propylene glycol propyl ether	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
P2-010	Ethyl thioglycolate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-011	Sodium oxalate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-012	2-Phospho-L-ascorbic acid trisodium salt	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-013	1-Bromo-4-chlorobutane	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-014	Sodium hydrogensulfite	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-015	Isobutyraldehyde	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-016	1-Naphthaleneacetic acid	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-017	Propyl 4-hydroxybenzoate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-019	Camphene	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
P2-020	Cyclopentanol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	

*N: Negative, P: Positive

Table 11.1. The IC_{50}s for test substances, relative controls and positive controls at laboratory A in the SIRC-CVS:TEA validation Phase III study

No.	Chemical Code	Test Substance (IC50 μg/mL)			Relative Control (IC50 μg/mL)			Positive Control (IC50 μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
1	P3-003	212.8	259.2	236.0	1069.3	1081.9	1075.6	93.7	90.2	92.0
2	P3-005	>5000	>5000	>5000	1057.7	1275.5	1166.6	86.7	95.5	91.1
3	P3-010	1323.3	1653.3	1488.3	1040.3	1053.7	1047.0	88.3	91.4	89.9
4	P3-012	1460.9	1541.2	1501.1	1040.1	1088.5	1064.3	87.3	93.8	90.6
5	P3-019	155.8	202.5	179.2	1096.7	1219.7	1158.2	86.3	90.6	88.5
6	P3-020	1347.4	1588.5	1468.0	1076.0	1044.6	1060.3	85.6	94.4	90.0
7	P3-022	<39.1	42.4	<42.4	1095.4	1159.1	1127.3	86.9	90.8	88.9
8	P3-024	151.8	182.9	167.4	1039.0	1095.2	1067.1	89.2	91.4	90.3
9	P3-027	484.9	869.1	677.0	1040.5	1417.7	1229.1	86.7	91.2	89.0
10	P3-028	<39.1	<39.1	<39.1	1037.2	1101.0	1069.1	89.9	90.5	90.2
11	P3-029	42.2	46.0	44.1	1073.7	1082.1	1077.9	89.8	91.5	90.7
12	P3-033	>5000	>5000	>5000	1010.5	1257.2	1133.9	94.0	85.9	90.0
13	P3-042	<39.1	<39.1	<39.1	1206.6	1133.1	1169.9	83.7	92.2	88.0
14	P3-045	117.7	128.7	123.2	1031.8	1121.7	1076.8	78.1	91.9	85.0
15	P3-073	444.1	470.6	457.4	1085.6	1084.0	1084.8	80.3	90.7	85.5
16	P3-074	52.1	47.5	49.8	1056.3	1063.6	1060.0	88.2	85.2	86.7
17	P3-075	<39.1	<39.1	<39.1	1203.1	1010.6	1106.9	87.0	91.2	89.1
18	P3-076	946.3	761.9	854.1	1038.1	1054.5	1046.3	94.2	80.6	87.4
19	P3-077	>5000	>5000	>5000	1194.4	1253.6	1224.0	91.5	92.0	91.8
20	P3-078	1941.1	2253.7	2097.4	1068.9	1138.0	1103.5	96.8	91.6	94.2
21	P3-079	>5000	>5000	>5000	1033.5	1412.3	1222.9	84.2	92.7	88.5
22	P3-080	1082.2	1666.5	1374.4	1010.2	1030.0	1020.1	90.9	85.8	88.4
23	P3-081	84.6	352.0	218.3	1114.0	1130.4	1122.2	90.8	91.2	91.0
24	P3-082	777.3	857.3	817.3	1152.5	1335.8	1244.2	85.7	91.7	88.7
25	P3-083	>5000	>5000	>5000	1090.9	1168.3	1129.6	92.1	93.3	92.7
26	P3-084	4903.1	>5000	>4903	1073.7	1446.4	1260.1	87.3	89.7	88.5
27	P3-085	3331.8	3672.4	3502.1	1036.1	1149.1	1092.6	84.4	92.8	88.6
28	P3-086	2243.5	3624.5	2934.0	1119.6	1151.0	1135.3	92.8	92.3	92.6

No.	Chemical Code	Test Sbstance (IC_{50} μg/mL)			Relative Control (IC_{50} μg/mL)			Positive Control (IC_{50} μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
29	P3-087	>5000	3648.0	>3648	1032.8	1408.9	1220.9	87.6	88.0	87.8
30	P3-088	>5000	>5000	>5000	1085.9	1201.1	1143.5	86.6	90.2	88.4
31	P3-089	>5000	>5000	>5000	1059.5	1076.6	1068.1	90.7	93.2	92.0
32	P3-090	<39.1	<39.1	<39.1	1172.0	1186.0	1179.0	89.1	90.8	90.0
33	P3-093	682.6	866.2	774.4	1053.8	1186.7	1120.3	93.0	93.1	93.1
34	P3-094	1429.5	1504.2	1466.9	1043.0	1277.7	1160.4	87.2	95.8	91.5
35	P3-095	1864.4	1696.9	1780.7	1149.4	1065.1	1107.3	91.4	92.4	91.9
36	P3-096	94.3	67.0	80.7	1058.7	1040.7	1049.7	88.1	89.5	88.8
37	P3-097	132.4	274.5	203.5	1085.7	1103.2	1094.5	88.7	84.6	86.7
38	P3-098	190.0	168.8	179.4	1146.3	1024.9	1085.6	87.1	89.4	88.3
39	P3-099	1133.6	1574.3	1354.0	1016.0	1209.4	1112.7	86.8	92.3	89.6
40	P3-100	2043.9	2606.8	2325.4	1031.6	1100.9	1066.3	91.0	91.0	91.0

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

Table 11.2. The IC50s for test substances, relative controls and positive controls at laboratory B in the SIRC-CVS:TEA validation Phase III study

No.	Chemical Code	Test Substance (IC50 μg/mL)			Relative Control (IC50 μg/mL)			Positive Control (IC50 μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
1	P3-001	119.6	122.6	121.1	1673.8	1571.9	1622.9	89.8	90.4	90.1
2	P3-003	695.2	672.8	684.0	1352.7	1038.2	1195.5	93.9	91.4	92.7
3	P3-005	>5000	>5000	>5000	1077.8	1260.8	1169.3	87.3	86.8	87.1
4	P3-008	17.7	22.8	20.3	1186.9	1573.0	1380.0	91.6	95.4	93.5
5	P3-010	626.8	535.2	581.0	1394.2	1488.5	1441.4	91.8	91.4	91.6
6	P3-012	814.2	768.8	791.5	1089.7	1433.6	1261.7	89.4	86.9	88.2
7	P3-019	265.5	187.4	226.5	1193.4	1296.8	1245.1	92.3	87.1	89.7
8	P3-020	2923.4	2017.9	2470.7	1026.6	1305.7	1166.2	79.6	85.8	82.7
9	P3-024	71.7	63.1	67.4	1155.3	1095.6	1125.5	92.4	89.7	91.1
10	P3-028	6.9	11.7	9.3	1455.3	1580.9	1518.1	86.8	93.5	90.2
11	P3-029	<39.1	<39.1	<39.1	1141.6	1274.1	1207.9	80.8	88.6	84.7
12	P3-033	4864.9	4126.6	4495.8	1120.4	1081.2	1100.8	92.1	85.3	88.7
13	P3-043	163.3	191.9	177.6	1572.9	1387.2	1480.1	78.1	91.5	84.8
14	P3-046	783.5	346.3	564.9	1281.8	1239.3	1260.6	92.8	91.3	92.1
15	P3-047	1599.2	1570.6	1584.9	1282.4	1430.4	1356.4	91.9	89.3	90.6
16	P3-048	2203.1	2105.0	2154.1	1298.6	1277.3	1288.0	91.9	92.6	92.3
17	P3-049	772.6	414.8	593.7	1668.1	1571.9	1620.0	78.4	89.7	84.1
18	P3-050	>5000	>5000	>5000	1275.1	1154.2	1214.7	92.1	86.7	89.4
19	P3-051	128.7	312.5	220.6	1334.1	1571.0	1452.6	94.9	93.1	94.0
20	P3-052	92.1	98.3	95.2	1302.2	1534.7	1418.5	94.4	89.0	91.7
21	P3-053	720.4	213.4	466.9	1068.6	1704.3	1386.5	81.6	92.8	87.2
22	P3-054	195.5	169.9	182.7	1319.0	1133.4	1226.2	89.0	91.1	90.1
23	P3-055	17.3	20.6	19.0	1071.6	1527.1	1299.4	89.9	89.8	89.9
24	P3-056	>5000	>5000	>5000	1359.1	1262.4	1310.8	87.0	84.8	85.9
25	P3-057	>5000	>5000	>5000	1173.1	1365.7	1269.4	92.3	92.5	92.4
26	P3-058	11.3	13.9	12.6	1188.3	1569.8	1379.1	87.3	88.7	88.0
27	P3-059	>5000	>5000	>5000	1101.0	1408.1	1254.6	88.9	89.5	89.2
28	P3-060	1343.6	1473.8	1408.7	1103.5	1431.3	1267.4	78.4	87.0	82.7

No.	Chemical Code	Test Substance (IC50 μg/mL)			Relative Control (IC50 μg/mL)			Positive Control (IC50 μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
29	P3-061	620.5	604.4	612.5	1084.0	1028.6	1056.3	89.5	82.7	86.1
30	P3-062	1729.4	1824.4	1776.9	1291.7	1472.4	1382.1	92.5	89.7	91.1
31	P3-063	>2500	>2500	>2500	1251.8	1457.5	1354.7	88.9	90.2	89.6
32	P3-064	1619.0	1403.1	1511.1	1262.8	1329.4	1296.1	89.9	90.0	90.0
33	P3-065	1604.1	1429.4	1516.8	1396.4	1067.3	1231.9	88.5	88.7	88.6
34	P3-066	>315*	>315*	>315*	1684.9	1646.6	1665.8	87.3	96.1	91.7
35	P3-067	875.3	807.7	841.5	1257.5	1405.5	1331.5	78.1	92.0	85.1
36	P3-068	1584.6	1468.4	1526.5	1176.9	1395.8	1286.4	93.3	87.9	90.6
37	P3-069	1276.0	1587.5	1431.8	1112.0	1368.8	1240.4	93.8	90.6	92.2
38	P3-070	3.6	14.0	8.8	1553.3	1683.6	1618.5	80.3	91.1	85.7
39	P3-071	97.5	70.7	84.1	1445.1	1194.8	1320.0	95.5	90.0	92.8
40	P3-072	57.2	60.1	58.7	1076.2	1605.6	1340.9	93.4	91.4	92.4

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

***: Not obtained at IC50 value due to precipitation**

Table 11.3. The IC50s for test substances, relative controls and positive controls at laboratory C in the SIRC-CVS:TEA validation Phase III study

No.	Chemical Code	Test Substance (IC50 μg/mL)			Relative Control (IC50 μg/mL)			Positive Control (IC50 μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
1	P3-002	>2500	>2500	>2500	1628.0	1753.1	1690.6	126.1	123.5	124.8
2	P3-003	>2500	>2500	>2500	1177.8	1413.7	1295.8	87.5	102.0	94.8
3	P3-004	105.8	244.3	175.1	1085.2	1618.1	1351.7	123.8	126.5	125.2
4	P3-005	>5000	>5000	>5000	1256.9	1375.1	1316.0	109.0	119.6	114.3
5	P3-006	845.8	1302.6	1074.2	1248.6	1555.9	1402.3	129.5	126.0	127.8
6	P3-007	77.4	35.4	56.4	1181.1	1747.4	1464.3	136.5	129.9	133.2
7	P3-009	>2500	>2500	>2500	1256.9	1665.8	1461.4	109.0	111.9	110.5
8	P3-010	3464.6	2748.7	3106.7	1831.1	1108.6	1469.9	120.6	87.5	104.1
9	P3-011	<39.1	<39.1	<39.1	1285.6	1418.2	1351.9	180.8	137.3	159.1
10	P3-012	3210.0	2765.9	2988.0	1851.8	1415.3	1633.6	117.1	119.5	118.3
11	P3-013	>5000	>5000	>5000	1186.4	1123.9	1155.2	125.7	140.6	133.2
12	P3-014	>5000	>5000	>5000	1400.1	1064.4	1232.3	114.8	133.4	124.1
13	P3-015	328.0	218.1	273.1	1071.9	1250.0	1161.0	141.6	133.2	137.4
14	P3-016	<39.1	40.4	<40.4	1017.5	1013.8	1015.7	140.1	130.6	135.4
15	P3-017	>2500	>2500	>2500	1353.9	1365.5	1359.7	123.7	138.3	131.0
16	P3-018	>5000	>5000	>5000	1154.1	1269.4	1211.8	116.7	121.1	118.9
17	P3-019	285.1	246.0	265.6	1159.4	1913.3	1536.4	121.2	118.8	120.0
18	P3-020	1946.0	2991.2	2468.6	1864.2	1573.0	1718.6	129.6	113.2	121.4
19	P3-021	<39.1	39.8	<39.8	1115.0	1166.5	1140.8	120.2	143.2	131.7
20	P3-023	1938.6	1664.5	1801.6	1340.7	1025.1	1182.9	107.1	128.3	117.7
21	P3-024	172.9	55.3	114.1	1182.3	1678.2	1430.3	136.1	90.9	113.5
22	P3-025	>5000	>5000	>5000	1017.1	1112.3	1064.7	137.2	124.9	131.1
23	P3-026	<39.1	<39.1	<39.1	1674.1	1106.5	1390.3	120.2	129.0	124.6
24	P3-028	<39.1	<39.1	<39.1	1822.5	1787.8	1805.2	116.7	82.6	99.7
25	P3-029	55.7	33.2	44.5	1786.4	1433.9	1610.2	128.0	113.9	121.0
26	P3-030	<19.5	<19.5	<19.5	1061.0	1169.4	1115.2	124.9	136.4	130.7
27	P3-031	85.9	86.5	86.2	1259.6	1112.6	1186.1	111.5	123.1	117.3
28	P3-032	41.7	55.9	48.8	1279.5	1369.2	1324.4	123.9	129.1	126.5

No.	Chemical Code	Test Sbstance (IC50 μg/mL)			Relative Control (IC50 μg/mL)			Positive Control (IC50 μg/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
29	P3-033	>5000	>5000	>5000	1133.0	1794.7	1463.9	114.7	83.9	99.3
30	P3-034	>2500	>2500	>2500	1244.8	1743.9	1494.4	141.3	98.9	120.1
31	P3-035	103.3	184.5	143.9	1269.4	1754.2	1511.8	105.9	109.2	107.6
32	P3-036	931.4	940.2	935.8	1418.2	1676.3	1547.3	148.0	119.4	133.7
33	P3-037	>2500	>2500	>2500	1389.2	1181.2	1285.2	114.0	122.7	118.4
34	P3-038	1786.6	2253.1	2019.9	1070.7	1288.2	1179.5	121.6	119.0	120.3
35	P3-039	919.1	922.5	920.8	1286.3	1143.1	1214.7	126.8	131.7	129.3
36	P3-040	62.5	56.2	59.4	1173.4	1116.6	1145.0	134.0	123.1	128.6
37	P3-041	<39.1	<39.1	<39.1	1456.5	1159.6	1308.1	138.8	146.3	142.6
38	P3-044	3114.8	2076.0	2595.4	1801.2	1154.5	1477.9	118.4	127.2	122.8
39	P3-091	<39.1	<39.1	<39.1	1356.1	1241.5	1298.8	129.1	135.6	132.4
40	P3-092	149.6	443.1	296.4	1193.8	1143.7	1168.8	119.0	121.4	120.2

*: If cell viability was greater than 50% at maximal concentration of 5,000 μg/mL, the result for that test chemical was IC50 > 5,000 μg/mL. Also, if the cell viability was less than 50% at a minimal concentration of 39.1 μg/mL, the result for that test chemical was IC50 < 39.1 μg/mL. IC50 at other maximal and minimal concentrations of test chemicals were expressed in the same manner.

Table 12. Inter-laboratory reproducibility of the SIRC-CVS:TEA method in the Phase II study

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P2-001	Piperonylbutoxide	P	P	P	1
P2-002	2,5-Dimethylhexanediol	N	N	N	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	Ammonium nitrate	P	P	P	1
P2-005	Potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-Trichlorocarbanilide	P	P	P	1
P2-007	1-Bromohexane	P	P	P	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	Propylene glycol propyl ether	N	N	N	1
P2-010	Ethyl thioglycolate	P	P	P	1
P2-011	Sodium oxalate	P	P	P	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-Bromo-4-chlorobutane	P	P	P	1
P2-014	Sodium hydrogensulfite	P	P	P	1
P2-015	Isobutyraldehyde	P	P	P	1
P2-016	1-Naphthaleneacetic acid	P	P	P	1
P2-017	Propyl 4-hydroxybenzoate	P	P	P	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	P	P	P	1
P2-019	Camphene	P	P	P	1
P2-020	Cyclopentanol	N	N	N	1

* N: Negative, P: Positive, 1: The results from all three laboratories were concordant.

Table 13. Inter-laboratory reproducibility of the SIRC-CVS:TEA method in the Phase III study

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P3-003	Dipropyl disulfide	P	P	N	0
P3-005	2-(2-Ethoxyethoxy)ethanol	N	N	N	1
P3-010	n,n-Dimethylguanidine sulfate	N	P	N	0
P3-012	Polyethylene hydrogenated castor oil (40E.O.)	N	P	N	0
P3-019	Diethyl toluamide	P	P	P	1
P3-020	4-Nitrobenzoic acid	N	N	N	1
P3-024	2-Amino-3-hydroxy pyridine	P	P	P	1
P3-028	Tetraethylene glycol	P	P	P	1
P3-029	Dodecanoic acid	P	P	P	1
P3-033	gamma-Butyrolactone	N	N	N	1

* N: Negative, P: Positive, 1: All laboratories' judge agreed, 0: Only two laboratories' judge agreed

Table 14. Eye irritation potential of test substances in the SIRC-CVS:TEA validation Phase III study

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation
P3-001	B	2-Ethoxyethyl methacrylate	P	P	P
P3-002	C	iso-Octylthioglycolate	N	N	N
P3-003	A/B/C	Dipropyl disulfide	P/P/N	P/P/N	P
P3-004	C	1-Bromo-octane	P	P	P
P3-005	A/B/C	2-(2-Ethoxyethoxy)ethanol	N/N/N	N/N/N	N
P3-006	C	Dioctyl ether	P	P	P
P3-007	C	3-Phenoxybenzyl alcohol	P	P	P
P3-008	B	Glycidyl methacrylate	P	P	P
P3-009	C	2-Ethylhexylthioglycolate	N	N	N
P3-010	A/B/C	n,n-Dimethylguanidine sulfate	N/P/N	N/P/N	N
P3-011	C	6-Hydroxy-2,4,5-triaminopyrimidine Sulfate	P	P	P
P3-012	A/B/C	Polyethylene hydrogenated castor oil (40E.O.)	N/P/N	N/P/N	N
P3-013	C	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-ｙｌ）-4-（1,1,3,3-tetramethylbutyl)phenol)	N	N	N
P3-014	C	Cellulose, 2-(2-hydroxy-3-(trimethylammonio) propoxy) ethyl ether chloride	N	N	N
P3-015	C	3,4-Dimethoxy benzaldehyde	P	P	P
P3-016	C	3-Chloropropionitrile	P	P	P
P3-017	C	2-Methyl-1-pentanol	N	N	N
P3-018	C	Ethyl-2-methylacetoacetate	N	N	N
P3-019	A/B/C	Diethyl toluamide	P/P/P	P/P/P	P
P3-020	A/B/C	4-Nitrobenzoic acid	N/N/N	N/N/N	N
P3-021	C	Sodium chloroacetate	P	P	P
P3-022	A	2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	P	P	P
P3-023	C	3,3-Dithiodipropionic acid	N	N	N
P3-024	A/B/C	2-Amino-3-hydroxy pyridine	P/P/P	P/P/P	P
P3-025	C	Sodium benzoate	N	N	N
P3-026	C	Methylthioglycolate	P	P	P
P3-027	A	3-(2-Aminoethylamino)propyl]trimethoxysilane	P	P	P
P3-028	A/B/C	Tetraethylene glycol	P/P/P	P/P/P	P
P3-029	A/B/C	Dodecanoic acid	P/P/P	P/P/P	P
P3-030	C	1,2-Benzisothiazol-3(2H)-one	P	P	P

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation
P3-031	C	2-Hydroxy-1,4-naphthoquinone	P	P	P
P3-032	C	Disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	P	P	P
P3-033	A/B/C	gamma-Butyrolactone	N/N/N	N/N/N	N
P3-034	C	1-Methylpropyl benzene	N	N	N
P3-035	C	4-(Methylmercapto)benzaldehyde	P	P	P
P3-036	C	1,9-Decaine	P	P	P
P3-037	C	2,4-Dimethyl-3-pentanol	N	N	N
P3-038	C	1-Ethyl-3-methylimidazolium ethylsulfate	N	N	N
P3-039	C	1,2,4-Triazole,sodium salt	P	P	P
P3-040	C	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl) bis[2,6-dibromophenol]	P	P	P
P3-041	C	Benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCL	P	P	P
P3-042	A	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	P	P	P
P3-043	B	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	P	P	P
P3-044	C	Isopropyl acetoacetate	N	N	N
P3-045	A	(3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	P	P	P
P3-046	B	1-Octanol	P	P	P
P3-047	B	2-Benzyloxyethanol	N	N	N
P3-048	B	Butanol	N	N	N
P3-049	B	Isobutyl alcohol	P	P	P
P3-050	B	Isopropyl alcohol	N	N	N
P3-051	B	Myristyl alcohol	P	P	P
P3-052	B	Hexyl cinnamic aldehyde	P	P	P
P3-053	B	n-Butanal	P	P	P
P3-054	B	Monoethanolamine	P	P	P
P3-055	B	m-Phenylenediamine	P	P	P
P3-056	B	Ethyl acetate	N	N	N
P3-057	B	Isopropyl myristate	N	N	N
P3-058	B	Methoxyethyl acrylate	P	P	P
P3-059	B	Methyl acetate	N	N	N
P3-060	B	Methyl cyanoacetate	N	N	N

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation
P3-061	B	Imidazole	P	P	P
P3-062	B	Pyridine	N	N	N
P3-063	B	Isopropyl bromide	N	N	N
P3-064	B	Cyclohexanone	N	N	N
P3-065	B	2-Methylbutyric acid	N	N	N
P3-066	B	Calcium thioglycolate trihydrate	–	–	–
P3-067	B	Citric acid	P	P	P
P3-068	B	Potassium sorbate	N	N	N
P3-069	B	Sodium salicylate	N	N	N
P3-070	B	Distearyldimethyl ammonium chloride	P	P	P
P3-071	B	n-Lauroylsarcosine sodium salt	P	P	P
P3-072	B	Sodium lauryl sulfate	P	P	P
P3-073	A	Triton X-100 (5%)	P	P	P
P3-074	A	2-Ethylhexyl p-dimethylaminobenzoate	P	P	P
P3-075	A	Promethazine hydrochloride	P	P	P
P3-076	A	2-Ethyl-1-hexanol	P	P	P
P3-077	A	3-Methoxy-1.2-propanediol	N	N	N
P3-078	A	Cyclohexanol	N	N	N
P3-079	A	Ethanol	N	N	N
P3-080	A	n-Hexanol	N	N	N
P3-081	A	3,3-Dimethylpentane	P	P	P
P3-082	A	Methyl cyclopentane	P	P	P
P3-083	A	Toluene	N	N	N
P3-084	A	Acetone	N	N	N
P3-085	A	Gluconolactone	N	N	N
P3-086	A	Methyl amyl ketone (2-heptanol)	N	N	N
P3-087	A	Methyl ethyl ketone (2-butanone)	N	N	N
P3-088	A	Methyl isobutyl ketone(4-methyl 2-pentanol)	N	N	N
P3-089	A	Glycerol	N	N	N
P3-090	A	Cetylpyridinium bromide	P	P	P
P3-091	C	Triton X-100	P	P	P
P3-092	C	Tween20	P	P	P

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation
P3-093	A	Sodium hydroxide	P	P	P
P3-094	A	Glycolic acid	N	N	N
P3-095	A	3,3-Dithiodipropionic acid	N	N	N
P3-096	A	Sucrose fatty acid ester	N	N	N
P3-097	A	methyl para-Hydroxybenzoate	P	P	P
P3-098	A	Silicic acid	P	P	P
P3-099	A	Benzyl alcohol	P	P	P
P3-100	A	Lactic acid	N	N	N

*N: Negative, P: Positive, NA: Not applicable

** Eye irritation potential of common test substances were expressed as a representative of three laboratories.

Table 15. Overall analysis by the judgment based on IC_{50} value of Triethanolamine (TEA) in UN GHS classification system in a bottom-up approach and top-down approach

Regulatory System	a Bottom-up Approach	a Top-down Approach
Accuracy	55.2% (64/116)	53.4% (62/116)
Sensitivity	60.0% (42/70)	71.4% (20/28)
Specificity	47.8% (22/46)	47.7% (42/88)
False Negative Rate	40.0% (28/70)	28.6% (8/28)
False Positive Rate	52.2% (24/46)	52.3% (46/88)

Table 16. Overall analysis by the judgement based on IC50 values in UN GHS classification system in a bottom-up approach

Regulatory System	Judgement by IC50 value of triethanolamine	Judgement by IC50 at 1600 ug/mL
Accuracy	55.2% (64/116)	58.9% (66/112)
Sensitivity	60.0% (42/70)	69.1% (47/68)
Specificity	47.8% (22/46)	43.2% (19/44)
False Negative Rate	40.0% (28/70)	30.9% (21/68)
False Positive Rate	52.2% (24/46)	56.8% (25/44)
Positive Predictive	63.6% (42/66)	65.3% (47/72)
Negative Predictive	44.0% (22/50)	47.5% (19/40)

Table 17. Cut-off values and their rational for selection as a criteria of the applicability domain

Property of interest	Inclusion criteria	Rationale for selection	References
Physical state	Solids and liquids only		
Molecular weight	≧180	The criteria were considered reasonable by the VMT.	Appendix 6
Purity	≧95%		
Water solubility	<1.0–10.0 g/L 10.0–100.0 g/L	Poorly or Somewhat soluble Soluble	SciFinder
Log D	≦2.88	generally less than 3.0	
Vapor pressure	≦6.0 kPa	Criteria used in SIRC-STE	ENV/JM/TG/RD (2013)19
PKa	<5.0		

Table 18. List of the test substances used in the Phase II and Phase III studies of SIRC-CVS:TEA validation and their *in vitro* judgments

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
Phase II Study														
P2-001	Piperonylbutoxide	51- 03- 6	Sigma-Aldrich	Liquid	338.44	90	0.021	4.75	5.31E-07	Ether	INCI	No	III	Positive
P2-002	2,5-Dimethylhexanediol	110-03-2	Sigma-Aldrich	Solid	146.23	97	13	0.76	4.37E-03	Alcohol	No	1	I	Negative
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	Sigma-Aldrich	Liquid	176.25	≧98.5	72	0.8	7.48E-04	Alcohol, Ether	INCI	2	III	Negative
P2-004	Ammonium nitrate	6484-52-2	Sigma-Aldrich	Solid	80.04	≧98	-	-	-	Inorganic salt	INCI	2	III	Positive
P2-005	Potassium tetrafluoroborate	14075-53-7	Sigma-Aldrich	Solid	125.9	96	-	-	-	Inorganic salt, Halogen compound	No	No	IV	Negative
P2-006	3,4,4'-Trichlorocarbanilide	101-20-2	Sigma-Aldrich	Solid	315.58	99	1.00E-04	6.07	8.89E-06	Amide, Halogen compound	INCI	No	IV	Positive
P2-007	1-Bromohexane	111-25-1	Sigma-Aldrich	Liquid	165.07	≧98.0	0.069	3.85	5.33E-01	Halogen compound	No	No	IV	Positive
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	Sigma-Aldrich	Solid	424.66	98	3.60E-05	8.97	6.13E-10	Phenol compound	No	No	IV	Negative
P2-009	Propylene glycol propyl ether	1569-01-3	Sigma-Aldrich	Liquid	118.17	99	99	0.68	1.22E-01	Alcohol, Ether	INCI	2	II	Negative
P2-010	Ethyl thioglycolate	623-51-8	Sigma-Aldrich	Liquid	120.17	97	13	1.1	3.60E-01	Thiol compound, Ester	INCI	No	III	Positive
P2-011	Sodium oxalate	62-76-0	Sigma-Aldrich	Solid	134	≧99.5	-	-	-	Organic salt (Carboxylic acid salt)	INCI	1	I	Positive
P2-012	2-Phospho-L-ascorbic acid trisodium salt	66170-10-3	Sigma	Solid	322.05	≧95.0	-	-	-	Heterocyclic compound, Organic salt, Phosphorus compound	INCI	No	III	Negative
P2-013	1-Bromo-4-chlorobutane	6940-78-9	Sigma-Aldrich	Liquid	171.46	99	0.29	2.75	3.45E-01	Halogen compound	No	No	IV	Positive

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P2-014	Sodium hydrogensulfite	7631-90-5	Sigma-Aldrich	Solid	104.06	≧58.5	-	-	-	Inorganic salt	INCI	No	III	Positive
P2-015	Isobutyraldehyde	78-84-2	Sigma-Aldrich	Liquid	72.11	98	15	0.76	1.96E+01	Aldehyde	INCI	2	III	Positive
P2-016	1-Naphthaleneacetic acid	86-87-3	Wako Pure Chemical	Solid	186.21	≧95.0	120	-0.14	4.17E-07	Carboxylic acid, Polycyclic compound	No	1	I	Positive
P2-017	Propyl 4-hydroxybenzoate	94-13-3	Sigma-Aldrich	Solid	180.2	≧98.0	1.2	2.88	1.24E-04	Ester, Phenol	INCI	No	III	Positive
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	Sigma-Aldrich	Solid	294.11	98	0.19	1.84	6.09E-06	Halogen compound, Heterocyclic compound, Ester, Ketone	No	2	III	Positive
P2-019	Camphene	79-92-5	Sigma-Aldrich	Solid	136.23	95	0.011	4.24	4.51E-01	Hydrocarbon	INCI	2	III	Positive
P2-020	Cyclopentanol	96-41-3	Sigma-Aldrich	Liquid	86.13	99	85	0.75	3.29E-01	Alcohol	No	2	II	Negative
Phase III Study														
P3-001	2-Ethoxyethyl methacrylate	2370-63-0	Sigma-Aldrich	Liquid	158.19	99	17	1.44	1.08E-01	Methacrylate, Ester, Ether	No	No	IV	Positive
P3-002	iso-Octylthioglycolate	25103-09-7	Wako Pure Chemical	Liquid	204.33	≧98.0	-	-	-	Thio compound, Ester	INCI	No	IV	Negative
P3-003	Dipropyl disulfide	629-19-6	Sigma-Aldrich	Liquid	150.31	98	2.1	4.19	9.80E-02	Disulfide compound	No	No	IV	Positive
P3-004	1-Bromo-octane	111-83-1	Sigma-Aldrich	Liquid	193.12	99	0.011	4.87	0.45	Halogen compound	No	No	IV	Positive
P3-005	2-(2-Ethoxyethoxy)ethanol	111-90-0	Sigma-Aldrich	Liquid	134.17	≧99	590	-0.42	9.77E-03	Alcohol, Ether	INCI	No	III	Negative
P3-006	Dioctyl ether	629-82-3	Sigma-Aldrich	Liquid	242.44	99	5.80E-03	7.15	-	Ether	INCI	No	IV	Positive
P3-007	3-Phenoxybenzyl alcohol	13826-35-2	Sigma-Aldrich	Liquid	200.23	98	0.19	3.39	2.95E-07	Alcohol	No	No	III	Positive

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-008	Glycidyl methacrylate	106-91-2	Sigma-Aldrich	Liquid	142.15	97	17	0.34	-	Methacrylate, Ester	No	No	III	Positive
P3-009	2-Ethylhexylthioglycolate	7659-86-1	Sigma-Aldrich	Liquid	204.33	≧95.0	0.13	3.99	8.88E-04	Thiol compound, Ester	No	No	IV	Negative
P3-010	n,n-Dimethylguanidine sulfate	598-65-2	Sigma-Aldrich	Solid	272.33	97	-	-	-	Organic salt	No	No	III	Negative
P3-011	6-Hydroxy-2,4,5-triaminopyrimidine Sulfate	1603-02-7	Wako Pure Chemical	Solid	239.21	≧95.0	679	-4.86	-	Heterocyclic compound(salt)	No	No	IV	Positive
P3-012	Polyethylene hydrogenated castor oil (40E.O.)	61788-85-0	Sigma-Aldrich	Solid	About 400	-	-		-	Surfactant (nonionic)	INCI	No	IV	Negative
P3-013	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	103597-45-1	Sigma-Aldrich	Solid	658.87	99	3.70E-08	14.32	1.51E-25	Phenol, Heterocyclic compound	No	No	IV	Negative
P3-014	Cellulose, 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride	68610-92-4	Sigma-Aldrich	Solid	>257	-	-	-	-	Quaternary ammonium compound, Synthetic polymer	INCI	No	III	Negative
P3-015	3,4-Dimethoxy benzaldehyde	120-14-9	Sigma-Aldrich	Solid	166.17	99	1.6	1.37	4.88E-04	Aldehyde	No	No	III	Positive
P3-016	3-Chloropropionitrile	542-76-7	Wako Pure Chemical	Liquid	89.52	≧98.0	23	0.29	1.44E-01	Halogen compound, Nitrile compound	No	2	III	Positive
P3-017	2-Methyl-1-pentanol	105-30-6	Sigma-Aldrich	Liquid	102.17	99	12	1.70	2.23E-01	Fatty alcohol	No	2	III	Negative
P3-018	Ethyl-2-methylacetoacetate	609-14-3	Sigma	Liquid	144.17	90	23	0.72	9.15E-02	Ester, Ketone	No	2	III	Negative
P3-019	Diethyl toluamide	134-62-3	Sigma-Aldrich	Liquid	191.27	95	7.5	2.42	1.80E-04	Amide	INCI	2	III	Positive
P3-020	4-Nitrobenzoic acid	62-23-7	Sigma-Aldrich	Solid	167.12	≧98.0	999	-1.22	1.17E-06	Carboxylic acid	No	2	III	Negative
P3-021	Sodium chloroacetate	3926-62-3	Sigma-Aldrich	Solid	116.48	98	-	-	-	Organic salt (Carboxylic acid salt), Halogen Compound	No	2	III	Positive
P3-022	2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	18472-51-0	Wako Pure Chemical	Liquid	897.76	-	-	-	-	Organic salt, Halogen Compound	INCI	2	II	Positive

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-023	3,3-Dithiodipropionic acid	1119-62-6	Wako Pure Chemical	Solid	210.27	≧97.0	1000	-3.36	1.64E-09	Carboxylic acid, Thio compound	No	2	II	Negative
P3-024	2-Amino-3-hydroxy pyridine	16867-03-1	Sigma-Aldrich	Solid	110.11	98	15	-0.44	2.33E-07	Heterocyclic compound, Amine	INCI	2	III	Positive
P3-025	Sodium benzoate	532-32-1	Sigma-Aldrich	Solid	144.1	≧99.0	-	-	-	Organic salt (Carboxylic acid salt)	INCI	2	II	Negative
P3-026	Methylthioglycolate	2365-48-2	Sigma-Aldrich	Liquid	106.14	95	30	0.59	4.77E-01	Thio compound, Ester	INCI	1	II	Positive
P3-027	3-(2-Aminoethylamino)propyl]trimethoxysilane	1760-24-3	Chemos	Liquid	222.36	97	1000	-2.33	8.21E-04	Silicon compound	No	1	I	Positive
P3-028	Tetraethylene glycol	17831-71-9	Sigma-Aldrich	Liquid	302.32	-	30	0.53	6.71E-07	Acrylate, Ether, Ester	No	1	I	Positive
P3-029	Dodecanoic acid	143-07-7	Sigma-Aldrich	Solid	200.32	≧99	16	2.56	8.81E-05	Fatty acid	INCI	1	I	Positive
P3-030	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Wako Pure Chemical	Solid	151.18	≧97.0	0.56	1.95	-	Heterocyclic compound, Thio compound, Amide	INCI	1	I	Positive
P3-031	2-Hydroxy-1,4-naphthoquinone	83-72-7	Sigma-Aldrich	Solid	174.15	97	31	-0.74	4.60E-06	Phenol compound	INCI	2	III	Positive
P3-032	Disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	27344-41-8	Wako Pure Chemical	Solid	562.56	≧98.0	-	-	-	Sulfonic acid	INCI	1	I	Positive
P3-033	gamma-Butyrolactone	96-48-0	Sigma-Aldrich	Liquid	86.09	≧99	70	-0.63	3.60E-02	Heterocyclic compound, Ketone	INCI	2	II	Negative
P3-034	1-Methylpropyl benzene	135-98-8	Wako Pure Chemical	Liquid	134.22	≧99	0.011	4.09	2.27E-01	Hydrocarbon(aromatic)	No	No	IV	Negative
P3-035	4-(Methylmercapto)benzaldehyde	3446-89-7	Sigma-Aldrich	Liquid	152.21	95	0.4	2.21	1.11E-03	Thio compound, Aldehyde	No	No	IV	Positive
P3-036	1,9-Decaine	1647-16-1	Sigma-Aldrich	Liquid	138.25	98	6.40E-04	4.99	2.79E-01	Alkene	No	No	IV	Positive
P3-037	2,4-Dimethyl-3-pentanol	3970-62-5	Sigma-Aldrich	Liquid	116.2	97	8.8	1.96	3.77E-01	Fatty alcohol	No	No	III	Negative

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-038	1-Ethyl-3-methylimidazolium ethylsulfate	342573-75-5	Alfa Aesar	Liquid	236.29	99	-	-	-	Heterocyclic compound, Inorganic salt	No	No	III	Negative
P3-039	1,2,4-Triazole,sodium salt	41253-21-8	Sigma-Aldrich	Solid	91.05	90	-	-	-	Heterocyclic compound	No	1	I	Positive
P3-040	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl) bis[2,6-dibromophenol]	4430-25-5	Sigma-Aldrich	Solid	986.55	85	4.60E-03	9.72	7.93E-23	Halogen compound, Phenol, Sulfonic acid	INCI	1	I	Positive
P3-041	Benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene) methyl-2-methy HCL	3248-91-7	Sigma-Aldrich	Solid	365.9	-	-	-	-	Organic salt	INCI	1	I	Positive
P3-042	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	72956-09-3	LKT. Labs,Inc	Solid	406.47	≧98	0.053	2.69	6.17E-19	Polycyclic compound, Alcohol	No	No	IV	Positive
P3-043	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	33089-61-1	LKT. Labs,Inc	Solid	293.41	97	2.20E-03	5.59	3.43E-09	Triazapentadien compound	No	No	IV	Positive
P3-044	Isopropyl acetoacetate	542-08-5	Wako Pure Chemical	Liquid	144.17	≧95.0	23	0.72	9.15E-02	Ester, Ketone	No	2	III	Negative
P3-045	(3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	76855-69-1	Sigma-Aldrich	Solid	287.43	98	0.4	2.37	3.43E-06	Silicon compound	No	2	II	Positive
P3-046	1-Octanol	111-87-5	Wako Pure Chemical	Liquid	130.23	≧98.0	1.2	2.88	1.52E-02	Fatty alcohol	INCI	2	II	Positive
P3-047	2-Benzyloxyethanol	622-08-2	Wako Pure Chemical	Liquid	152.19	≧97.0	26	1.11	1.19E-03	Alcohol, Ether	INCI	2	II	Negative
P3-048	Butanol	71-36-3	Wako Pure Chemical	Liquid	74.12	≧99.0	48	0.84	1.14E+00	Alcohol	INCI	1	I	Negative
P3-049	Isobutyl alcohol	78-83-1	Sigma-Aldrich	Liquid	74.12	≧99.0	68	0.68	2.19E+00	Alcohol	No	1	I	Positive
P3-050	Isopropyl alcohol	67-63-0	Wako Pure Chemical	Liquid	60.1	≧99.9	141	0.17	1.08E+01	Alcohol	INCI	2	III	Negative
P3-051	Myristyl alcohol	112-72-1	Wako Pure Chemical	Solid	214.39	≧97.0	5.80E-04	5.93	1.96E-04	Fatty alcohol	INCI	2	III	Positive
P3-052	Hexyl cinnamic aldehyde	101-86-0	Wako Pure Chemical	Liquid	216.32	≧97.0	0.039	4.87	9.29E-05	Aldehyde	INCI	2	II	Positive

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-053	n-Butanal	123-72-8	Wako Pure Chemical	Liquid	72.11	≧98.0	14	0.91	1.28E+01	Aldehyde	No	2	III	Positive
P3-054	Monoethanolamine	141-43-5	Sigma-Aldrich	Liquid	61.08	≧99.0	1000	-4.08	6.11E-02	Alkanolamine	INCI	2	III	Positive
P3-055	m-Phenylenediamine	108-45-2	TCI	Solid	108.14	>98.0	77	-0.19	4.28E-04	Amine	INCI	1	I	Positive
P3-056	Ethyl acetate	141-78-6	Sigma-Aldrich	Liquid	88.11	99.8	39	0.79	1.49E+01	Ester	INCI	No	III	Negative
P3-057	Isopropyl myristate	110-27-0	Wako Pure Chemical	Liquid	270.45	≧95.0	2.60E-03	7.25	4.39E-05	Ester	INCI	No	IV	Negative
P3-058	Methoxyethyl acrylate	3121-61-7	Wako Pure Chemical	Liquid	130.14	≧98.0	59	0.51	4.83E-01	Acrylate, Ether, Ester	No	1	II	Positive
P3-059	Methyl acetate	79-20-9	Sigma-Aldrich	Liquid	74.08	99.5	81.5	0.28	4.91E+01	Ester	INCI	2	II	Negative
P3-060	Methyl cyanoacetate	105-34-0	Sigma-Aldrich	Liquid	99.09	99	1000	-2.96	2.92E-02	Ester, Nitrile compound	No	2	II	Negative
P3-061	Imidazole	288-32-4	Sigma-Aldrich	Solid	68.08	99	228	-0.7	3.20E-03	Heterocyclic compound, Amine	INCI	1	I	Positive
P3-062	Pyridine	110-86-1	Sigma-Aldrich	Liquid	79.1	≧99.0	893	0.83	3.04E+00	Heterocyclic compound	No	1	I	Negative
P3-063	Isopropyl bromide	75-26-3	Wako Pure Chemical	Liquid	122.99	≧97.0	1.8	2.16	2.73E+01	Halogen compound	No	No	IV	Negative
P3-064	Cyclohexanone	108-94-1	Sigma-Aldrich	Liquid	98.14	99.8	15	0.82	3.99E-01	Ketone, Hydrocarbon(cyclic)	No	No	III	Negative
P3-065	2-Methylbutyric acid	116-53-0	Sigma-Aldrich	Liquid	102.13	≧98	1000	-1.14	7.39E-02	Carboxylic acid	No	1	I	Negative
P3-066	Calcium thioglycolate trihydrate	5793-98-6	TCI	Solid	184.22	>94.0	-	-	-	Thio compound, Organic salt(Carboxylic acid salt)	No	1	I	-
P3-067	Citric acid	77-92-9	Sigma-Aldrich	Solid	192.12	≧99.5	999	-6.91	7.64E-06	Carboxylic acid	INCI	n.a.	n.a.	Positive
P3-068	Potassium sorbate	24634-61-5	Sigma-Aldrich	Solid	150.22	≧98.0	-	-	-	Organic salt (Carboxylic acid salt)	INCI	n.a.	n.a.	Negative

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-069	Sodium salicylate	54-21-7	Wako Pure Chemical	Solid	160.1	≧99.5	-	-	-	Organic salt (Carboxylic acid salt), Phenol	INCI	1	I	Negative
P3-070	Distearyldimethyl ammonium chloride	107-64-2	TCI	Solid	586.5	>95.0	-	-	-	Quaternary ammonium compound	INCI	1	I	Positive
P3-071	n-Lauroylsarcosine sodium salt	137-16-6	Wako Pure Chemical	Solid	293.38	≧95.0	-	-	-	Surfactant (anionic)	INCI	2	III	Positive
P3-072	Sodium lauryl sulfate	151-21-3	Wako Pure Chemical	Solid	288.38	≧95.0	-	-	-	Surfactant (anionic)	INCI	2	III	Positive
P3-073	Triton X-100 (5%)	9002-93-1	Sigma-Aldrich	Liquid	324.41	-	-	-	-	Surfactant (nonionic)	INCI	2	III	Positive
P3-074	2-Ethylhexyl p-dimethylaminobenzoate	21245-02-3	Wako Pure Chemical	Liquid	277.4	≧97.0	4.70E-03	5.41	6.09E-07	PABA derivative	INCI	No	IV	Positive
P3-075	Promethazine hydrochloride	58-33-3	Sigma-Aldrich	Solid	320.88	98	-	-	-	Heterocyclic compound, Organic salt	No	1	I	Positive
P3-076	2-Ethyl-1-hexanol	104-76-7	Wako Pure Chemical	Liquid	130.23	≧98.0	1.7	2.72	-	Fatty alcohol	No	2	II	Positive
P3-077	3-Methoxy-1.2-propanediol	623-39-2	TCI	Liquid	106.12	>98.0	843	-0.94	-	Alcohol, Ether	No	No	IV	Negative
P3-078	Cyclohexanol	108-93-0	Sigma-Aldrich	Liquid	100.16	≧95.0	44	1.28	1.17E-01	Alcohol	No	1	I	Negative
P3-079	Ethanol	64-17-5	Wako Pure Chemical	Liquid	46.068	≧99.5	183	-0.18	1.10E+01	Alcohol	INCI	2	I	Negative
P3-080	n-Hexanol	111-27-3	Sigma-Aldrich	Liquid	102.17	≧99.0	8.8	1.86	1.26E-01	Alcohol	INCI	2	II	Negative
P3-081	3,3-Dimethylpentane	562-49-2	Sigma-Aldrich	Liquid	100.2	99	8.20E-03	4.02	1.02E+01	Hydrocarbon	No	No	IV	Positive
P3-082	Methyl cyclopentane	96-37-7	TCI	Liquid	84.16	≧96.0	0.084	3.17	1.67E+01	Hydrocarbon	No	No	III	Positive
P3-083	Toluene	108-88-3	Wako Pure Chemical	Liquid	92.14	≧99.5	0.32	2.72	3.69E+00	Hydrocarbon (aromatic)	INCI	2	III	Negative
P3-084	Acetone	67-64-1	Sigma-Aldrich	Liquid	58.08	≧99.5	94.7	-0.04	4.64E+01	Ketone	INCI	2	II	Negative

Code No.	Chemical Name	CAS No.	Supplier	Physicality	Molecular Weight	Purity (%)	Water solubility (g/L, pH7)	Log D (pH7)	Vapor Pressure (kPa,25℃)	Final Chemical Class	INCI Listing	GHS	EPA	*In vitro* Judgment
P3-085	Gluconolactone	90-80-2	Wako Pure Chemical	Solid	178.14	≧97.0	999	-3.47	1.01E-10	Polyol	INCI	No	IV	Negative
P3-086	Methyl amyl ketone (2-heptanol)	110-43-0	Wako Pure Chemical	Liquid	114.19	≧98.0	5.0	2	6.31E-01	Ketone	No	No	III	Negative
P3-087	Methyl ethyl ketone (2-butanone)	78-93-3	TCI	Liquid	72.11	≧99.0	47	0.47	1.53E+01	Ketone	INCI	2	III	Negative
P3-088	Methyl isobutyl ketone(4-methyl 2-pentanol)	108-10-1	Sigma-Aldrich	Liquid	72.11	≧99.0	12	1.33	2.43E+00	Ketone	INCI	No	III	Negative
P3-089	Glycerol	56-81-5	Wako Pure Chemical	Liquid	92.09	≧99.0	715	-1.85	3.09E-05	Polyol	INCI	No	IV	Negative
P3-090	Cetylpyridinium bromide	140-72-7	Sigma-Aldrich	Solid	384.44	≧97.0	-	-	-	Surfactant (cationic)	No	1	I	Positive
P3-091	Triton X-100	9002-93-1	Sigma-Aldrich	Liquid	324.41	-	-	-	-	Surfactant (nonionic)	INCI	1	I	Positive
P3-092	Tween20	9005-64-5	Sigma-Aldrich	Liquid	346.46	-	-	-	-	Surfactant (nonionic)	INCI	No	III	Positive
P3-093	Sodium hydroxide	1310-73-2	Wako Pure Chemical	Solid	40	≧97.0	-	-	-	Alkali	INCI	1	I	Positive
P3-094	Glycolic acid	79-14-1	Sigma-Aldrich	Solid	76.05	≧98.0	1000	-4.62	-	Carboxylic acid	INCI	2	III	Negative
P3-095	See P3-023													
P3-096	Sucrose fatty acid ester	Non	TCI	Solid	>342.3	-	-	-	-	Polyol, Ester	No	2	II	Positive
P3-097	methyl para-Hydroxybenzoate	99-76-3	Wako Pure Chemical	Solid	152.15	≧99.0	5.6	1.86	7.40E-04	Ester, Phenol	INCI	2	II	Positive
P3-098	Silicic acid	7699-41-4	Wako Pure Chemical	Solid	78.1	-	-	-	-	Silicon compound	No	No	IV	Positive
P3-099	Benzyl alcohol	100-51-6	Sigma-Aldrich	Liquid	108.14	≧98.5	47	1.06	2.11E-02	Alcohol	INCI	1	I	Negative
P3-100	Lactic acid	50-21-5	Wako Pure Chemical	Liquid	90.08	≧85.0	1000	-4.2	2.00E-03	Carboxylic acid	INCI	1	I	Negative

Table19. Analysis classified by chemical class (GHS, Bottom-up, TEA)

Regulatory System	Alcohol	Carboxylic acid	Ester	Ether	Halogen compound	Heterocyclic compound
Accuracy	33.3% (7/21)	28.6% (2/7)	55.6% (10/18)	40.0% (4/10)	63.6% (7/11)	75.0% (9/12)
Sensitivity	25.0% (4/16)	28.6% (2/7)	60.0% (6/10)	40.0% (2/5)	100.0%(5/5)	75.0% (6/8)
Specificity	60.0% (3/5)	0.0% (0/0)	50.0% (4/8)	40.0% (2/5)	33.3% (2/6)	75.0% (3/4)
False Negative Rate	75.0% (12/16)	71.4% (5/7)	40.0% (4/10)	60.0% (3/5)	0.0% (0/5)	25.0% (2/8)
False Positive Rate	40.0% (2/5)	0.0% (0/0)	50.0% (4/8)	60.0% (3/5)	66.7% (4/6)	25.0% (1/4)

Regulatory System	Hydrocarbon	Ketone	Organic salt	Phenol	Surfactant	Thiol compound
Accuracy	50.0% (3/6)	44.4% (4/9)	77.8% (7/9)	71.4% (5/7)	85.7% (6/7)	57.1% (4/7)
Sensitivity	50.0% (1/2)	16.7% (1/6)	71.4% (5/7)	75.0% (3/4)	100.0%(5/5)	66.7% (2/3)
Specificity	50.0% (2/4)	100.0%(3/3)	100.0%(2/2)	66.7% (2/3)	50.0% (1/2)	50.0% (2/4)
False Negative Rate	50.0% (1/2)	83.3% (5/6)	28.6% (2/7)	25.0% (1/4)	0.0% (0/5)	33.3% (1/3)
False Positive Rate	50.0% (2/4)	0.0% (0/3)	0.0% (0/2)	33.3% (1/3)	50.0% (1/2)	50.0% (2/4)

Table 20.1. Analysis classified by state (GHS, Bottom-up, TEA); Liquid and solid

Regulatory System	Liquid	Solid
Accuracy	44.1% (30/68)	70.8% (34/48)
Sensitivity	42.1% (16/38)	81.3% (26/32)
Specificity	46.7% (14/30)	50.0% (8/16)
False Negative Rate	57.9% (22/38)	18.8% (6/32)
False Positive Rate	53.3% (16/30)	50.0% (8/16)

Table 20.2. Analysis after cut Molecular weight 180 (GHS, Bottom-up, TEA)

Regulatory System	Analysis after Cut mw ≥180	Analysis after Cut mw <180
Accuracy	72.1% (31/43)	45.2% (33/73)
Sensitivity	95.5% (21/22)	43.8% (21/48)
Specificity	47.6% (10/21)	48.0% (12/25)
False Negative Rate	4.5% (1/22)	56.3% (27/48)
False Positive Rate	52.4% (11/21)	52.0% (13/25)

Table 20.3. Analysis after cut Molecular weight 180 and purity ≧80% (GHS, Bottom-up, TEA)

Regulatory System	Analysis after Cut mw ≥180	Analysis after Cut mw <180
Accuracy	71.0% (23/32)	45.2% (33/73)
Sensitivity	93.8% (15/16)	43.8% (21/48)
Specificity	50.0% (8/16)	48.0% (12/25)
False Negative Rate	6.3% (1/16)	56.2% (27/48)
False Positive Rate	50.0% (8/16)	52.0% (13/25)

Table 20.4. Analysis classified by state in water (10.0 g/L) (GHS, Bottom-up, TEA)

Regulatory System	Water Solubility ≥10.0 g/L	Water Solubility < 10.0 g/L
Accuracy	44.0% (22/50)	50.0% (19/38)
Sensitivity	38.5% (15/39)	84.6% (11/13)
Specificity	63.6% (7/11)	32.0% (8/25)
False Negative Rate	61.5% (24/39)	15.4% (2/13)
False Positive Rate	36.4% (4/11)	68.0% (17/25)

Table 20.5. Analysis after cut log D (2.88) (GHS, Bottom-up, TEA)

Regulatory System	logD ≥2.88	logD < 2.88
Accuracy	43.5% (10/23)	47.7% (31/65)
Sensitivity	100.0%(5/5)	44.7% (21/47)
Specificity	27.8% (5/18)	55.6% (10/18)
False Negative Rate	0.0% (0/5)	55.3% (26/47)
False Positive Rate	72.2% (13/18)	44.4% (8/18)

Table 20.6. Analysis after cut vapor pressure (6.0kPa)(GHS, Bottom-up, TEA)

Regulatory System	Vapor pressure ≥6.0 kPa	Vapor pressure < 6.0 kPa
Accuracy	36.4% (4/11)	48.6% (34/70)
Sensitivity	28.6% (2/7)	52.4% (22/42)
Specificity	50.0% (2/4)	42.9% (12/28)
False Negative Rate	71.4% (5/7)	47.6% (20/42)
False Positive Rate	50.0% (2/4)	57.1% (16/28)

Table 20.7. Analysis after cut pKa (5.0pKa)(GHS, Bottom-up, TEA)

Regulatory System	pKa ≥5.0	pKa < 5.0
Accuracy	51.3% (20/39)	40.0% (4/10)
Sensitivity	46.2% (12/26)	40.0% (4/10)
Specificity	61.5% (8/13)	0.% (0/0)
False Negative Rate	53.8% (14/26)	60.0% (6/10)
False Positive Rate	38.5% (5/13)	0.% (0/0)
Positive Predictive	70.6% (12/17)	100.0%(4/4)
Negative Predictive	36.4% (8/22)	0.0% (0/6)

Table 21.1. Analysis of categories: Alcohol

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity (%)	GHS	*In vitro* Judgment
P3-045	Ethanol	64-17-5	46.068	≥99.5	2	Negative
P3-049	Isopropyl alcohol	67-63-0	60.1	≥99.9	2	Negative
P3-015	Butanol	71-36-3	74.12	≥99.0	1	Negative
P3-022	Isobutyl alcohol	78-83-1	74.12	≥99.0	1	Positive
P2-020	Cyclopentanol	96-41-3	86.13	99	2	Negative
P3-018	Cyclohexanol	108-93-0	100.16	≥95.0	1	Negative
P3-064	2-Methyl-1-pentanol	105-30-6	102.17	99	2	Negative
P3-048	n-Hexanol	111-27-3	102.17	≥99.0	2	Negative
P3-093	3-Methoxy-1.2-propanediol	623-39-2	106.12	>98.0	No	Negative
P3-014	Benzyl alcohol	100-51-6	108.14	≥98.5	1	Negative
P3-073	2,4-Dimethyl-3-pentanol	3970-62-5	116.2	97	No	Negative
P2-009	Propylene glycol propyl ether	1569-01-3	118.17	99	2	Negative
P3-054	1-Octanol	111-87-5	130.23	≥98.0	2	Positive
P3-046	2-Ethyl-1-hexanol	104-76-7	130.23	≥98.0	2	Positive
P3-009	2-(2-Ethoxyethoxy)ethanol	111-90-0	134.17	≥99	No	Negative
P2-002	2,5-Dimethylhexaediol	110-03-2	146.23	97	1	Negative
P3-044	2-Benzyloxyethanol	622-08-2	152.19	≥97.0	2	Negative
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	176.25	≥98.5	2	Negative
P3-097	3-Phenoxybenzyl alcohol	13826-35-2	200.23	98	No	Positive
P3-053	Myristyl alcohol	112-72-1	214.39	≥97.0	2	Positive
P3-069	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol	72956-09-3	406.47	≥98	No	Positive

Red: No correct predictive capacity with in vitro assay,

Table 21.2. Analysis of categories: Ester

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity (%)	GHS	*In vitro* Judgment
P3-050	Methyl acetate	79-20-9	74.08	99.5	2	Negative
P3-077	Ethyl acetate	141-78-6	88.11	99.8	No	Negative
P3-051	Methyl cyanoacetate	105-34-0	99.09	99	2	Negative
P3-026	Methylthioglycolate	2365-48-2	106.14	95	1	Positive
P2-010	Ethyl thioglycolate	623-51-8	120.17	97	No	Positive
P3-024	Methoxyethyl acrylate	3121-61-7	130.14	≥98.0	1	Positive
P3-082	Glycidyl methacrylate	106-91-2	142.15	97	No	Positive
P3-059	Ethyl-2-methylacetoacetate	609-14-3	144.17	90	2	Negative
P3-062	Isopropyl acetoacetate	542-08-5	144.17	≥95.0	2	Negative
P3-037	methyl para-Hydroxybenzoate	99-76-3	152.15	≥99.0	2	Positive
P3-076	2-Ethoxyethyl methacrylate	2370-63-0	158.19	99	No	Positive
P2-017	Propyl 4-hydroxybenzoate	94-13-3	180.2	≥98.0	No	Positive
P3-096	iso-Octylthioglycolate	25103-09-7	204.33	≥98.0	No	Negative
P3-079	2-Ethylhexylthioglycolate	7659-86-1	204.33	≥95.0	No	Negative
P3-087	Isopropyl myristate	110-27-0	270.45	≥95.0	No	Negative
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	294.11	98	2	Positive
~~P3-002~~	~~Tetraethylene glycol~~	~~17831-71-9~~	~~302.32~~	~~-~~	~~1~~	~~Positive~~
~~P3-040~~	~~Sucrose fatty acid ester~~	~~Non~~	~~>342.3~~	~~-~~	~~2~~	~~Positive~~

Red: No correct predictive capacity with in vitro assay, Dark: Not used to analyze

Table 21.3. Analysis of categories:Ether

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity (%)	GHS	*In vitro* Judgment
P3-093	3-Methoxy-1.2-propanediol	623-39-2	106.12	>98.0	No	Negative
P2-009	Propylene glycol propyl ether	1569-01-3	118.17	99	2	Negative
P3-024	Methoxyethyl acrylate	3121-61-7	130.14	≥98.0	1	Positive
P3-009	2-(2-Ethoxyethoxy)ethanol	111-90-0	134.17	≥99	No	Negative
P3-044	2-Benzyloxyethanol	622-08-2	152.19	≥97.0	2	Negative
P3-076	2-Ethoxyethyl methacrylate	2370-63-0	158.19	99	No	Positive
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	176.25	≥98.5	2	Negative
P3-075	Dioctyl ether	629-82-3	242.44	99	No	Positive
~~P3-002~~	~~Tetraethylene glycol~~	~~17831-71-9~~	~~302.32~~	~~-~~	~~1~~	~~Positive~~
P2-001	Piperonylbutoxide	51- 03- 6	338.44	90	No	Positive

Red: No correct predictive capacity with in vitro assay, Dark: Not used to analyze

Table 21.4. Analysis of categories: Ketone

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity (%)	GHS	*In vitro* Judgment
P3-042	Acetone	67-64-1	58.08	≥99.5	2	Negative
P3-052	Methyl ethyl ketone (2-butanone)	78-93-3	72.11	>99.0	2	Negative
P3-095	Methyl isobutyl ketone(4-methyl 2-pentanol)	108-10-1	72.11	≥99.0	No	Negative
P3-004	gamma-Butyrolactone	96-48-0	86.09	≥99	2	Negative
P3-070	Cyclohexanone	108-94-1	98.14	99.8	No	Negative
P3-059	Ethyl-2-methylacetoacetate	609-14-3	144.17	90	2	Negative
P3-062	Isopropyl acetoacetate	542-08-5	144.17	≥95.0	2	Negative
P3-094	Methyl amyl ketone (2-heptanol)	110-43-0	114.19	≥98.0	No	Negative
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	294.11	98	2	Positive

Red: No correct predictive capacity with in vitro assay,

Table 21.5. Analysis of categories: hetelocyclic compounds

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity	GHS	*In vitro* Judgment
P3-021	Imidazole	288-32-4	68.08	99	1	Positive
P3-031	Pyridine	110-86-1	79.1	≥99.0	1	Negative
P3-004	gamma-Butyrolactone	96-48-0	86.09	≥99	2	Negative
P3-028	1,2,4-Triazole,sodium salt	41253-21-8	91.05	90	1	Positive
P3-003	2-Amino-3-hydroxy pyridine	16867-03-1	110.11	98	2	Positive
P3-013	1,2-Benzisothiazol-3(2H)-one	2634-33-5	151.18	≥97.0	1	Positive
P3-080	1-Ethyl-3-methylimidazolium ethylsulfate	342573-75-5	236.29	99	No	Negative
P3-084	6-Hydroxy-2,4,5-triamino pyrimidine Sulfate	1603-02-7	239.21	≥95.0	No	Positive
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	294.11	98	2	Positive
P3-030	Promethazine hydrochloride	58-33-3	320.88	98	1	Positive
P2-012	2-Phospho-L-ascorbic acid trisodium salt	66170-10-3	322.05	≥95.0	No	Negative
P3-090	2,2'-Methylene-bis-(6-(2H benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl) phenol)	103597-45-1	658.87	99	No	Negative

Red: No correct predictive capacity with in vitro assay,

Table 21.6. Analysis of categories: carboxylic acid(containing salt)

Code No.	Chemical Name	CAS No.	Molecular Weight	Purity (%)	GHS	*In vitro* Judgment
P3-047	Glycolic acid	79-14-1	76.05	≥98.0	2	Negative
P3-023	Lactic acid	50-21-5	90.08	≥85.0	1	Negative
P3-025	2-Methylbutyric acid	116-53-0	102.13	≥98	1	Negative
P3-066	Sodium chloroacetate	3926-62-3	116.48	98	2	Positive
P2-011	Sodium oxalate	62-76-0	134	≥99.5	1	Positive
P3-055	Sodium benzoate	532-32-1	144.1	≥99.0	2	Negative
~~P3-038~~	~~Potassium sorbate~~	~~24634-61-5~~	~~150.22~~	~~≥98.0~~	~~n.a.~~	~~Negative~~
P3-033	Sodium salicylate	54-21-7	160.1	≥99.5	1	Negative
P3-006	4-Nitrobenzoic acid	62-23-7	167.12	≥98.0	2	Negative
~~P3-016~~	~~Calcium thioglycolate trihydrate~~	~~5793-98-6~~	~~184.22~~	~~>94.0~~	~~1~~	~~n.a.~~
P2-016	1-Naphthaleneacetic acid	86-87-3	186.21	≥95.0	1	Positive
~~P3-035~~	~~Citric acid~~	~~77-92-9~~	~~192.12~~	~~≥99.5~~	~~n.a.~~	~~Positive~~
P3-001	Dodecanoic acid	143-07-7	200.32	≥99	1	Positive
P3-060	3,3-Dithiodipropionic acid	1119-62-6	210.27	≥97.0	2	Negative

Red: No correct predictive capacity with in vitro assay, Dark: Not used to analyze

Table 22. Analysis after cut Molecular weight <180 for alcohol, ester, ether, ketone heterocyclic compound and carboxylic acid , and purity ≧80% (GHS, Bottom-up, TEA).

Regulatory System	Analysis in applicability domain
Accuracy	64.9% (37/57)
Sensitivity	92.3% (24/26)
Specificity	41.9% (13/31)
False Negative Rate	7.6% (2/26)
False Positive Rate	58.1% (18/31)

Figures for SIRC-CVS:TEA validation version 7.8

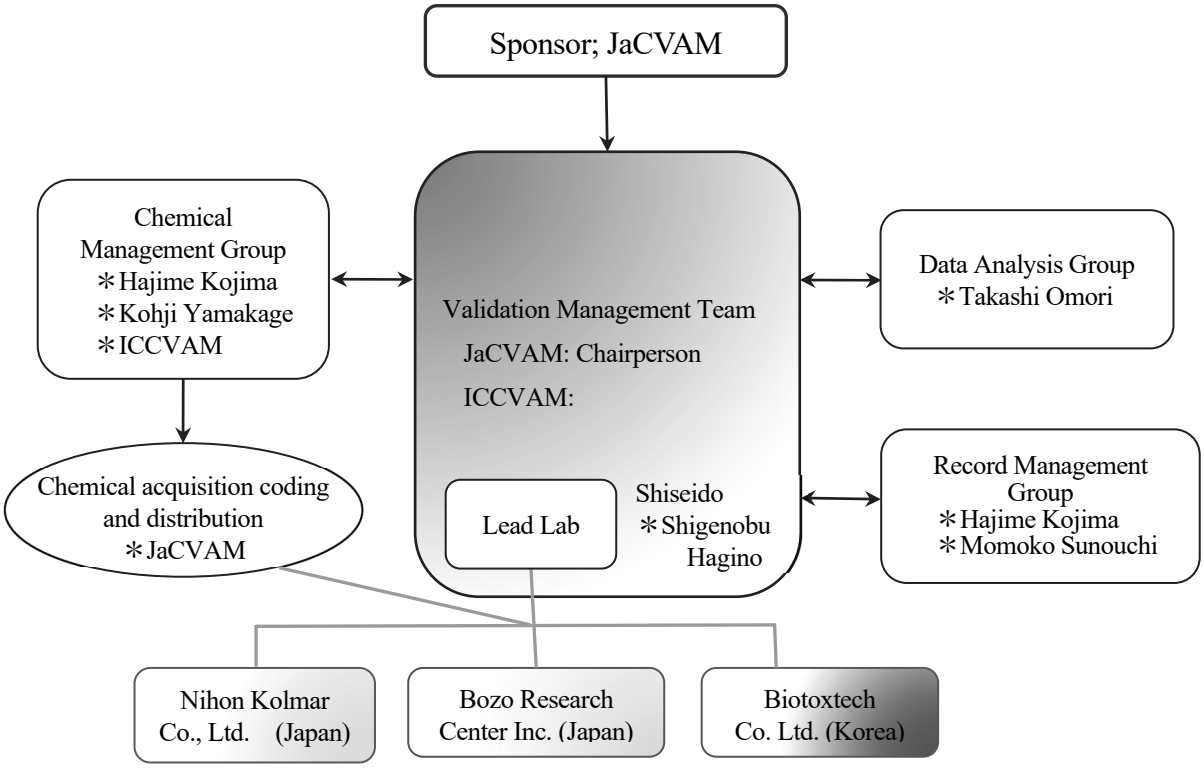


Fig. 1. Study organization for SIRC-CVS:TEA validation study

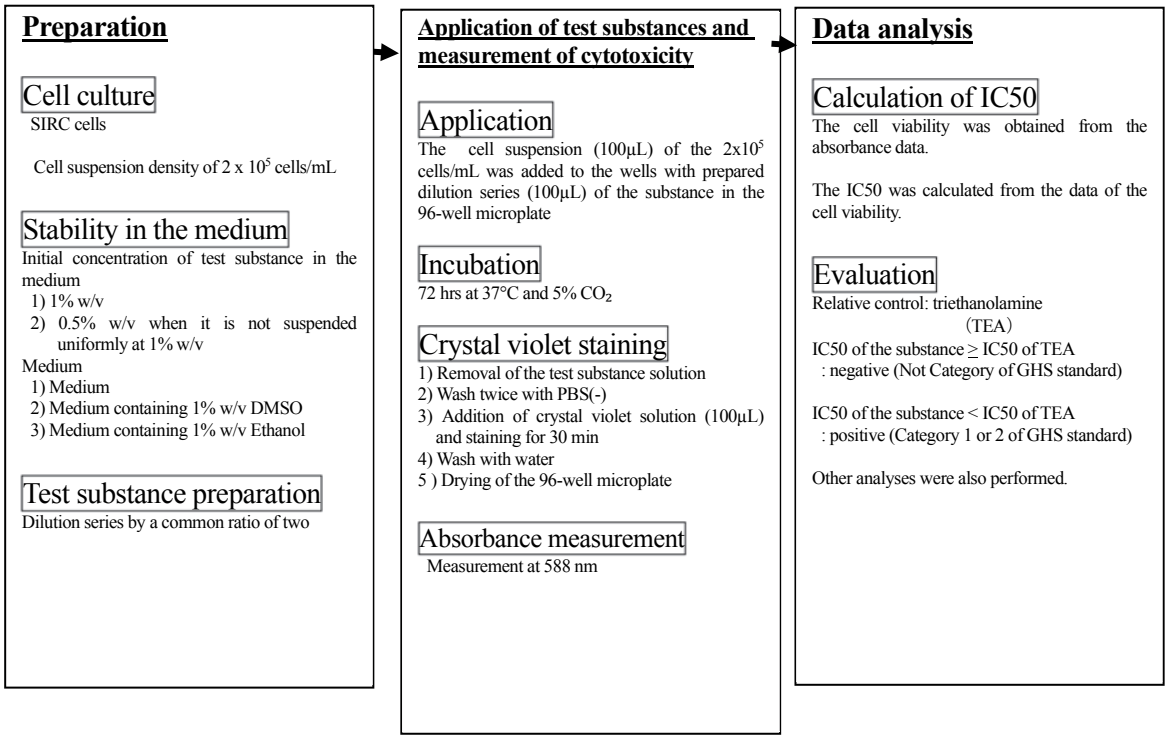


Fig. 2. SIRC-CVS:TEA test procedure

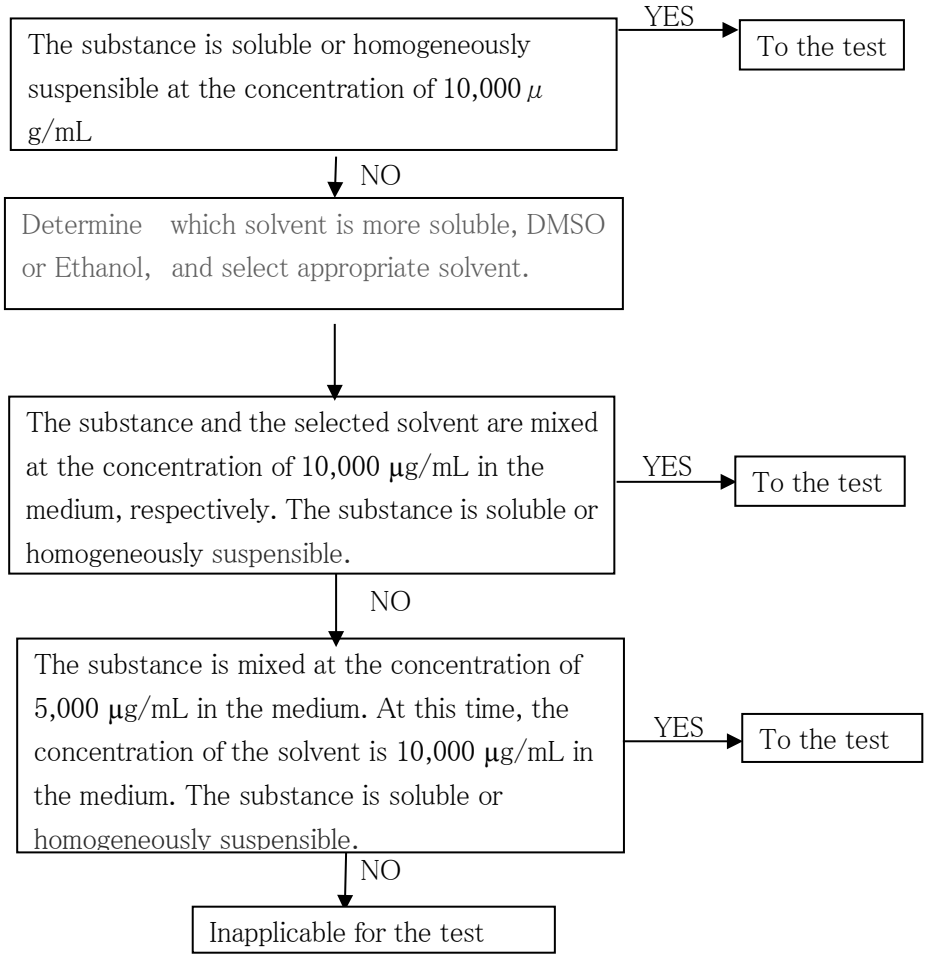


Fig. 3. Flow chart of examination of stability for the substance in the medium

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
C	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
D	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	N C	PBS
E	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
F	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
G	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Fig. 4.1. Layout of 96-well microplates

PBS: 200 μL of PBS(-)
NC: Medium, 10,000 μg/mL DMSO-medium solution or 10,000 μg/mL ethanol-medium solution of 100 μL
S: A 1:1 serial dilution (by adding 100 μL)
R: A 1:1 serial dilution of the relative control (by adding 100 μL)
P: A 1:1 serial dilution of the positive control (by adding 100 μL).
The dilution series of the test substance was made using medium, 10,000 μg/mL DMSO-medium solution or 10,000 μg/mL ethanol-medium solution. The dilution series of positive control and relative control was made using medium.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		■	■	■	■	■	■	■	■	■	■	
C		■	■	■	■	■	■	■	■	■	■	
D		■	■	■	■	■	■	■	■	■	■	
E		■	■	■	■	■	■	■	■	■	■	
F		■	■	■	■	■	■	■	■	■	■	
G		■	■	■	■	■	■	■	■	■	■	
H												

■ : Cell suspension (100 μL)

Fig. 4.2. Addition of cell suspension

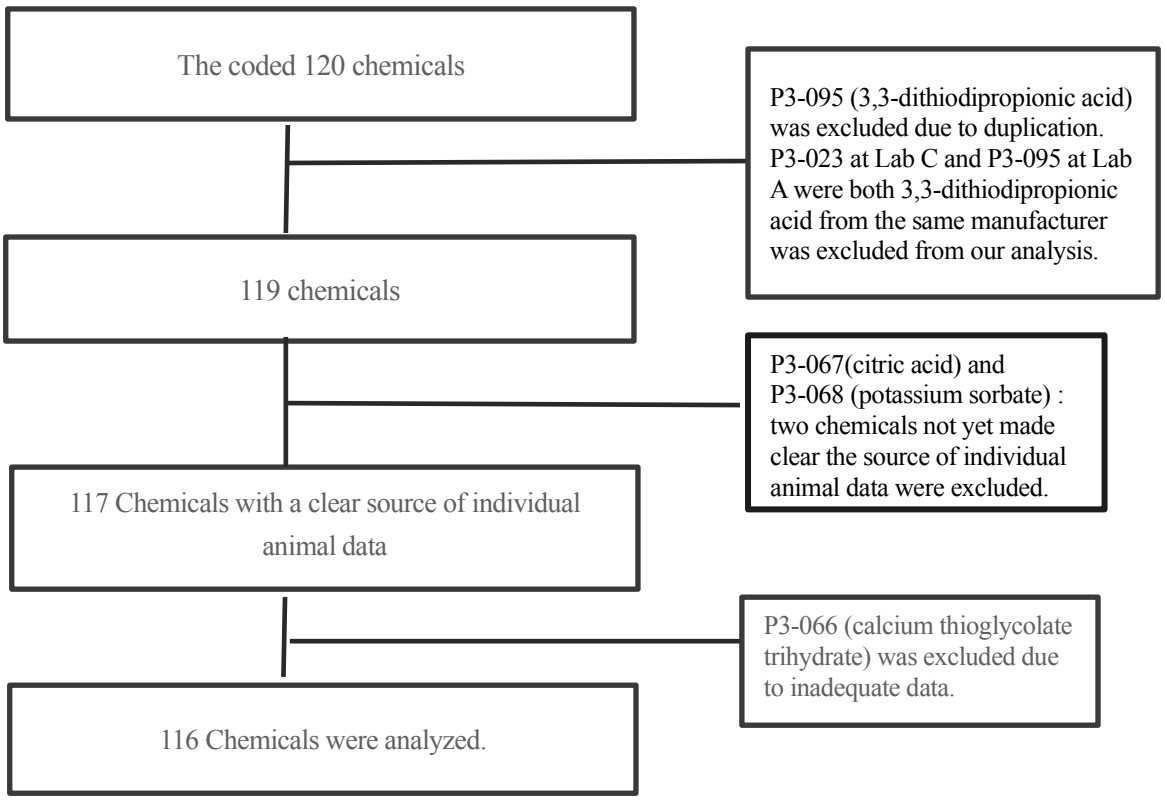


Fig.5. Evaluation of predictive capacity for the SIRC-CVS validation study

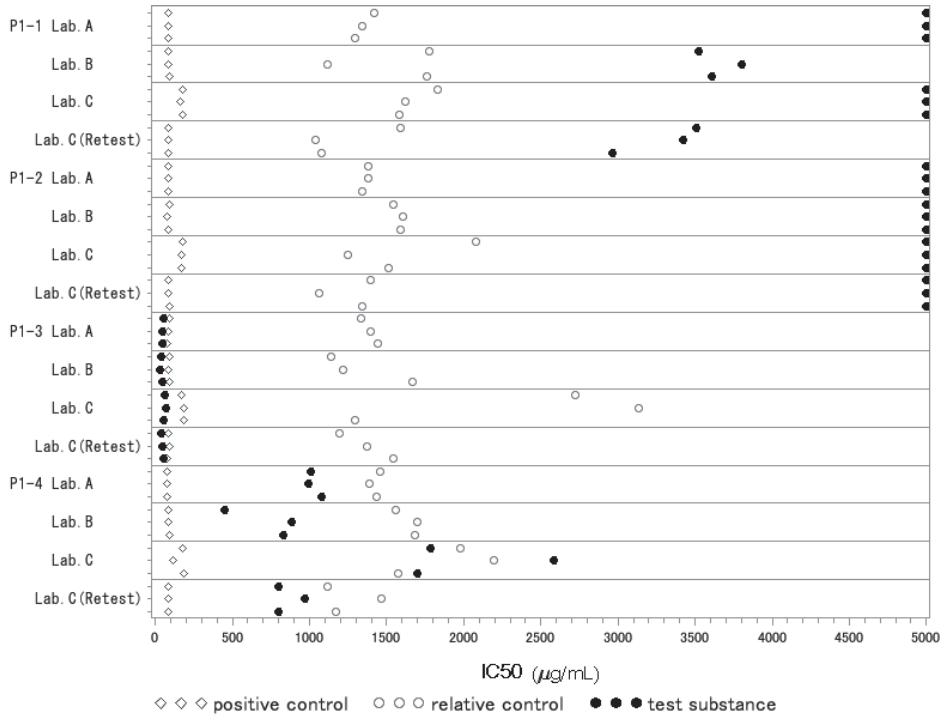
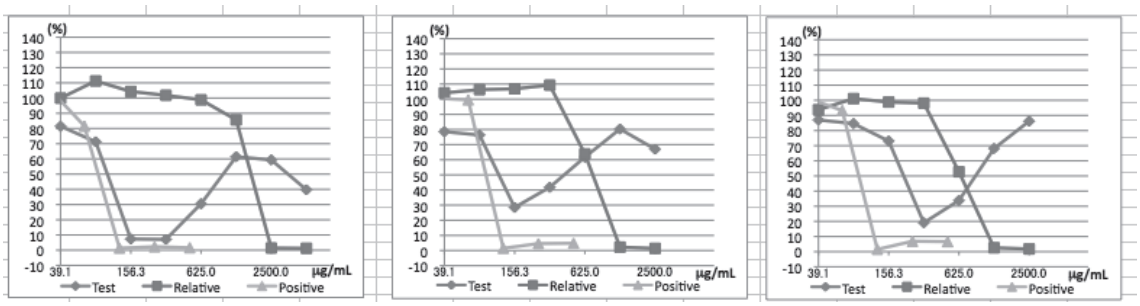


Fig. 6. A comparison of test substances, reference control, and positive control at the three participating laboratories

P1-1: ethyl-2-methyl acetoacetate, P1-2: safflower oil,
P1-3: 3-chloropropionitrile, P1-4: sodium dehydroacetate

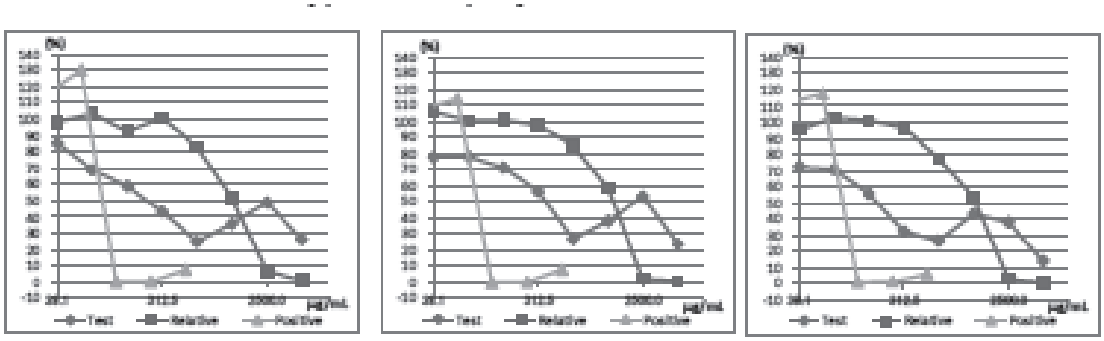
SA008 Solvent: DMSO

Run 1 Run 2 Run 3



SB001 Solvent: Medium

Run 1 Run 2 Run 3



SC006 Solvent: Medium

Run 1 Run 2 Run 3

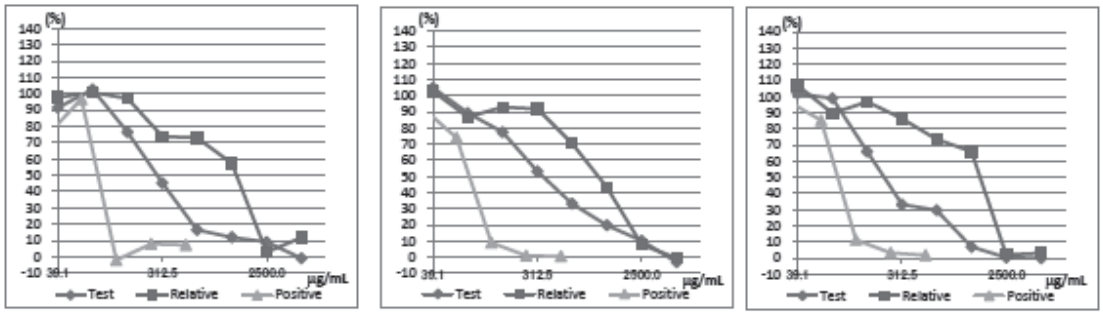
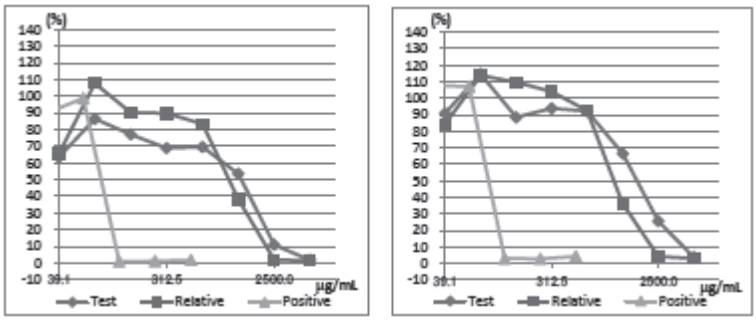


Fig.7. Dose response curves of P2-001

SA90

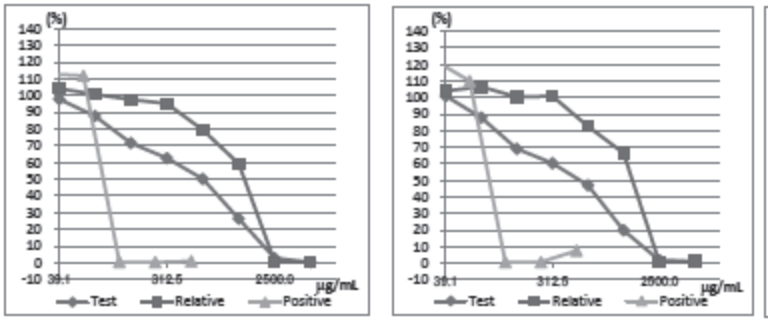
Run 1 Run 2



Solvent: Medium

SB71

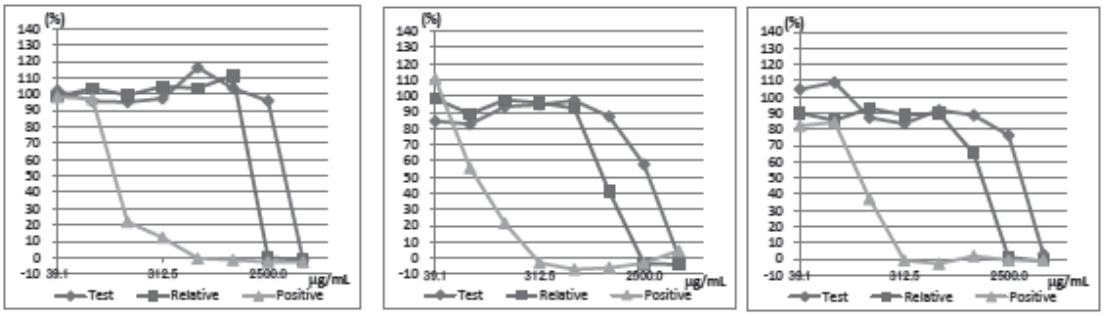
Run 1 Run 2



Solvent: Medium

SC63

Run 1 Run 2 Run 3

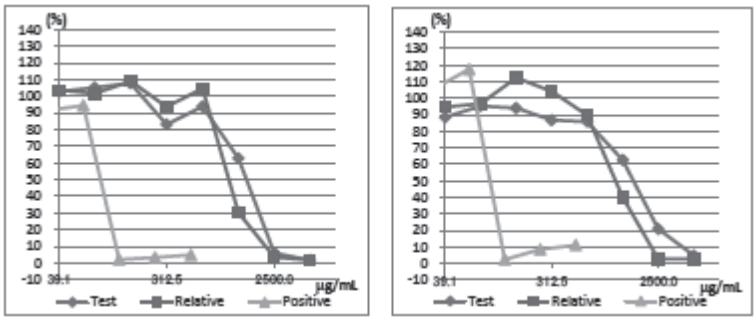


Solvent:

Fig.8-1. Dose response curves of P3-010

SA84

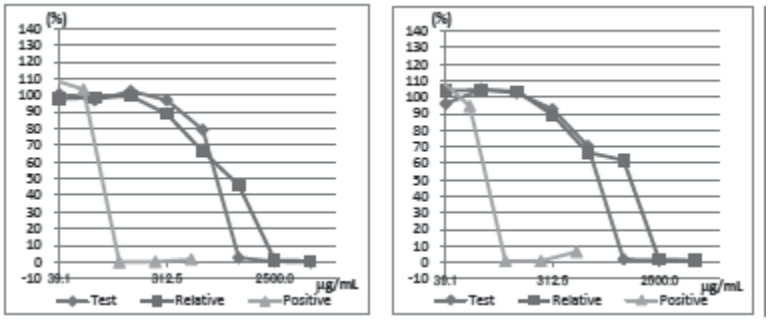
Run 1 Run 2



Solvent: Medium

SB77

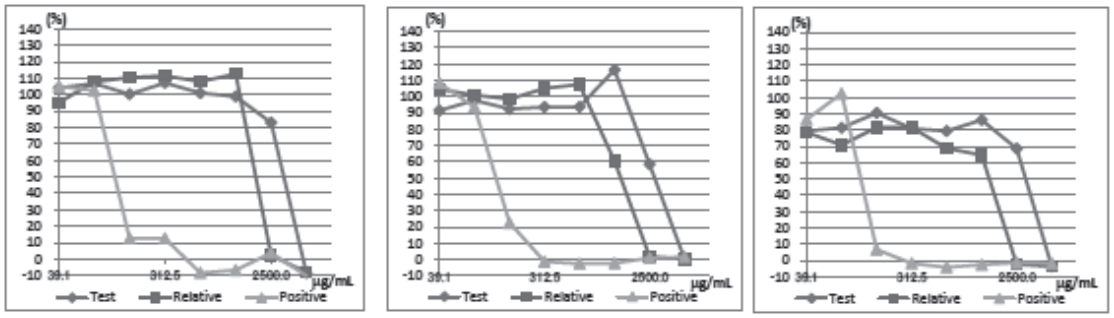
Run 1 Run 2



Solvent: Medium

SC64

Run 1 Run 2 Run 3

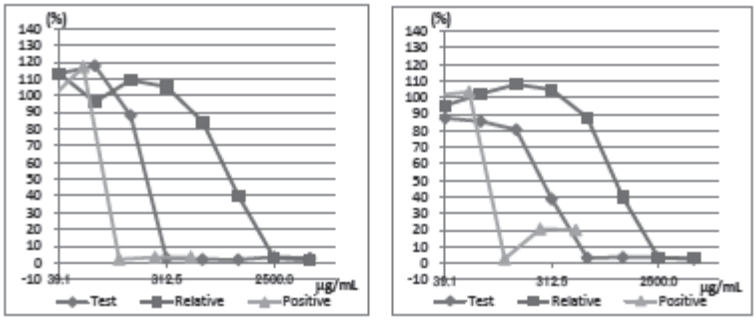


Solvent: Medium

Fig.8-2. Dose response curves of P3-012

SA82

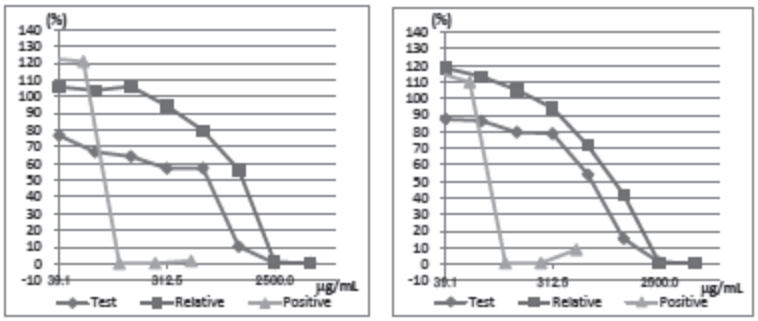
Run 1 Run 2



Solvent: Medium

SB79

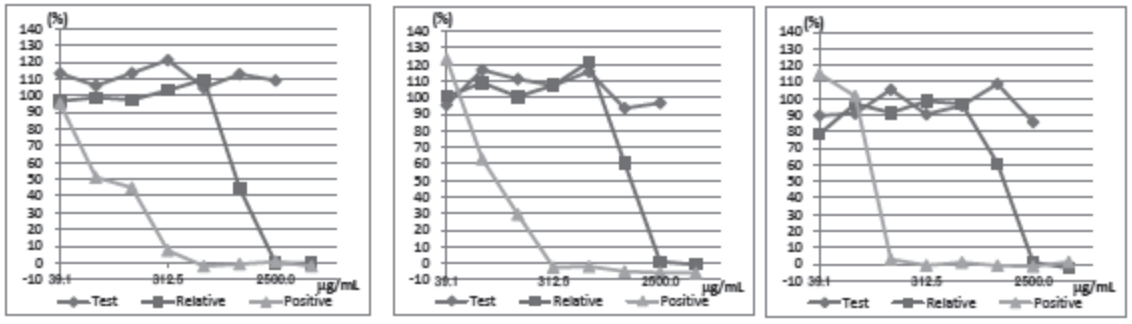
Run 1 Run 2



Solvent: Ethanol

SC61

Run 1 Run 2 Run 3



Solvent: DMSO

Fig.8-3. Dose response curves of P3-003

Run 1 Run 2

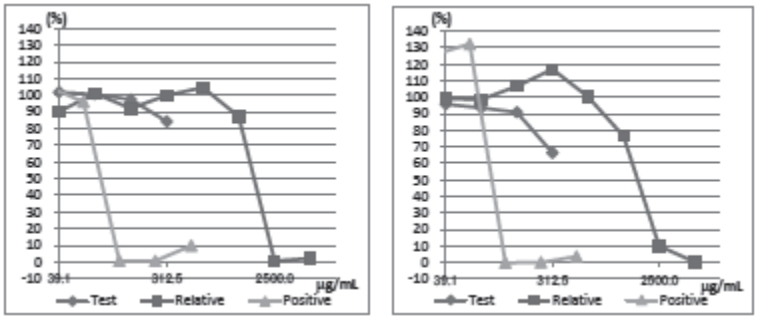


Fig.9. Dose response curves of P3-066 at Lab.B

P3-066: SB94 Solvent: Medium

2011 年 9 月 15 日

眼刺激性試験代替法「SIRC 細胞毒性試験」
説明資料

（株）資生堂
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目次

1. 緒言

化粧品についての動物を用いる安全性試験に対する反対運動は 1980 年代に、眼刺激性を予測するためのウサギを用いる Draize の眼刺激性試験（Draize 試験）や急性毒性を予測する LD50 試験を中心に日米欧においてほぼ同時に活発化し，動物実験廃止へ向けた活動は化粧品業界にとって重要な課題となった(板垣ら, 2008)。

1990 年度に設置された厚生科学研究班の「新規原料配合化粧品の安全性評価のための試験法の研究」では日本化粧品工業連合会（粧工連）が参画し、眼刺激性試験をインビトロ試験に代替可能か否かが検討された。この調査、研究が基盤となり、1991 年度に発足した厚生科学研究班の「新規化粧品原料配合化粧品の安全性評価のための試験法の研究」において、眼刺激性試験代替法のバリデーション研究が実施された。参加施設は当時の国立衛生試験所（現在は国立医薬品食品衛生研究所）と粧工連傘下 18 企業にキットメーカー、大学等を加え、計 29 施設であった。その結果は 1999 年に公表された「代替法を用いて化粧品原料の眼刺激性を評価するにあたっての指針（案）」に結びついた。この指針案では、培養細胞を用いる試験で無毒性と判断された物質は、化粧品製剤への濃度 10%までの配合に対しインビボで眼に対する障害性が少ないと予測できることおよびその場合にインビボ試験を省略できるとしている（大野, 1999, Ohno, 2004）。

厚生科学研究のバリデーションでは、SIRC 細胞毒性試験が高い施設間再現性を有することや濃度 10%における Draize 試験の最大平均評価点（MAS）15 点を境界とする分類に対し高い予測能を有することが報告されている（Tani et al., 1999）。しかし、試験法の公的な認知のためには化学物質原体について眼刺激性評価の国際標準である Globally Harmonized System of Classification and Labelling of Chemicals (GHS)に基づく予測ができることが必要であり、なおかつ第三者による評価がなされる必要があった。そのため、厚生科学研究で得られたデータの再解析を中心とし、データを追加して研究が進められた。そして、SIRC 細胞毒性試験は GHS 基準における無刺激性物質(NI)を同定できる原体評価用の試験法として JaCVAM へ提案された。

この方法の第三者評価が JaCVAM の眼刺激性試験代替法評価委員会により実施された結果、試験法の限界を考慮したうえで使用すれば無刺激性物質を同定する事は可能であると判断された。しかしながら、本法の正確性と信頼性を厳密に評価するには追加のバリデーション研究が必要であるとの見解が示された。

2. 代替しようとする試験法の名称および代替法の名称

代替しようとする試験法は Draize 試験であり、その代替法の候補は SIRC 細胞毒性試験である。

3. Draize 試験に関する資料

ヒトの眼に対する刺激を予測するため、ウサギを用いる Draize 試験（Draize,1959）が広く用いられてきた。Draize 試験は被験物質をウサギの結膜嚢に投与し、一定時間毎に角膜、虹彩および結膜の障害を経時的に肉眼判定し評価する方法である。当試験は安全性試験の中でも特に残酷な印象を与えるとして、動物愛護の観点から代替法の開発と法規制への組み込みが望まれている（大野,1996）。

眼の重篤な損傷性、刺激性については、GHSに判定基準があり、区分 1、区分 2A、区分 2B およびこれらに該当しないもの（無刺激性物質;NI）に分類される。その際に用いられる Draize 試験のスコア方法を Table 1 に、GHS の分類方法を Table 2 にそれぞれ示す。

なお、GHS においては、分類のための試験を行う前に、化学物質の眼に対する重篤な損傷性または眼刺激性を判定するために、いくつかの要因を考慮することとされている。そのうちの一つが pH であり、pH≦2 および≧11.5 など極端な pH は、特に明らかな緩衝能力をともなっている場合、眼に対する重篤な損傷作用があることが示唆されている。そのような物質は眼に明らかな作用を生じると予測され区分 1 に分類される。

Table 1 Scale for scoring ocular lesions in the Draize eye test

(1) Cornea	
(A) Opacity-degree of density (area most dense taken for reading)	
No Opacity	0
Scattered or diffuse area, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
(B) Area of cornea involved	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
A × B × 5	Total maximum = 80
(2) Iris	
(A) Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
A × 5	Total maximum = 10
(3) Conjunctivae	
(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
(B) Chemosis	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
(C) Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
Score (A + B + C) × 2	Total maximum = 20

The table is the same as that reported by Draize et al. (1959)

Table 2 GHS classification of serious eye damage / eye irritation

Caterory of GHS	Criteria
1	An eye irritant Category 1 (irreversible effects on the eye) is a test material that produces: (a) at least in one animal effects on the cornea, iris or conjunctiva that are not expected to reverse or have not fully reversed within an observation period of normally 21 days; and/or (b) at least in 2 of 3 tested animals, a positive response of: (i) corneal opacity $\geq$ 3; and/or (ii) iritis > 1.5; calculated as the mean socres following grading at 24, 48 and 72 hours after installation of the test material
2	An eye irritant Category 2A (irritating to eyes) is a test material that produces: (a) at least in 2 of 3 tested animals a positive response of: (i) corneal opacity $\geq$ 1; and/or (ii) iritis $\geq$ 1; and/or (iii) conjunctival redness $\geq$ 2; and/or (iv) conjunctival oedema (chemosis) $\geq$ 2 calculated as the mean socres following grading at 24, 48 and 72 hours after installation of the test material, and which fully reverses within an observation period of normally 21 days. Within this category an eye irritant is considered mildly irritating to eyes (Category 2B) when the effects listed above are fully reversible within 7 days of observation.

The table is the same as the third revised edition of the GHS (2009).

Draize 試験は、化粧品や医薬部外品の原料や製品の評価に使用されてきているが、それらの状況については Annex 1に記載した。

4. SIRC 細胞毒性試験の原理

代替法開発は、複雑な生体反応の解析をもとに、試験管レベルで生体反応の一部を再現する（板垣ら, 2008）。大野（1996）は、Draize 試験から得られる情報と代替法から得られる情報を分類し、細胞毒性は角膜上皮細胞の変性、剥離の情報と対応するとしている。すなわち、細胞毒性試験は角膜の障害を予測することを想定している。一方、角膜の評価点は Draize 試験の総評価点 110 点のうち、80 点を占めているため、角膜の評価点が総評価点に対応すると考えられる（Hagino et al, 1991）。GHS における NI は角膜に反応がほぼ認められない程度であると考えられることから、角膜の障害を予測できる細胞毒性試験は GHS における NI を同定するうえで、適切な代替試験法であると考えられる。

なお、化粧品、薬用化粧品は角膜に反応がほぼ認められないということを一つの判断基準としており、化粧品、薬用化粧品の原料を評価する目的で考える場合においても、角膜の障害を予測できる細胞毒性試験は適切な代替試験法であると考えられる。

細胞の種類として、ウサギ角膜由来細胞（SIRC 細胞）が必須かどうかについては、厚生科学研究で、SIRC 細胞に加えて、ヒト子宮頸部由来上皮癌細胞（HeLa 細胞）、チャイニーズハムスター肺由来線維芽細胞(CHL 細胞)などの試験が行われた。結論として、細胞間で差が認められないとされ、これらのうちのどの細胞でも用いることが可能とされている。この 3 種の細胞の中から SIRC 細胞を取り上げる理由は、眼刺激性試験代替法として用いた論文が多いことと、インビボで用いられる組織と同じ「ウサギ角膜」に由来していることによる。

細胞毒性のエンドポイントは、厚生科学研究で、クリスタルバイオレット染色（CVS）法、Neutral red 取り込み(NRU)法、MTT 法による測定が行われた。結論として、エンドポイント間に差は認められないとされ、これらのうちのどの細胞毒性のエンドポイントも用いることが可能とされている。この 3 種のエンドポイントの中から CVS 法を取り上げる理由は、エンドポイントアッセイを同一の 96 ウェルマイクロプレート上で行うことができ、他の方法に比較して操作が簡便であることによる。また、操作終了後の着色した 96 ウェルマイクロプレートを特別な条件を設定せずに室温で長期間保存できるという

利点もある。

5. 厚生科学研究による SIRC 細胞毒性試験のバリデーションデータの解析 ［研究 1］

5.1.目的

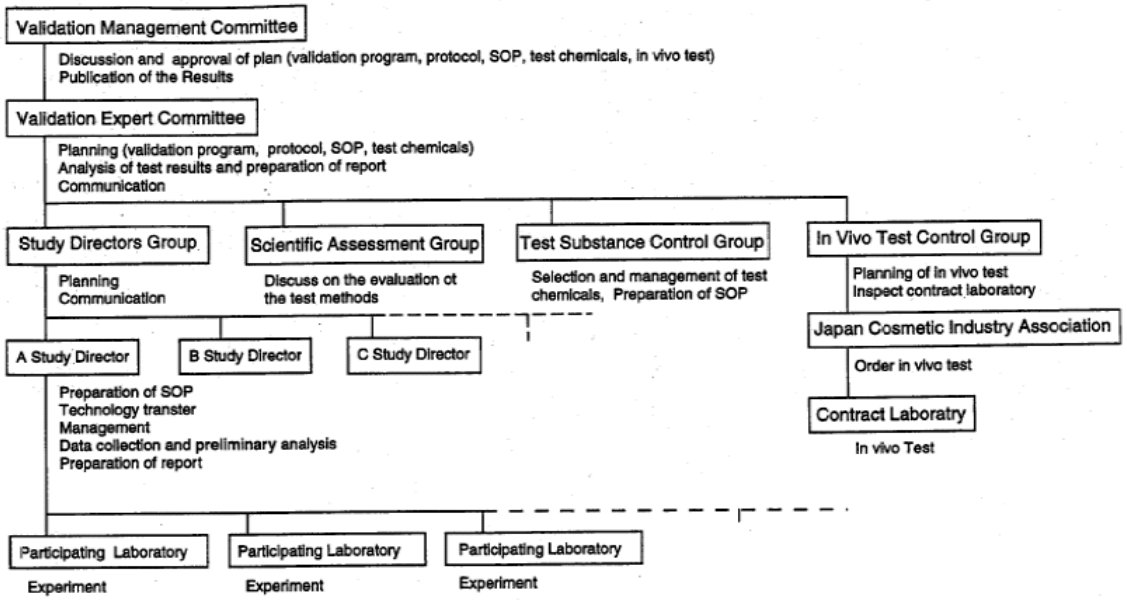
1991 年度に発足した厚生科学研究班の「新規化粧品原料配合化粧品の安全性評価のための試験法の研究」において、12 種の眼刺激性試験代替法のバリデーションが同時に実施された。その中の一つが CVS 法による SIRC 細胞毒性試験である。今回、この SIRC 細胞毒性試験が眼刺激性評価における GHS の NI を同定する試験法という観点で、施設内の再現性、施設間の再現性を再解析した。

5.2.バリデーションの組織, 参加施設等

バリデーションの組織を Fig. 1 に示す。この組織図は 12 種の試験法の評価が行われた厚生科学研究によるバリデーションの全体に関わるものである。

バリデーション運営委員会を最高決定機関とし、その下に執行機関として実行委員会を置き、さらにその下に試験責任者グループ、科学評価グループ、被験物質管理グループおよびインビボ試験管理グループを置いた。試験責任者グループの下には各試験責任者を置いた。試験法毎に試験責任者を置き、CVS 法による SIRC 細胞毒性試験にも試験責任者を置いた。試験責任者の実施施設がリードラボとしての役割を担った。試験責任者は、SOP の取りまとめ、技術移転、マネージメント、データの集計と処理、報告資料の作成を行った。なお、図に示したものは第二次および第三次バリデーションの組織であり、第一次バリデーションにおいては試験責任者グループを設置しなかった。すなわち、第一次バリデーションの組織は運営委員会の下に実行委員会、被験物質管理委員会、インビボ試験管理委員会を置き、実行委員会の下に試験責任者が設置された。試験責任者の実施施設はいずれの段階においてもリードラボの役割を担った。

Fig. 1 Organization of the second and third validations



The figure is the same as that reported by Ohno et al.(1999). The facilities of the study directors had the role of lead laboratory.

12 種の試験法のバリデーションへの参加施設を示す。参加施設は一定の技術レベルで試験が行えるようにするため、GLP についての講義および技術講習会（1992 年 10 月）を国立衛生試験所（当時）において実施した。参加者は GLP の原理を尊重して試験を実施した。バリデーションにおける試験は SOP に従って行われた。被験物質は被験物質管理グループがコード化し、各施設に配布した。データのチェックは各施設が行い、それをリードラボが集計、そしてその集計にミスが無いかを各施設の試験担当者がチェックした。関連する書類は試験責任者および各施設からの試験担当者がチェックした。バリデーション終了後、すべてのデータは国立衛生試験所（当時）に保管された（Ohno et al, 1999）。そして、試験終了から 5 年以上経過した時点で、廃棄された。

Table 3 List of the co-operating organizations for the Japanese validaton

Administrative organizations	**Japan Cosmetic Industry Corporation**
Ministry of Health and Welfare	Shiseido Safety & Analytical Res. Center
National Institute of Health Sciences	POLA Corp.
(Div. Pharmacol. Div. Toxicol.	Kanebo Ltd
and Div. Genetics Mutagen.)	KOSE Corp.
	Lion Corp.
Universities	KAO Corp.
Yokohama-City University	SUNSTAR Inc.
Showa University	OPPEN Cosmetic Co. Ltd
	NOEVIR Co. Ltd
Kit suppliers	Kaminomoto Co. Ltd
Oriental Yeast Co. Ltd	Procter & Gamble Far East, Inc.
Kurabo Industries, Ltd	Nippon Menard Cosmetic Co. Ltd
Invitro International Japan, Ltd	Yakult Central Institute for Microbiological Res.
Toyobo Co., Ltd	Ajinomoto Co. Inc.
	Cow Brand Soap Kyoshinsha Co., Ltd
Others	Hoyu Co. Ltd
RIKEN Gene Bank	CLUB COSMETICS Co. Ltd
Japan Seigiken Research Centre Co. Ltd	Nippon Shikizai Inc.

The table is the same as that reported by Ohno et al.(1999).

CVS 法による SIRC 細胞毒性試験のバリデーションへの参加は、第一次バリデーションでは 6 施設（資生堂、ポーラ、カネボウ、花王、メナード、国立衛生試験所・薬理部）であり、リードラボは資生堂が務めた。第二次バリデーションは 6 施設（資生堂、ポーラ、サンスター、メナード、ホーユー、理研ジーンバンク）であり、リードラボはポーラが務めた。第三次バリデーションは 5 施設（資生堂、ポーラ、サンスター、ホーユー、理研ジーンバンク）であり、リードラボはポーラが務めた。

Table 4 List of the participation of organization

First valication	Shiseido	Pola	Kanebo	Menard	NIHS	Kao			
Second validation	Shiseido	Pola		Menard			Sunstar	Hoyu	Riken gene bank
Third validation	Shiseido	Pola					Sunstar	Hoyu	Riken gene bank

NIHS : National Institute of Health Sciences

5. 3.SIRC 細胞毒性試験のプロトコール

厚生科学研究のバリデーションで用いたプロトコールにおける主な特徴を表 5 に示す。項目は後述する SIRC 細胞毒性試験の（JaCVAM による第三者評価時の）提案プロトコールと異なる点を選んだ。被験物質の調製手順については Fig.2 に示す。

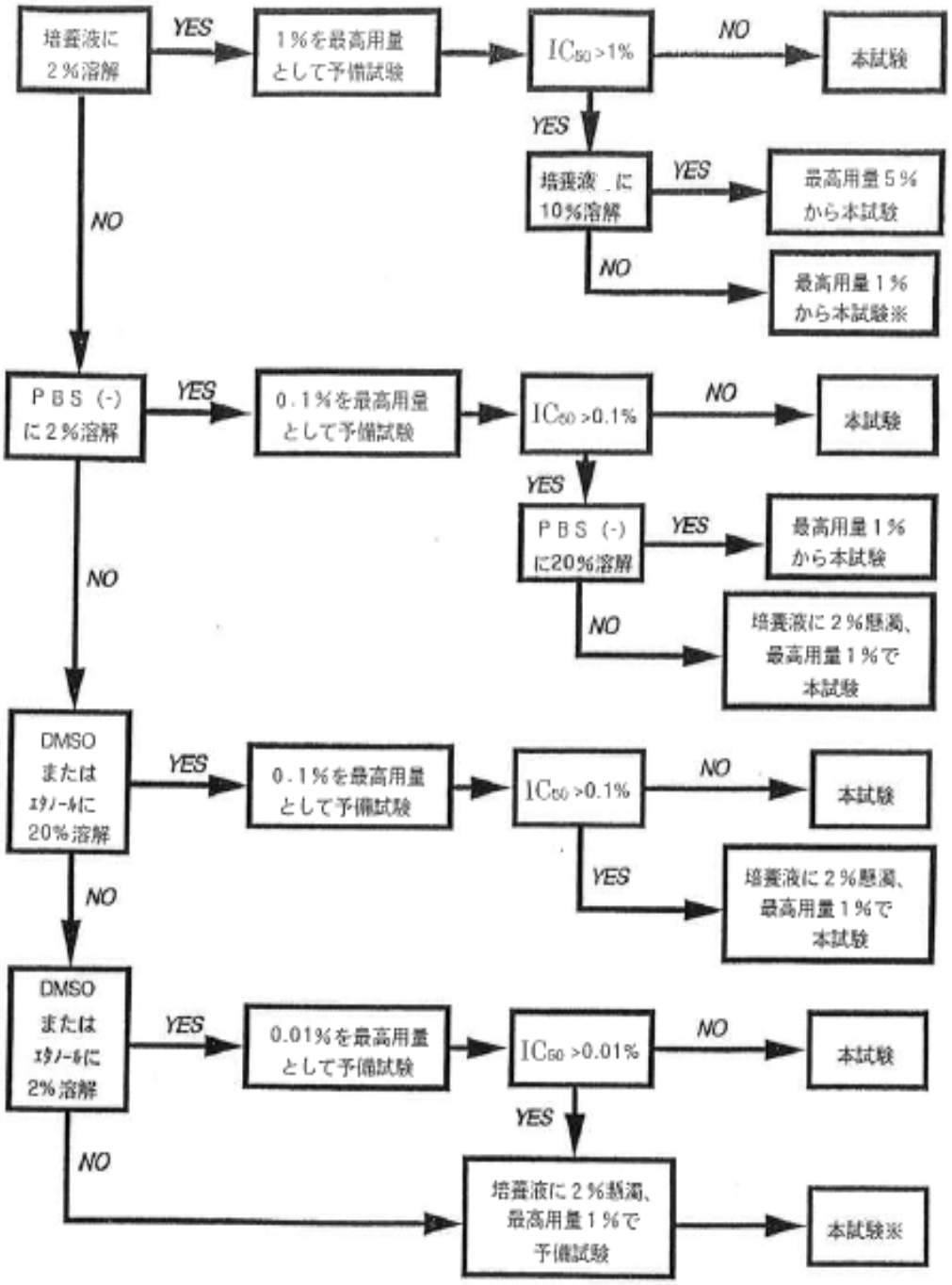
表 5 厚生科学研究のバリデーションで用いられた SIRC 細胞毒性試験プロトコールの特徴

項目	厚生科学研究のバリデーション時のプロトコール
培養液	10%仔牛血清（CS）を添加した MEM 培養液を用いる。
被験物質由来の汚染への対処	培養液および PBS(-)で調製する被験物質は、液体の場合無菌フィルターを用いて、固体の場合エタノール（添加後、蒸散）を用いて滅菌を行う。
被験物質の調製	図を用いて被験物質の調製手順を細かく設定(Fig.2 参照)。
予備試験	実施
被験物質の希釈系列	第一次バリデーションでは公比 2 を用いる。第二次バリデーションでは細胞生存率が 20～80%の間に少なくとも 3 点が入る希釈系列とし、最小で公比 1.1 まで実施する。第三次バリデーションでは細胞生存率が 20～80%の間に少なくとも 1 点が入る希釈系列とし、最小で公比 1.1 まで実施する。
陽性対照を用いた試験成立の条件	なし
比較対照物質を用いた試験成立の条件	なし
陰性対照を用いた試験成立の条件	なし
洗浄の際の PBS(-)の量	0.2mL/ウェル
マイクロプレートリーダーの測定波長	590nm 付近の吸光度を測定する。
IC50 計算法	片対数グラフに濃度-反応曲線を作成し、陰性対照の 50%となる濃度を求める。または解析ソフトを用いる。
結果の評価法	なし （バリデーションにおける試験終了後に、MAS15 から回帰直線を用いて外挿した細胞毒性の IC50 値を基準に設定し評価した。#）
試験の繰り返し	記載なし
Neutral red 取り込み試験を実施した後のプレートの使用	第一次バリデーションでは、Neutral red 取り込み試験を実施した後のプレートを使用せずに試験を実施する。 第二次と第三次バリデーションにおいては Neutral red 取り込み試験を実施した後のプレートを用いて、Crystal violet 染色試験を実施する$。

#：当時はバリデーションの方法論が今日のように確立されていなかった。

$：Neutral red 取り込み試験を実施した後のプレートを使用した場合もしない場合も、値は変わらない。このことは、Neutral red 取り込み試験を実施した後のプレートを使用しない第一次バリデーションにおける SLS の IC50 値と使用した第二、第三次バリデーションにおける SLS の IC50 に差がないことから確認された。また、第一次バリデーションの被験物質のデータと第二、第三次バリデーションの被験物質のデータは、いずれも Neutral red 取り込み試験のデータに極めて近似していることからも確認された。（Itagaki et al, 1995, Tani et al, 1999）

Fig. 2 細胞毒性試験における被験物質の調製手順(小島, 1999)



5.4.バリデーションに供した被験物質

バリデーション研究に供した 39 被験物質を Table 6 に示す。

被験物質 39 物質の内訳は、化学的クラスで分類すると、界面活性剤が 18 物質、それ以外の有機化合物が 19 物質、無機化合物が 2 物質であった。界面活性剤以外の有機化合物の内訳は、アルコール 3 物質、カルボン酸 3 物質、アルカノールアミン 3 物質、有機塩 3 物質、ポリオール 2 物質、エステル 2 物質、色素 1 物質、PABA 誘導体 1 物質、アミン 1 物質であった。また、存在状態（固体または液体）で分類すると、固体 20 物質、液体 15 物質、水溶液 4 物質であった。

この 39 物質のうちインビボが原体で実施されたものは 18 物質、10%水溶液で実施されたものは 35 物質、1%水溶液で実施されたものは 3 物質、0.1%水溶液で実施されたものは 1 物質であった。複数の濃度段階を設けた物質があり、3 濃度で実施されたものは 3 物質、2 濃度で実施されたものは 12 物質、1 濃度で実施されたものは 24 物質であった。

インビボが原体で実施された 18 物質の内訳は、界面活性剤 3 物質、無機化合物 2 物質、アルコール 2 物質、ポリオール 2 物質、エステル 2 物質、有機塩 2 物質、アルカノールアミン 1 物質、PABA 誘導体 1 物質、色素 1 物質、アミン 1 物質、カルボン酸 1 物質であった。

Table 6 List of the test substances and their characteristics

Substance	CAS	Class	Physical state	MW
Isotonic sodium chloride solution	7647-14-5	Inorganics	Solution	58.4
Polyoxyethylene hydrogenated caster oil (60 E.O.)	61788-85-0	Surfactants	Solid	-
Polyoxyethylene sorbitan monolaurate (20 E.O.)	9005-64-5	Surfactants	Liquid	346.5
Polyethyleneglycol monolaurate (10 E.O.)	9004-81-3	Surfactants	Liquid	-
Sodium N-lauryl sarcosinate (30% solution)	137-16-6	Surfactants	Solution	311.4
Sodium hydrogenated tallow L-glutamate	68187-34-8	Surfactants	Solid	-
Sodium dodecyl sulfate	151-21-3	Surfactants	Solid	288.4
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	3088-31-1	Surfactants	Solution	274.4
Polyoxyethylene octylphenylether (10 E.O.)	9002-93-1	Surfactants	Liquid	324.4
Benzalkonium chloride	8001-54-5	Surfactants	Solid	283.9
Sucrose fatty scid ester	-	Surfactants	Solid	-
Glycerin	56-81-5	Polyols	Liquid	92.1
Acid red 92	18472-87-2	Color additives	Solid	829.6
Polyoxyethylene sorbitan monooleate (20E.O.)	9005-65-6	Surfactants	Liquid	-
Calcium thioglycolate	814-71-1	Organic salts	Solid	130.2
Distearyldimethylammonium chloride	107-64-2	Surfactants	Solid	586.5
2-Ethylhexyl p-dimethylamino benzonate	21245-02-3	PABA derivatives	Liquid	277.4
Cetylpyridinium chloride	123-03-5	Surfactants	Solid	340
Methyl p-hydroxybenzoate	99-76-3	Esters	Solid	152.2
Isopropyl myristate	110-27-0	Esters	Liquid	270.5
Polyethylene glycol 400	25322-68-3	Polyols	Liquid	360～400
Silicic anhydride	7631-86-9	Inorganics	Solid	60.1
Benzyl alcohol	100-51-6	Alcohols	Liquid	108.1
Sodium salicylate	54-21-7	Organic salts	Solid	160.1
m-Phenylenediamine	108-45-2	Amines	Solid	108.1
Ethanol	64-17-5	Alcohols	Liquid	64.1
Monoethanolamine	141-43-5	Alkanolamines	Liquid	61.1
Triethanolamine	102-71-6	Alkanolamines	Liquid	149.2
Stearyltrimethylammonium chloride	112-03-8	Surfactants	Solid	348.1
Diisopropanolamine	110-97-4	Alkanolamines	Solid	133.2
Potassium laurate	10124-65-9	Surfactants	Solid	238.4
Cetyltrimethylammonium bromide	57-09-0	Surfactants	Solid	364.5
Acetic acid	64-19-7	Carboxylic acids	Liquid	60.1
Butanol	71-36-3	Alcohols	Liquid	74.1
Chlorhexidine gluconate (20% solution)	18472-51-0	Organic salts	Solution	897.8
Domiphen bromide	538-71-6	Surfactants	Solid	414.5
Lactic acid	50-21-5	Carboxylic acids	Liquid	90.1
Glycolic acid	79-14-1	Carboxylic acids	Solid	76.1
Di (2-ethylhexyl) sodium sulfosuccinate	577-11-7	Surfactants	Solid	488.5

5.5.バリデーションにおける被験物質の Draize 試験データ

厚生科学研究のバリデーションにおける被験物質の原体での Draize 試験結果を Table 7 に示す。GHS で NI に分類されるものは 9 物質、1、2A または 2B に分類されるものは 9 物質であった。GHS 分類については厚生科学研究で行われたインビボデータ資料に基づき、「the third revised edition of the GHS」の方法を用いて求めた。表中において GHS の分類が 1or2A となっているが、これは厚生科学研究における Draize 試験の観察のデータが 14 日目までで 21 日目のデータが無いため 1 と 2A を区別できないためである。しかしながら、本検討は NI を同定することを目的とするため、区別せずに「1or2A」とした。GHS で NI に分類される場合を陰性（N; Negative）、それ以外に分類される場合を陽性（P; Positive）とした。

Table 7 Draize eye test results in the Japanese validation study（as is）

Substance (as is)	Physical state	MAS	GHS	*In vivo* classification in this study
2-Ethylhexyl p-dimethylamino benzonate	Liquid	0.0	NI	N
Isopropyl myristate	Liquid	0.0	NI	N
Isotonic sodium chloride solution	Liquid	0.0	NI	N
Silicic anhydride	Powder	2.7	NI	N
Polyethylene glycol 400	Liquid	4.0	NI	N
Glycerin	Liquid	4.7	NI	N
Polyoxyethylene sorbitan monooleate (20 E.O.)	Liquid	4.7	NI	N
Triethanolamine	Liquid	8.0	NI	N
Methyl p-hydroxybenzoate	Powder	8.7	NI	N
Sucrose fatty acid ester	Powder	28.3	1or2A	P
Benzyl alcohol	Liquid	31.0	1or2A	P
Ethanol	Liquid	32.7	1or2A	P
Acid red 92	Powder	71.0	1or2A	P
Calcium thioglycolate	Powder	79.7	1	P
m-Phenylenediamine	Powder	80.7	1or2A	P
Sodium salicylate	Powder	83.7	1or2A	P
Distearyldimethylammonium chloride	Powder	96.3	1	P
Lactic acid	Liquid	102.7	1	P

MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
P: Positive, N: Negative.

濃度 10%での Draize 試験結果に基づき、原体における GHS 区分が予測できる物質については、それらのデータに基づいて分類したので Table 8 に示す。すなわち、濃度 10%で刺激性が認められ区分 1、区分 2A または区分 2B に分類されているものは原体ではそれ以上の刺激が認められると予測した。該当する 16 物質の内訳は、界面活性剤 11 物質、カルボン酸 2 物質、アルコール 1 物質、アルカノールアミン 2 物質であった。なお、表中には ICCVAM の推奨する参照物質リストに記載されているデータを加えた。Polyoxyethylene octylphenylether (10 E.O.)は、ICCVAM の参照物質リストにおいて、原液で適用された場合に GHS 区分 1 へ分類されており、10%における 1or2A への分類との間に妥当な関係が確認された。Sodium dodecyl sulfate は ICCVAM の参照物質リストにおいて濃度 3%のデータに基づいて NI に分類されているが、厚生科学研究の濃度 10%のデータに基づくと 1or2A に分類されており、原体では 1or2A とした。

最終的に厚生科学研究のインビボデータにおいて、GHS で NI に分類されるものは 9 物質、それ以外に分類されるものは 25 物質、合計 34 物質であった。なお、34 物質の内訳は、界面活性剤 13 物質、アルコール 3 物質、アルカノールアミン 3 物質、カルボン酸 3 物質、無機化合物 2 物質、ポリオール 2 物質、エステル 2 物質、有機塩 3 物質、PABA 誘導体 1 物質、色素 1 物質、アミン 1 物質であった。

Table 8 Draize eye test results（as is）in the Japanese validation study, including the classification predicted from the result of 10% concentration

Substance	MAS	GHS classification based on the data of Japanese validation study	GHS classification by ICCVAM Recommended Reference Substance List	*In vivo* classification in this study
2-Ethylhexyl p-dimethylamino benzoate	0.0	NI		N
Isopropyl myristate	0.0	NI		N
Isotonic sodium chloride solution	0.0	NI		N
Silicic anhydride	2.7	NI		N
Polyethylene glycol 400	4.0	NI		N
Glycerin	4.7	NI		N
Polyoxyethylene sorbitan monooleate (20E.O.)	4.7	NI		N
Triethanolamine	8.0	NI		N
Methyl p-hydroxybenzoate	8.7	NI		N
Sucrose fatty acid ester	28.3	1or2A		P
Benzyl alcohol	31.0	1or2A		P
Ethanol	32.7	1or2A		P
Acid red 92	71.0	1or2A		P
Calcium thioglycolate	79.7	1		P
m-Phenylenediamine	80.7	1or2A		P
Sodium salicylate	83.7	1or2A		P
Distearyldimethylammonium chloride	96.3	1		P
Lactic acid	102.7	1	1 (100%)#	P
Sodium dodecyl sulfate*	15.0≦	1or2A	NI (3%)#	P
Diisopropanolamine*	23.0≦	1, 2Aor2B		P
Monoethanolamine*	23.3≦	1or2A		P
Glycolic acid*	25.0≦	1or2A		P
Sodium hydrogenated tallow L-glutamate*	26.7≦	1or2A		P
Chlorhexidine gluconate (20% solution)*	28.3≦	1or2A		P
Butanol*	34.0≦	1or2A	1 (10%)#	P
Potassium laurate*	38.0≦	1or2A	1 (10%)#	P
Polyoxyethylene octylphenylether (10 E.O.)*	41.3≦	1or2A	1 (100%)#	P
Di (2-ethylhexyl) sodium sulfosuccinate*	57.0≦	1or2A	1 (10%)#	P
Acetic acid*	68.0≦	1or2A	1 (10%)#	P
Cetyltrimethylammonium bromide*	76.7≦	1or2A	1 (10%)#	P
Benzalkonium chloride*	78.0≦	1or2A	1 (5%)#	P
Stearyltrimethylammonium chloride*	91.3≦	1		P
Cetylpyridinium chloride*	94.7≦	1		P
Domiphen bromide*	96.3≦	1or2A	1 (10%)#	P

* : The *in vivo* results of as is application were predicted by the data of 10% concentration.
# : Tested concentration is shown in parenthesis.
P: Positive, N: Negative.

5.6.SIRC 細胞毒性試験のバリデーション結果

5.6.1.施設内変動

厚生科学研究でバリデーションが行われた被験物質のうち原体における眼刺激性・GHS 分類が判明している物質について、SIRC 細胞毒性試験に基づくインビボ予測結果の施設内の再現性を確認した。施設内の繰り返し数は 2 回である。この 2 回は日をあらためて試験した結果である。

Table 9 は、各被験物質および各施設での細胞毒性試験の結果であり、下段に 2 回の細胞毒性試験結果を、上段にそれらの平均値示す。細胞毒性試験結果は、比較対照物質として Triethanolamine を用い、NI と予測された場合を陰性(N)、それ以外を陽性(P)とした。第 1 回目のそれぞれの被験物質の IC50 は第 1 回目の Triethanolamine の IC50 と比較して陽性と陰性を評価した。Triethanolamine を実施した 5 施設 Lab.A、B、G、H、I について施設内の再現性を検討したところ、施設内の 2 回の結果が異なることは無かった。したがって、施設内の再現性は良好と考えられた。

なお、Triethanolamine を実施していない施設については、評価を実施できなかった。仮に 5 施設の Triethanolamine の平均 IC50 である 2090 μg/mL で分類したところ、2 回の細胞毒性試験が異なる所は認められず、他の施設と評価が異なる所も認められなかった。

Table 9 Results of repeatability on the SIRC-CVS assay

Substance	*In vivo* Classification	IC50 (μg/mL)[$]								
		Lab.A	Lab.B	Lab.C	Lab.D	Lab.E	Lab.F	Lab.G	Lab.H	Lab.I
2-Ethylhexyl p-dimethylamino Benzoate	N	381 (P) 284 (P) 478 (P)	1193 (P) 1407 (P) 979 (P)		97.5 (NE) 95 (NE) 100 (NE)			570 (P) 670 (P) 470 (P)	484 (P) 643 (P) 325 (P)	120 (P) 140 (P) 100 (P)
Isopropyl myristate	N	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)		6000<(NE) 6000 (NE) 10000< (NE)			10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)
Isotonic sodium chloride solution	N	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)	50000< (NE) 100000< (NE) 50000< (NE)	10000< (NE) 10000< (NE) 10000< (NE)	500000< (NE) 500000< (NE) 500000< (NE)	500000< (NE) 500000< (NE) 500000< (NE)			
Silicic anhydride	N	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)		38750 (NE) 45000 (NE) 32500 (NE)			10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)
Polyethylene glycol 400	N	6854.5 (N) 6522 (N) 7187 (N)	50000< (N) 50000< (N) 50000< (N)		32750 (NE) 36000 (NE) 29500 (NE)			47500 (N) 48000 (N) 47000 (N)	34500 (N) 35000 (N) 34000 (N)	40000 (N) 40000 (N) 40000 (N)
Glycerin	N	12746 (N) 10343 (N) 15148 (N)	5347.5 (N) 5579 (N) 5116 (N)		6750 (NE) 7200 (NE) 6300 (NE)			5350 (N) 4900 (N) 5800 (N)	12500 (N) 12100 (N) 12900 (N)	27000 (N) 32000 (N) 22000 (N)
Polyoxyethylene sorbitan monooleate (20E.O.)	N	745 (P) 660 (P) 830 (P)	762 (P) 757 (P) 767 (P)		1075 (NE) 1100 (NE) 1050 (NE)			1075 (P) 1150 (P) 1000 (P)	710 (P) 745 (P) 675 (P)	1400 (P) 900 (P) 1900 (P)
Triethanolamine	N	1440 (N) 1580 (N) 1300 (N)	1430 (N) 1540 (N) 1320 (N)					1750 (N) 1850 (N) 1650 (N)	1993 (N) 1910 (N) 2075 (N)	3850 (N) 3200 (N) 4500 (N)
Methyl p-hydroxybenzoate	N	103 (P) 140 (P) 66 (P)	214 (P) 238 (P) 190 (P)		195 (NE) 240 (NE) 150 (NE)			257 (P) 275 (P) 239 (P)	215.5 (P) 235 (P) 196 (P)	255 (P) 220 (P) 290 (P)
Sucrose fatty acid ester	P	250 (P) 240 (P) 260 (P)	304 (P) 301 (P) 306 (P)		315 (NE) 315 (NE) 315 (NE)			292.5 (P) 290 (P) 295 (P)	294.5 (P) 300 (P) 289 (P)	257.5 (P) 255 (P) 260 (P)
Benzyl alcohol	P	1148 (P) 1038 (P) 1258 (P)	888.5 (P) 800 (P) 977 (P)		1100 (NE) 800 (NE) 1400 (NE)			1485 (P) 1700 (P) 1270 (P)	830 (P) 810 850 (P)	1675 (P) 1950 (P) 1400 (P)
Ethanol	P	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)					10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)
Acid red 92	P	230 (P) 220 (P) 240 (P)	231 (P) 221 (P) 241 (P)		332.5 (NE) 360 (NE) 305 (NE)			340 (P) 340 (P) 340 (P)	268.5 (P) 252 (P) 285 (P)	380 (P) 380 (P) 380 (P)
Calcium thioglycolate	P	300(P) 250 (P) 350 (P)	660(P) 622 (P) 698 (P)		420(NE) 380 (NE) 460 (NE)			287.5(P) 245 (P) 330 (P)	292.5(P) 265 (P) 320 (P)	600< (NE; Retest) 600< (NE) 1000< (NE)
m-Phenylenediamine[&]	P	167 (P) 170 (P) 163 (P)	73 (P) 62 (P) 84 (P)		255 (NE) 255 (NE) 212-256.6 (NE)			290 (P) 290 (P) 290 (P)	167 (P) 114 (P) 220 (P)	355 (P) 390 (P) 320 (P)
Sodium salicylate	P	840 (P) 770 (P) 910 (P)	559 (P) 579 (P) 539 (P)		950 (NE) 1100 (NE) 800 (NE)			1195 (P) 790 (P) 1600 (P)	635 (P) 365 (P) 905 (P)	1525 (P) 1700 (P) 1350 (P)
Distearyldimethylammonium chloride	P	18.5 (P) 20.0 (P) 17.0 (P)	43.8 (P) 42.4 (P) 45.3 (P)		35.5 (NE) 35 (NE) 36 (NE)			57 (P) 57 (P) 57 (P)	32.1 (P) 33.2 31 (P)	39.7 (P) 44.4 (P) 35.0 (P)
Lactic acid	P	994 (P) 917 (P) 1070 (P)	982 (P) 992 (P) 971 (P)					1315 (P) 1380 (P) 1250 (P)	1285 (P) 1240 (P) 1330 (P)	1575 (P) 1450 (P) 1700 (P)
Sodium dodecyl sulfate*	P	182 (P) 174 (P) 189 (P)	172 (P) 168 (P) 176 (P)	117 (NE) 117 (NE) 117 (NE)	190 (NE) 190 (NE) 190 (NE)	198 (NE) 201 (NE) 194 (NE)	149 (NE) 140 (NE) 157 (NE)			
Diisopropanolamine*	P	455 (P) 472 (P) 437 (P)	901 (P) 1040 (P) 761 (P)					720 (P) 820 (P) 620 (P)	170 (P) 170 (P) 170 (P)	1250 (P) 1300 (P) 1200 (P)
Monoethanolamine*	P	4.46 (P) 4.33 (P) 4.58 (P)	9.8 (P) 9.6 (P) 10.0 (P)					5.9 (P) 7.2 (P) 4.6 (P)	10.5 (P) 9.6 (P) 11.3 (P)	17.5 (P) 17.5 (P) 17.5 (P)
Glycolic acid*	P	914 (P) 938 (P) 889 (P)	682 (P) 558 (P) 806 (P)					890 (P) 880 (P) 900 (P)	778 (P) 820 (P) 735 (P)	1075 (P) 1050 (P) 1100 (P)
Sodium hydrogenated tallow L-glutamate*	P	143 (P) 142 (P) 143 (P)	118 (P) 115 (P) 120 (P)	113 (NE) 83.1 (NE) 143 (NE	90.8 (NE) 77.0 (NE) 104.5 (NE)	235 (NE) 250 (NE) 219 (NE)	1115 (NE) 1200 (NE) 1030 (NE)			
Chlorhexidine gluconate (20% solution)*	P	67.2 (P) 60.9 (P) 73.5 (P)	44.8 (P) 46.3 (P) 43.3 (P)					67.5 (P) 78.0 (P) 57.0 (P)	45.8 (P) 26.0 (P) 65.5 (P)	112.5 (P) 115.0 (P) 110.0 (P)
Butanol*	P	10000< (N) 10000< (N) 10000< (N)	4395 (N) 5190 (N) 3600 (N)					10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)	10000< (N) 10000< (N) 10000< (N)
Potassium laurate*	P	103 (P) 107 (P) 99 (P)	117 (P) 123 (P) 110 (P)					73 # 58 88	110 (P) 100 (P) 120 (P)	150 (P) 155 (P) 145 (P)
Polyoxyethylene octylphenylether (10 E.O.)*	P	26.7 (P) 25.1 (P) 28.3 (P)	38.0 (P) 42.7 (P) 33.2 (P)	23.3 (NE) 32.2 (NE) 14.3 (NE)	32.3 (NE) 17.5 (NE) 47.0 (NE)	51.0 (NE) 54.9 (NE) 47.0 (NE)	59.5 (NE) 54.0 (NE) 65.0 (NE)			
Di (2-ethylhexyl) sodium sulfosuccinate*	P	210 (P) 209 (P) 211 (P)	182 (P) 177 (P) 186 (P)					181 (P) 190 (P) 172 (P)	156 (P) 148 (P) 163 (P)	175 (P) 175 (P) 175 (P)
Acetic acid*	P	681(P) 671 (P) 691 (P)	691 (P) 652 (P) 730 (P)					690 (P) 700 (P) 680 (P)	795< # 795 1000<	820 (P) 850 (P) 790 (P)
Cetyltrimethylammonium bromide*	P	2.95 (P) 1.96 (P) 3.94 (P)	3.21(P) 3.31 (P) 3.10 (P)					1.72 (P) 1.28 (P) 2.15 (P)	2.3># 2.3> 2.3>	2.50 (P) 2.10 (P) 2.90 (P)
Benzalkonium chloride*	P	16.2 (P) 15.7 (P) 16.6 (P)	25.2 (P) 18.3 (P) 32.1 (P)	13.2 (NE) 13.6 (NE) 12.8 (NE)	15.5 (NE) 14.5 (NE) 16.5 (NE)	29.0 (NE) 28.3 (NE) 29.7 (NE)	15.0 (NE) 16.5 (NE) 13.5 (NE)			
Stearyltrimethylammonium chloride*	P	1.07 (P) 1.42 (P) 0.71 (P)	1.47 (P) 1.25 (P) 1.68 (P)					1.31 (P) 1.42 (P) 1.20 (P)	1.17 (P) 0.98 (P) 1.36 (P)	2.90 (P) 2.80 (P) 3.00 (P)
Cetylpyridinium chloride*	P	0.53 (P) 0.59 (P) 0.46 (P)	0.96 (P) 0.95 (P) 0.96 (P)		0.88 (NE) 0.94 (NE) 0.82 (NE)			2.55 (P) 1.8 (P) 3.3 (P)	2.245 (P) 2.6 (P) 1.89 (P)	2.85 (P) 2.3 (P) 3.4 (P)
Domiphen bromide*	P	13.4 (P) 12.10 (P) 14.7 (P)	11.4 (P) 10.8 (P) 11.90 (P)					7.55 (P) 7.70 (P) 7.40 (P)	13.4 (P) 12.9 (P) 13.9 (P)	14.8 (P) 13.0 (P) 16.5 (P)

#:Value was excluded from analysis due to deviation from SOP. (Tani et al., 1999)

*: The *in vivo* results of as is application was predicted from the data of 10% concentration.

$: The intralaboratory results show as two IC50 values in lower parts and the averages in upper parts.

P:Positive, N:Negative, NE:Could not be evaluated

Blank column: Not tested

5.6.2.施設間変動

厚生科学研究でバリデーションが行われた被験物質のうち、原体における眼刺激性・GHS 分類が判明している物質について、SIRC 細胞毒性試験に基づくインビボ予測結果から施設間の再現性を確認した。比較対照物質は Triethanolamine とし、各施設における 2 回のデータを用いた。細胞毒性試験結果に基づいて予測された GHS 分類 NI を陰性(N)、それ以外の GHS 分類を陽性(P)とした。Table 10 には、Triethanolamine を実施した 5 施設について、物質毎の評価を示した。

その結果、5 施設間の評価に異なる所は認められなかった。一方、Lab. C～Lab.Fについては比較対照物質である Triethanolamine のデータが無く、評価を実施できなかったが、念のため 5 施設（A～E）の Triethanolamine の IC50 の平均値である 2090 μg/mL を用いて仮に分類したところ、他の施設と評価が異なる所は認められなかった。

以上より、SIRC 細胞毒性試験の施設間の再現性は良好と考えられた。

Table 10 Results of interlaboratory reproducibility on the SIRC-CVS assay

Substance	*In vivo* classification	*In vitro* classification				
		Lab. A	Lab. B	Lab. G	Lab. H	Lab. I
2-Ethylhexyl p-dimethylamino benzoate	N	P	P	P	P	P
Isopropyl myristate	N	N	N	N	N	N
Isotonic sodium chloride solution	N	N	N			
Silicic anhydride	N	N	N	N	N	N
Polyethylene glycol 400	N	N	N	N	N	N
Glycerin	N	N	N	N	N	N
Polyoxyethylene sorbitan monooleate (20E.O.)	N	P	P	P	P	P
Triethanolamine	N	N	N	N	N	N
Methyl p-hydroxybenzoate	N	P	P	N	N	N
Sucrose fatty acid ester	P	P	P	P	P	P
Benzyl alcohol	P	P	P	P	P	P
Ethanol	P	N	N	N	N	N
Acid red 92	P	P	P	P	P	P
Calcium thioglycolate	P	P	P	P	P	
m-Phenylenediamine#	P					
Sodium salicylate	P	P	P	P	P	P
Distearyldimethylammonium chloride	P	P	P	P	P	P
Lactic acid	P	P	P	P	P	P
Sodium dodecyl sulfate*	P	P	P			
Diisopropanolamine*	P	P	P	P	P	P
Monoethanolamine*	P	P	P	P	P	P
Glycolic acid*	P	P	P	P	P	P
Sodium hydrogenated tallow L-glutamate*	P	P	P			
Chlorhexidine gluconate (20% solution)*	P	P	P	P	P	P
Butanol*	P	N	N	N	N	N
Potassium laurate*	P	P	P		P	P
Polyoxyethylene octylphenylether (10 E.O.)*	P	P	P			
Di (2-ethylhexyl) sodium sulfosuccinate*	P	P	P	P	P	P
Acetic acid*	P	P	P	P		P
Cetyltrimethylammonium bromide*	P	P	P	P		P
Benzalkonium chloride*	P	P	P			
Stearyltrimethylammonium chloride*	P	P	P	P	P	P
Cetylpyridinium chloride*	P	P	P	P	P	P
Domiphen bromide*	P	P	P	P	P	P

P:Positive, N:Negative

*: The *in vivo* results of as is application was predicted from the data of 10% concentration.

#:M-Phenylenediamine was excluded from analysis due to instability. (Tani et al., 1999)

Blank column: NT(Not tested) or NE (Could not be evaluated)

6. SIRC 細胞毒性試験の追加試験および無刺激性物質検出能力の解析[研究 2]

6.1.目的

1991 年度に発足した厚生科学研究班の「新規化粧品原料配合化粧品の安全性評価のための試験法の研究」において 12 種の眼刺激性試験代替法のバリデーション研究が実施され、その中の CVS 法による SIRC 細胞毒性試験について GHS の NI を同定する試験法として再解析された。その結果、再現性は施設内および施設間で良好であることが確認された。

次の段階として、GHS の NI を同定する試験法としてインビボを予測できるか否かを検討する必要があるが、そのためには更に多くの物質のデータが必要であった。特に、GHS 分類における 2B の物質のデータが不足していた。また、厚生科学研究のデータに基づく解析では、NI 以外が 25 物質に対し、NI は 9 物質であり、NI の追加も必要と思われた。そこで、ICCVAM Recommended Reference Substance List(2006)に収載されている 2B および NI の化学物質(計 27 物質)全てについて SIRC 細胞毒性試験を実施した。また、原体(100%)でのインビボの報告がある化粧品原料 41 品について SIRC 細胞毒性試験を実施した。厚生科学研究におけるデータに追加データを加え、SIRC 細胞毒性試験が GHS の NI を同定する能力について検討した。

6.2.研究施設

本研究が行われた研究施設は GLP 適合施設では無く、GLP に従った試験は実施していない。被験物質はコード化されずに試験が行われた。しかしながら、被験物質の管理および測定データの処理を含む試験操作については、施設内で QA チェックが行われ、生データが適切に最終結果に反映されていることなどを確認した。

6.3.プロトコール

6.3.1.試験に用いたプロトコール

試験に用いたプロトコールは Annex 2 参照。

6.3.2.(JaCVAM による第三者評価時の)提案プロトコール(Annex 3)

- 細胞
  - ウサギ角膜由来細胞(SIRC; Statens Seruminstitut Rabbit Cornea: ATCC No.CCL-60)を ATCC(American Type Culture Collection)から入手する。
  - 細胞はマイコプラズマ汚染がないことを確認する。
- 培養液と培養条件
  - 10%牛胎児血清(FBS)と 200mM L-Glutamine を添加し、Sodium bicarbonate で中和した MEM 培養液を用い、37℃、5%CO_2 で培養する。培養液には適切な抗生物質を用いることができる。例えば、Antibiotic-Antimycotic (GIBCO BRL)または Penicillin Streptmycin (GIBCO BRL)を培養液中へ 1%の濃度になるように加える。この場合の最終濃度は、Penicillin 100U/mL, Streptmycin 100 μg/mL, (Antibiotic-Antimycotic においてはこれに加え Amphotericin B 250ng/mL)である。
  - 凍結保存した細胞は、解凍後、試験に用いる前に 1 回以上継代し、良好な増殖を示すことを確認する。
  - 96 ウェルプレートに播種する細胞の最終濃度は 1x10^5 個/mL とする。
- 被験物質の調製
  - 被験物質は用時に調製する。
  - 被験物質は培養液に 10000 μg/mL の濃度に溶解または均一に懸濁させて被験物質液とする。被験物質を溶解または懸濁させる際に、ミキサー、加温機や超音波処理機を用いることができる。また、DMSO および Ethanol を溶媒として用い、培養液中に溶解または均一に懸濁させることができる。溶媒を用いる際、被験物質液中の DMSO および Ethanol の最高濃度は 10000 μg/mL とする。最終的な被験物質の最高試験濃度は 5000 μg/mL、溶媒の試験濃度は 5000 μg/mL とする。なお、被験物質適用後に沈殿などが認められた場合、該当する濃度は均一に懸濁していなかったものとする。

・DMSO、Ethanol 以外の溶媒を用いる場合は、媒体が化学的に安定であること、細胞毒性を示さない濃度であること，被験物質と反応しないことを使用前に確認する。

・被験物質の濃度
・被験物質の最高試験濃度を 5000μg/mL とし、公比 2 で4段階以上の濃度を設ける。これより細かい公比を設けることができる。（少なくとも 5000μg/mL から公比 2 で 4 段階取れば比較対照物質 Triethanolamine の IC50 の範囲である 1000～5000μg/mL をカバーすることが可能である）(Annex 4,5)

・対照および試験成立基準
・陽性対照
・陽性対照として Sodium dodecyl sulfate（SDS）（CAS:151-21-3）を用いる。標準的なプロトコールで試験された SDS の IC50 は、50～250μg/mL の範囲であり、これを試験成立の条件とする(Annex 5)。
・比較対照
・GHS における NI を同定する比較対照物質として Triethanolamine（CAS：102-71-6）を用いる。標準的なプロトコールで試験された Triethanolamine の IC50 は、1000～5000μg/mL の範囲であり、これを試験成立の条件とする。
・陰性対照
・陰性対照として、培養液、10000μg/mL DMSO 培養液溶液または 10000μg/mL Ethanol 培養液溶液を用いる。これらは被験物質を溶解または懸濁させる際に用いた溶媒によって選択する。標準的なプロトコールで試験された場合の吸光度は 0.4 を越えており、これを試験成立の条件とする。

・試験操作
・被験物質等の細胞への適用
・96 ウェルマイクロプレート上に被験物質、陽性対照、比較対照の希釈系列(0.1mL/ウェル)を作製する。また、陰性対照のウェル、並びに細胞を添加しないウェル（例えば、PBS(-)を 0.2mL/ウェル添加）を作製する。
・2 x10^5 個/mL の SIRC 細胞浮遊液を、被験物質、陽性対照、比較対照および陰性対照のウェルに 0.1mL ずつ添加する。
・被験物質が揮発し周囲のウェルへ影響を与える可能性を考慮する場合、ウェルを覆うマイクロプレートシーリングテープを貼付することができる。なお、被験物質が他のウェルに影響を与える場合には、希釈して再試験をすることができる。
・培養
・約 20 分間の室温放置後、炭酸ガスインキュベーター中に移し、37℃、5%CO_2 で約 72 時間培養する。
・Crystal violet による細胞染色と吸光度測定
・反転により培養液を捨てる。
・PBS(-)液により 2 回洗浄する。
（PBS(-)液を 0.2～0.25mL/ウェル入れ、穏やかに撹拌後反転により捨てる）
・0.4%Crystal violet methanol 溶液を 0.1mL/ウェル添加し、室温で 30 分間放置する。
・水道水にて緩やかに洗浄した後、風乾する。
・マイクロプレートリーダーを用いて 588nm の吸光度を測定する。波長は 570nm～595nm の範囲内で設定することができる。
・陰性対照の吸光度を 100%とし、50%の吸光度を示す濃度(IC50)を算出する。
・IC50 の算出にあたっては、生存率 50%をはさむ 2 濃度とその濃度における細胞生存率から式 LogIC50=[(50-y1)logx2-(50-y2)logx1]/(y2-y1) を用いて算出する。（※記号は、被験物質濃度 x1（低濃度側）、 x2（高濃度側）におけるそれぞれの細胞生存率を y1，y2 で

示す。Log は常用対数である。）
また、片対数グラフに濃度-反応曲線を作成し、陰性対照の 50%となる濃度を求めても良い。適切な解析ソフトがあればそれを用いても良い。
・被験物質の濃度 5000μg/mL で細胞生存率が 50%以下にならない場合は IC50>5000μg/mL とする。試験した最低濃度で細胞生存率が 50%未満の場合は、IC50 は試験した最低濃度未満とする。

・結果の評価
・比較対照物質 Triethanolamine の IC50 と比較し、GHS で NI に分類される物質を予測する。Triethanolamine の IC50 以上を陰性とし、NI に分類されると予測する。
・試験は 2 回を繰り返して行い、その結果に基づき評価する。2 回の評価結果が異なった場合には同様に 3 回目を実施し、2 回の同じ評価結果を採用し、その結果に基づき評価する。

6.3.3.試験に用いたプロトコールと（JaCVAM による第三者評価時の）提案プロトコールとの違い

表 11 試験に用いたプロトコールと提案するプロトコールとの違い

項目	試験時のプロトコール	提案するプロトコール
陽性対照に基づく試験合格基準	陽性対照(SDS)の IC50 値が設定した範囲に収まることを試験成立の条件とする。その範囲は、厚生科学研究で得られた SDS の平均 IC50±3SD（99%信頼区間）である 77.7～258.7μg/mL を合格基準とする。	陽性対照として SDS を設定する。標準的なプロトコールで試験された SDS の IC50 が 50～250μg/mL の範囲であり、これを試験成立の条件とする。
2 試験間での陽性対照のばらつきに基づく試験合格基準	2 試験間での陽性対照（SDS）の IC50 値が±2 倍以内に収まることを合格基準とする。	基準を設けない
左右の陰性対照のばらつきに基づく試験合格基準	体系的に試験精度を見極めるために、96 穴マイクロプレートの左右に陰性対照を設定し、両者の吸光度が同様であることを確認する。左右の陰性対照の平均吸光度が全体の平均吸光度の 15%以内(平均値±15%)に収まることを試験の合格基準とする。	基準を設けない
マイクロプレートシーリングテープの貼付	物質が揮発性し周囲のウェルへ影響を与える可能性を考慮し、ウェルを覆うマイクロプレートシーリングテープを貼付する。	被験物質が揮発し周囲のウェルへ影響を与える可能性を考慮する場合、ウェルを覆うマイクロプレートシーリングテープを貼付することができる(Annex 6)。

6.4.被験物質

本研究で用いた物質を Table 12 に示す。ICCVAM Recommended Reference Substance List に収載されている物質のうち NI と 2B に分類されている 27 物質（No.1～27）を用いた。更に原体（100%）での *in vivo* の報告があり、かつ培養液に溶解または懸濁可能な化粧品原料 41 物質（No.28～68）を用いた。これら 68 物質の内訳は、界面活性剤 12 物質、エステル 10 物質、アルコール 9 物質、ポリオール7物質、芳香族化合物 5 物質、有機塩 5 物質、ベンゾフェノン 2 原料、オイル 2 物質、無機塩 2 物質、エーテル2物質、ニトリル 1 物質、酸 1 物質、アルデヒド 1 物質、有機

金属 1 物質、ヘテロサイクリック化合物 1 物質、ハロゲン化炭化水素 1 原料、ケトン 1 物質、チオール 1 物質、ジオキソラン 1 物質、炭化水素 1 物質、トリアセテート 1 物質、アミン 1 物質であった。また、これらを存在状態で分類すると、固体 26 物質、液体 40 物質、水溶液 2 物質であった。

Table 12 The 68 substances

	Substance	CAS	Class	Physical state	MW
1	Ethyl-2-methyl acetoacetate	609-14-3	Esters	Liquid	144.2
2	Ammonium nitrate	6484-52-2	Inorganic salts	Solid	80.0
3	Butyl Dipropasol Solvent	29911-27-1	Ethers	Liquid	176.3
4	3-Chloropropionitrile	542-76-7	Nitriles	Liquid	89.5
5	Cyclopentanol	96-41-3	Alcohols	Liquid	86.1
6	3,3-Dithiodipropionic acid	1119-62-6	Acids	Solid	210.3
7	Hexyl cinnamic aldehyde	101-86-0	Aldehydes	Liquid	216.3
8	N-Lauroylsarcosine sodium salt	137-16-6	Surfactants	Solid	293.4
9	Maneb	12427-38-2	Organic metals	Solid	265.3
10	2-Methyl-1-pentanol	105-30-6	Alcohols	Liquid	102.2
11	Propasol Solvent P	1569-01-3	Ethers	Liquid	118.2
12	6-Methyl purine	2004-03-7	Heterocyclic compounds	Solid	134.1
13	2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	96568-04-6	Esters	Solid	280.1
14	Triton X-100	9002-93-1	Surfactants	Liquid	250.4
15	iso-Octyl acrylate	29590-42-9	Esters	Liquid	184.3
16	tetra-Aminopyrimidine sulfate	5392-28-9	Organic salts	Solid	238.2
17	2,4-Difluoronitrobenzene	446-35-5	Aromatics	Liquid	159.1
18	n,n-Dimethylguanidine sulfate	598-65-2	Organic salts	Solid	272.3
19	2-(n-Dodecylthio)ethanol	1462-55-1	Alcohols	Liquid	206.3
20	iso-Propyl bromide	75-26-3	Halogenated hydrocarbon	Liquid	123.0
21	Di-iso-butyl ketone	108-83-8	Ketones	Liquid	142.2
22	iso-Octylthioglycolate	25103-09-7	Thiols	Liquid	204.3
23	2,4-Pentanediol	625-69-4	Polyols	Liquid	104.2
24	2,2-Dimethyl-3-pentanol	3970-62-5	Alcohols	Liquid	116.2
25	Potassium tetrafluoroborate	14075-53-7	Inorganic salts	Solid	125.9
26	3-Methoxy-1,2-propanediol	623-39-2	Polyols	Liquid	106.1
27	Toluene	108-88-3	Aromatics	Liquid	92.1
28	2-Bromo-2-Nitropropane-1,3-Diol	52-51-7	Polyols	Solid	200.0
29	Benzalkonium chloride	8001-54-5	Surfactants	Solid	283.9
30	Benzophenone-1	131-56-6	Benzophenones	Solid	214.2
31	Benzophenone-2	131-55-5	Benzophenones	Solid	246.2
32	Butoxyethanol	111-76-2	Alcohols	Liquid	118.2
33	Butylene glycol	107-88-0	Polyols	Liquid	90.1
34	Cetrimonium chloride	112-02-7	Surfactants	Solid	320.0
35	Cetyl alcohol	36653-82-4	Alcohols	Solid	242.4
36	Chlorhexidine digluconate 20% solution	18472-51-0	Organic salts	Solution	897.8
37	Chlorophene	120-32-1	Aromatics	Solid	218.7
38	Chloroxylenol	88-04-0	Aromatics	Solid	156.6
39	Diethylhexyl adipate	103-23-1	Esters	Liquid	370.6
40	Diisopropyl adipate	6938-94-9	Esters	Liquid	230.3
41	Dioctyl sodium sulfosuccinate	577-11-7	Surfactants	Solid	488.5
42	Ethylhexyl palmitate	29806-73-3	Esters	Liquid	368.6
43	Hexylene glycol	107-41-5	Polyols	Liquid	118.2
44	Isocetyl stearate	25339-09-7	Esters	Liquid	508.9
45	Isopropyl Myristate	110-27-0	Esters	Liquid	270.45
46	Isopropyl Palmitate	142-91-6	Esters	Liquid	298.5
47	Lauramide DEA	120-40-1	Surfactants	Solid	287.4
48	Methoxyisopropyl acetate	108-65-6	Esters	Liquid	132.2
49	Oleyl alcohol	143-28-2	Alcohols	Liquid	268.5
50	PEG-40 stearate	9004-99-3	Surfactants	Solid	-
51	Phenethyl alcohol	60-12-8	Alcohols	Liquid	122.2
52	Phenoxyethanol	122-99-6	Alcohols	Liquid	138.2
53	Phytantriol	74563-64-7	Polyols	Liquid	330.6
54	Propylene carbonate	108-32-7	Dioxolanes	Liquid	102.1
55	Resorcinol	108-46-3	Aromatics	Solid	110.1
56	Safflower (Carthamus tinctorius) oil	8001-23-8	Oils	Liquid	-
57	Sesame (Sesamum indicum) oil	8008-74-0	Oils	Liquid	-
58	Sodium dehydroacetate	4418-26-2	Organic salts	Solid	190.1
59	Sodium naphthalenesulfonate	532-02-5	Organic salts	Solid	230.2
60	Sodium stearate	822-16-2	Surfactants	Solid	306.5
61	Sorbitan oleate	1338-43-8	Surfactants	Liquid	428.6
62	Sorbitan sesquioleate	8007-43-0	Surfactants	Liquid	1175.7
63	Squalane	111-01-3	Hydrocarbons	Liquid	422.8
64	Stearalkonium chloride	122-19-0	Surfactants	Solid	424.2
65	TEA-Lauryl sulfate 40% solution	139-96-8	Surfactants	Solution	415.6
66	Triacetin	102-76-1	Triacetates	Liquid	218.2
67	Triethylene glycol	112-27-6	Polyols	Liquid	150.2
68	Triisopropanolamine	122-20-3	Amines	Solid	191.3

6.5.被験物質の Draize 試験データに基づく分類

被験物質についての GHS 分類データを Table 13-1 および 13-2 に示す。27 物質(No.1～27)については ICCVAM Recommended Reference Substance List により分類した。化粧品原料 41 物質(No.28～68)については、論文並びにグローバルな化学物質データベースのデータに基づいて分類した(Annex 7)。用いたデータベースは IUCLID (International Uniform ChemicaL Information Database)、SIDS (Screening Information Data Set)、ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals)であった。論文に示されたデータは、角膜、虹彩、結膜への影響の有無や MAS 等が記載されているため、角膜に反応が認められない場合または MAS 15点以下を NI として分類した。IUCLID および SIDS においては Not (Non) irritating 、Slightly irritating の結果となっているもの、または MAS15 点以下を NI と分類した。データベースの記載内容を調べ、GHS 分類に関わるデータを考慮して分類した。ECETOC は直接 GHS 基準で分類した。NI を陰性(N)、それ以外を陽性(P)で示した。なお、被験物質の希釈液で陽性の結果が得られている場合には、眼刺激の濃度依存性に基づいて、原体の眼刺激性を陽性と判別した。

Table 13-1 GHS classification of the substances

	Substance	GHS classification by ICCVAM Recommended Reference Substance List	The classification used in this study
1	Ethyl-2-methyl acetoacetate	2B	P
2	Ammonium nitrate	2B	P
3	Butyl Dipropasol Solvent	2B	P
4	3-Chloropropionitrile	2B	P
5	Cyclopentanol	2B	P
6	3,3-Dithiodipropionic acid	2B	P
7	Hexyl cinnamic aldehyde	2B (12.5%)*	P
8	N-Lauroylsarcosine sodium salt	2B	P
9	Maneb	2B	P
10	2-Methyl-1-pentanol	2B	P
11	Propasol Solvent P	2B	P
12	6-Methyl purine	2B	P
13	2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	2B	P
14	Triton X-100	1(100%), 2B (5%), NI (1%)	P
15	iso-Octyl acrylate	NI	N
16	tetra-Aminopyrimidine sulfate	NI	N
17	2,4-Difluoronitrobenzene	NI	N
18	n,n-Dimethylguanidine sulfate	NI	N
19	2-(n-Dodecylthio)ethanol	NI	N
20	iso-Propyl bromide	NI	N
21	Di-iso-butyl ketone	NI	N
22	iso-Octylthioglycolate	NI	N
23	2,4-Pentanediol	NI	N
24	2,2-Dimethyl-3-pentanol	NI	N
25	Potassium tetrafluoroborate	NI	N
26	3-Methoxy-1,2-propanediol	NI	N
27	Toluene	NI	N

P: Positive, N: Negative.

*: Tested concentration is shown in parenthesis.

Table 13-2 GHS classification of the substances

	Substance	GHS classification by ICCVAM Recommended Reference Substance List	GHS classification predicted from the previous paper	GHS classification obtained from Japanese validation study	GHS classification obtained from grobal chemical databases	The classification used in this study
28	2-Bromo-2-Nitropropane-1,3-Diol		2B, 2A or 1			P
29	Benzalkonium chloride	1 (5%)*	2B, 2A or 1	2A or 1 (10%)	1(10%)&	P
30	Benzophenone-1		2B			P
31	Benzophenone-2		2B			P
32	Butoxyethanol		1		2B or 2A#, 2B or 2A$	P
33	Butylene glycol		NI		NI#	N
34	Cetrimonium chloride		2B, 2A or 1			P
35	Cetyl alcohol		NI		NI#	N
36	Chlorhexidine digluconate		2Aor1			P
37	Chlorophene		2Aor1			P
38	Chloroxylenol		2B, 2A or 1			P
39	Diethylhexyl adipate		NI		NI#, NI$	N
40	Diisopropyl adipate		NI			N
41	Dioctyl sodium sulfosuccinate		2B, 2A or 1		2A#,	P
42	Ethylhexyl palmitate		NI			N
43	Hexylene glycol		2B, 2A or 1		2B, 2A or 1#, NI, 2B or 2A$	P
44	Isocetyl stearate		NI			N
45	Isopropyl Myristate		NI	NI	NI#	N
46	Isopropyl Palmitate		NI			N
47	Lauramide DEA		2A or 1			P
48	Methoxyisopropyl acetate		2B, 2A or 1		NI#, 2B or 2A$	P
49	Oleyl alcohol		NI			N
50	PEG-40 stearate		NI			N
51	Phenethyl alcohol		2B, 2A or 1		2B or 2A#	P
52	Phenoxyethanol		2A		2B or 2A#, 2B, 2A or 1$	P
53	Phytantriol		2B, 2A or 1			P
54	Propylene carbonate		NI		2B or 2A#	P
55	Resorcinol		2B, 2A or 1		2A or 1#	P
56	Safflower (Carthamus tinctorius) oil		NI			N
57	Sesame (Sesamum indicum) oil		NI			N
58	Sodium dehydroacetate		NI			N
59	Sodium naphthalenesulfonate		2A or 1			P
60	Sodium stearate		NI			N
61	Sorbitan oleate		NI			N
62	Sorbitan sesquioleate		NI			N
63	Squalane		NI			N
64	Stearalkonium chloride		2A or 1			P
65	TEA-Lauryl sulfate		2A or 1		2A or 1#	P
66	Triacetin		NI		NI#, NI$	N
67	Triethylene glycol		NI		NI#	N
68	Triisopropanolamine		2B, 2A or 1			P

P: Positive, N: Negative.
*: Tested concentration is shown in parenthesis.
#: It was classified on the basis of the data from the IUCLID.
$: It was classified on the basis of the data from SIDS.
&: It was classified on the basis of the data from ECETOC Technical report No.48 (2).

6.6. 68 種の被験物質の SIRC 細胞毒性試験

68 物質の SIRC 細胞毒性試験結果について Table 14 に示す。No.4 の 3-Chloropropionitrile は、最高試験濃度 5000 μg/mL で揮発による陰性対照ウェルへの影響が認められ、500 μg/mL で試験した結果、陽性を示した。No.5 の Cyclopentanol は、1 回目の試験が陽性対照の試験成立基準に適合しなかったため再試験を行った結果、陰性を示した。No.9 の Maneb については、溶媒を用いても均一な懸濁が得られず試験を実施できなかった。No.17 の 2,4-Difluoronitrobenzene は最高試験濃度 5000 および 500 μg/mL で揮発による陰性対照ウェルへの影響が認められ、50 μg/mL で試験した結果、陽性を示した(Annex 8)。

これらのデータは、SIRC 細胞による GHS の NI の予測についての検討（後述）に供した。

Table14 Results of the 68 substances

No.	Substance	IC50 of th first measurement		IC50 of the second measurement		*In vitro*	*In vivo*	Evaluation
		Substance	Triethanolamine	Substance	Triethanolamine			
1	Ethyl-2-methyl acetoacetate	2978.4	2164.2	3410.9	1620.4	N	P	False negative
2	Ammonium nitrate	1999.0	2000.5	1439.6	1808.3	P	P	True positive
3	Butyl Dipropasol Solvent	2729.9	1675.7	3646.0	1401.5	N	P	False negative
4	3-Chloropropionitrile	47.2	1757.2	50.1	1604.0	P	P	True positive
5	Cyclopentanol	2684.1	1656.6	2366.4	1687.6	N	P	False negative
6	3,3-Dithiodipropionic acid	1436.8	1940.1	1313.8	1674.5	P	P	True positive
7	Hexyl cinnamic aldehyde	49.1	1709.2	125.5	1704.8	P	P	True positive
8	N-Lauroylsarcosine sodium salt	53.3	2228.9	55.1	1694.8	P	P	True positive
9	Maneb	Could not be tested		Could not be tested			P	Could not be tested
10	2-Methyl-1-pentanol	1665.9	1558.9	1688.2	1386.8	N	P	False negative
11	Propasol Solvent P	3889.9	1868.2	3816.8	1663.4	N	P	False negative
12	6-Methyl purine	<39.1	1669.9	<39.1	1576.9	P	P	True positive
13	2,6-Dichloro-5-fluoro-beta -oxo-3-pyridinepropanoate	<39.1	1932.9	84.2	1461.8	P	P	True positive
14	Triton X-100	<39.1	1945.1	<39.1	1599.5	P	P	True positive
15	iso-Octyl acrylate	327.7	1424.0	98.1	1251.7	P	N	False positive
16	Tetra-Aminopyrimidine sulfate	97.8	1666.2	85.7	1347.1	P	N	False positive
17	2,4-Difluoronitrobenzene	30.4	1012.3	36.2	1595.4	P	N	False positive
18	n,n-Dimethylguanidine sulfate	1380.8	1526.8	1018.5	1690.2	P	N	False positive
19	2-(n-Dodecylthio)ethanol	<39.1	1501.7	169.6	1448.5	P	N	False positive
20	iso-Propyl bromide	>5000	1763.3	>5000	1206.8	N	N	True negative
21	Di-iso-butyl ketone	>5000	1773.9	>5000	1808.9	N	N	True negative
22	iso-Octylthioglycolate	399.6	1614.6	219.1	1452.7	P	N	False positive
23	2,4-Pentanediol	>5000	1435.9	3126.7	1295.2	N	N	True negative
24	2,2-Dimethyl-3-pentanol	1399.8	1500.2	976.2	1429.1	P	N	False positive
25	Potassium tetrafluoroborate	4595.1	1525.0	>5000	1683.3	N	N	True negative
26	3-Methoxy-1,2-propanediol	>5000	1820.5	>5000	1451.3	N	N	True negative
27	Toluene	>5000	1349.7	>5000	1782.0	N	N	True negative
28	2-Bromo-2-Nitropropane-1,3-Diol	<39.1	1786.8	<39.1	1757.9	P	P	True positive
29	Benzalkonium chloride	<39.1	1664.1	<39.1	1118.3	P	P	True positive
30	Benzophenone-1	52.4	1338.9	92.9	1452.3	P	P	True positive
31	Benzophenone-2	49.4	2145.3	76.0	1669.1	P	P	True positive
32	Butoxyethanol	2099.4	1861.3	2275.0	1330.7	N	P	False negative
33	Butylene glycol	>5000	1770.2	>5000	1488.4	N	N	True negative
34	Cetrimonium chloride	<39.1	1611.9	<39.1	1534.3	P	P	True positive
35	Cetyl alcohol	<39.1	1550.9	<39.1	2290.9	P	N	False positive
36	Chlorhexidine digluconate 20% solution	<39.1	1408.8	<39.1	1437.1	P	P	True positive
37	Chlorophene	<39.1	1260.3	<39.1	1441.2	P	P	True positive
38	Chloroxylenol	81.1	1267.2	69.7	1374.6	P	P	True positive
39	Diethylhexyl adipate	>5000	1695.5	>5000	1354.3	N	N	True negative
40	Diisopropyl adipate	372.0	1495.1	333.9	1486.9	P	N	False positive
41	Dioctyl sodium sulfosuccinate	53.2	1339.4	55.5	1303.1	P	P	True positive
42	Ethylhexyl palmitate	>5000	1218.0	>5000	1662.7	N	N	True negative
43	Hexylene glycol	>5000	1484.0	>5000	1485.4	N	P	False negative
44	Isocetyl stearate	>5000	1468.0	>5000	1696.4	N	N	True negative
45	Isopropyl Myristate	>5000	1531.6	3606.0	1452.4	N	N	True negative
46	Isopropyl Palmitate	>5000	1222.7	>5000	1557.3	N	N	True negative
47	Lauramide DEA	<39.1	1737.8	<39	1555.9	P	P	True positive
48	Methoxyisopropyl acetate	2482.4	1662.5	4172.9	1647.2	N	P	False negative
49	Oleyl alcohol	<39.1	1706.2	<39	1283.2	P	N	False positive
50	PEG-40 stearate	288.9	1436.5	249.0	1700.4	P	N	False positive
51	Phenethyl alcohol	621.0	1446.6	753.3	1508.0	P	P	True positive
52	Phenoxyethanol	970.7	1471.9	1420.5	2276.3	N	P	False negative
53	Phytantriol	<39.1	1545.5	53.0	1565.2	P	P	True positive
54	Propylene carbonate	>5000	1584.5	>5000	1552.1	N	N	True negative
55	Resorcinol	401.3	1413.8	386.7	1498.2	P	P	True positive
56	Safflower (Carthamus tinctorius) oil	1786.3	1439.4	2644.7	1601.9	N	N	True negative
57	Sesame (Sesamum indicum) oil	>5000	1622.5	>5000	1009.0	N	N	True negative
58	Sodium dehydroacetate	827.1	1621.0	1012.7	1499.5	P	N	False positive
59	Sodium naphthalenesulfonate	1321.2	1464.9	639.4	1381.5	P	P	True positive
60	Sodium stearate	194.2	1857.2	337.9	1628.4	P	N	False positive
61	Sorbitan oleate	784.6 (test1) 3142.8 (test2)	1403.1（test1） 1446.3（test2）	866.0 (test3)	1424.0(test3)	P	N	False positive
62	Sorbitan sesquioleate	1439.8	1713.5	917.4	1781.1	P	N	False positive
63	Squalane	>5000	1513.6	>5000	1550.3	N	N	True negative
64	Stearalkonium chloride	<39.1	1631.5	<39.1	1341.0	P	P	True positive
65	TEA-Lauryl sulfate 40% solution	234.4	1825.7	241.6	1586.1	P	P	True positive
66	Triacetin	1470.1	1685.9	1482.6	1576.9	P	N	False positive
67	Triethylene glycol	>5000	1769.7	>5000	1446.2	N	N	True negative
68	Triisopropanolamine	845.5	1642.3	614.0	1549.9	P	P	True positive

6.7.SIRC 細胞毒性試験による NI の予測

SIRC 細胞毒性試験によって GHS 分類の NI とそれ以外を区別できるか否かについて、厚生科学研究の 33 物質のデータに追加試験で実施した 68 物質のデータを加えて検討した。NI を陰性(N)、それ以外を陽性(P)として示した。Benzalkonium chloride、Polyoxyethylene octylphenylether (10 E.O.)(別名 Triton X-100)、Isopropyl myristate および Di(2-ethylhexyl) sodium sulfosuccinate(別名 Dioctyl sodium sulfosuccinate)、の 4 物質は両者に共通であり、さらに追加試験で用いられた Maneb は難溶解性で試験できなかったため、実質的には 63 物質の追加となり、合計 96 物質で対応性を検討した。なお、Benzalkonium chloride、Polyoxyethylene octylphenylether (10 E.O.)、Isopropyl myristate および Di(2-ethylhexyl) sodium sulfosuccinate の結果については、厚生科学研究と追加試験の両者で結果は一致し、インビボにおける陽性、陰性を SIRC 細胞毒性試験は正しく予測した(Annex 9)。

96 物質中 65 物質の結果がインビトロとインビボで一致した。偽陰性は、Ethanol、Butanol、Ethyl-2-methyl acetoacetate、Butyl Dipropasol Solvent、Cyclopentanol 、2-Methyl-1-pentanol、Propasol Solvent P、Butoxyethanol、Hexylene glycol、Methoxyisopropyl acetate、Phenoxyethanol、Propylene carbonate であった。これらはアルコール 6 物質、エステル 2 物質、エーテル 2 物質、ポリオール 1 物質およびジオキソラン1物質であり、分子量は 180 未満の低分子であるという特徴を有していた。

ポリオールについてはインビボで陽性を示す 3 物質(2-Bromo-2-Nitropropane-1,3-Diol, Hexylene glycol, Phytantoriol)、インビボで陰性を示す6物質(Glycerol、Polyethylene glycol 400、2,4-Pentanediol, 3-Methoxy-1,2-propanediol 、Butylene glycol、Triethylene glycol)について検討され、8 物質でインビトロとインビボが対応したが、Hexylene glycol のみ偽陰性を示した。

偽陽性は、iso-Octyl acrylate、tetra-Aminopyrimidine sulfate、n,n-Dimethylguanidine sulfate、2-(n-Dodecylthio)ethanol 、iso-Octylthioglycolate 、2,2-Dimethyl-3-pentanol 、Cetyl alcohol 、Diisopropyl adipate、Oleyl alcohol、PEG-40 stearate、Sodium dehydroacetate、Sodium stearate、Sorbitan oleate、Sorbitan sesquioleate、Triacetin であった。

感度、特異度、偽陽性度、偽陰性度、一致度はそれぞれ 79%(44/56)、53%(21/40)、48%(19/40)、21%(12/56)、68%(65/96)であった。

なお、追加データに関しては、学会で報告されている結果と一部相違がみられたが、Annex 10 でその理由を示している。

Table 15 The eye irritancy of test samples predicted by the SIRC-CVS assay

		In vitro (Classification by SIRC-CVS assay using triethanolamine as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	Positive (1, 2Aor 2B)	(44) Sucrose fatty acid ester Benzyl alcohol Acid red 92 Calcium thioglycolate Sodium salicylate Distearyldimethylammonium chloride Lactic acid Sodium dodecyl sulfate* Diisopropanolamine* Monoethanolamine* Glycolic acid* Sodium hydrogenated tallow L-glutamate* Chlorhexidine gluconate (20% solution)* Potassium laurate* Polyoxyethylene octylphenylether (10 E.O.)* Di (2-ethylhexyl) sodium sulfosuccinate* Acetic acid* Cetyltrimethylammonium bromide* Benzalkonium chloride* Stearyltrimethylammonium chloride* Cetylpyridinium chloride* Domiphen bromide* --- Ammonium nitrate 3-Chloropropionitrile 3,3-Dithiodipropionic acid Hexyl cinnamic aldehyde N-Lauroylsarcosine sodium salt 6-Methyl purine 2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate 2-Bromo-2-Nitropropane-1,3-Diol Benzophenone-1 Benzophenone-2 Cetrimonium chloride Chlorhexidine digluconate Chlorophene Chloroxylenol Lauramide DEA Phenethyl alcohol Phytantriol Resorcinol Sodium naphthalenesulfonate Stearalkonium chloride TEA-Lauryl sulfate 40% solution Triisopropanolamine	(12) Ethanol Butanol* --- Ethyl-2-methyl acetoacetate Butyl Dipropasol Solvent Cyclopentanol 2-Methyl-1-pentanol Propasol Solvent P Butoxyethanol Hexylene glycol Methoxyisopropyl acetate Phenoxyethanol Propylene carbonate
	Negative (NI)	(19) 2-Ethylhexyl p-dimethylamino benzoate Polyoxyethylene sorbitan monooleate (20E.O.) Methyl p-hydroxybenzoate --- iso-Octyl acrylate tetra-Aminopyrimidine sulfate 2,4-Difluoronitrobenzene n,n-Dimethylguanidine sulfate 2-(n-Dodecylthio)ethanol iso-Octylthioglycolate 2,2-Dimethyl-3-pentanol Cetyl alcohol Diisopropyl adipate Oleyl alcohol PEG-40 stearate Sodium dehydroacetate Sodium stearate Sorbitan oleate Sorbitan sesquioleate Triacetin	(21) Isopropyl myristate Isotonic sodium chloride solution Silicic anhydride Polyethylene glycol 400 Glycerin Triethanolamine --- iso-Propyl bromide Di-iso-butyl ketone 2,4-Pentanediol Potassium tetrafluoroborate 3-Methoxy-1,2-propanediol Toluene Butylene glycol Diethylhexyl adipate Ethylhexyl palmitate Isocetyl stearate Isopropyl Palmitate Safflower (Carthamus tinctorius) oil Sesame (Sesamum indicum) oil Squalane Triethylene glycol

*: The *in vivo* results of as is application was predicted from the data of 10% concentration.

Table 16 Predictive capacity of the SIRC-CVS assay

	N	Sensitivity	Specificity	False positive rate	False negative rate	Concordance
SIRC-CVS assay vs Draize eye test	96	79% (44/56)	53% (21/40)	48% (19/40)	21% (12/56)	68% (65/96)

次に、SIRC 細胞毒性試験による化学物質・原体の眼刺激性の予測に関し、被験物質の適用範囲を限定して検討した(Annex 11)。当検討については、ICCVAMにおいて牛角膜混濁および透過性試験(BCOP)、摘出鶏眼試験(ICE)を評価する際に行われており、これらの試験の OECD ガイドラインでも特定の物質群を除外することが記載されている。被験物質の適用範囲から除外した物質群は、アルコール、エステル、エーテルで、なおかつ分子量 180 未満の低分子とした。このうち低分子のアルコールについては、今回のものと評価方法は異なるものの、SIRC 細胞毒性試験において偽陰性を示す物質群として ohno ら(1999)により既に報告されている。

該当する 14 被験物質を除外して対応性を検討した結果、82 物質中 63 物質の結果がインビトロとインビボで一致した。偽陰性は Hexylene glycol(Annex 12) と Propylene carbonate、2 物質であった。感度、特異度、偽陽性度、偽陰性度、一致度はそれぞれ 95%(42/44)、55%(21/38)、45%(17/38)、5%(2/44)、77%(63/82)であった。このように適用除外となる物質群を考慮することは NI を予測するうえで不可欠であると思われた(Annex 13)。

以上より、SIRC 細胞毒性試験は、試験法の特性を理解して用いるならば、GHS の NI に分類される物質を予測できる試験法であると判断した。

Table 17 The eye irritancy of test samples predicted by the SIRC-CVS assay

		In vitro (Classification by SIRC-CVS assay using triethanolamine as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	Positive (1, 2Aor 2B)	(42) Sucrose fatty acid ester ~~Benzyl alcohol~~ Acid red 92 Calcium thioglycolate Sodium salicylate Distearyldimethylammonium chloride Lactic acid Sodium dodecyl sulfate* Diisopropanolamine* Monoethanolamine* Glycolic acid* Sodium hydrogenated tallow L-glutamate* Chlorhexidine gluconate (20% solution)* Potassium laurate* Polyoxyethylene octylphenylether (10 E.O.)* Di (2-ethylhexyl) sodium sulfosuccinate* Acetic acid* Cetyltrimethylammonium bromide* Benzalkonium chloride* Stearyltrimethylammonium chloride* Cetylpyridinium chloride* Domiphen bromide* --- Ammonium nitrate 3-Chloropropionitrile 3,3-Dithiodipropionic acid Hexyl cinnamic aldehyde N-Lauroylsarcosine sodium salt 6-Methyl purine 2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate 2-Bromo-2-Nitropropane-1,3-Diol Benzophenone-1 Benzophenone-2 Cetrimonium chloride Chlorhexidine digluconate Chlorophene Chloroxylenol Lauramide DEA ~~Phenethyl alcohol~~ Phytantriol Resorcinol Sodium naphthalenesulfonate Stearalkonium chloride TEA-Lauryl sulfate 40% solution Triisopropanolamine	(2) ~~Ethanol~~ ~~Butanol*~~ --- ~~Ethyl-2-methyl acetoacetate~~ ~~Butyl Dipropasol Solvent~~ ~~Cyclopentanol~~ ~~2-Methyl-1-pentanol~~ ~~Propasol Solvent P~~ ~~Butoxyethanol~~ Hexylene glycol ~~Methoxyisopropyl acetate~~ ~~Phenoxyethanol~~ Propylene carbonate
	Negative (NI)	(17) 2-Ethylhexyl p-dimethylamino benzoate Polyoxyethylene sorbitan monooleate (20E.O.) ~~Methyl p-hydroxybenzoate~~ --- iso-Octyl acrylate tetra-Aminopyrimidine sulfate 2,4-Difluoronitrobenzene n,n-Dimethylguanidine sulfate 2-(n-Dodecylthio)ethanol iso-Octylthioglycolate ~~2,2-Dimethyl-3-pentanol~~ Cetyl alcohol Diisopropyl adipate Oleyl alcohol PEG-40 stearate Sodium dehydroacetate Sodium stearate Sorbitan oleate Sorbitan sesquioleate Triacetin	(21) Isopropyl myristate Isotonic sodium chloride solution Silicic anhydride Polyethylene glycol 400 Glycerin Triethanolamine --- iso-Propyl bromide Di-iso-butyl ketone 2,4-Pentanediol Potassium tetrafluoroborate 3-Methoxy-1,2-propanediol Toluene Butylene glycol Diethylhexyl adipate Ethylhexyl palmitate Isocetyl stearate Isopropyl Palmitate Safflower (Carthamus tinctorius) oil Sesame (Sesamum indicum) oil Squalane Triethylene glycol

*: The *in vivo* results of as is application was predicted from the data of 10% concentration.

Table 18 Predictive capacity of the SIRC-CVS assay

	N	Sensitivity	Specificity	False positive rate	False negative rate	Concordance
SIRC-CVS assay vs Draize eye test	82	95% (42/44)	55% (21/38)	45% (17/38)	5% (2/44)	77% (63/82)

6.8. SIRC 細胞毒性試験による NI の予測のまとめ

SIRC 細胞毒性試験は、先の厚生科学研究のバリデーション研究において施設間の再現性が高いことや Draize 試験の MAS15 点を境界とする分類に対する高い予測能が報告されている。しかしながら、眼刺激性試験の代替法としての妥当性を示すためには化学物質の原体を評価できることを確認する必要があった。そこで、厚生科学研究のデータを再解析し、さらに1施設で実施した追加のデータを加え、SIRC 細胞毒性試験が GHS の NI を予測可能か否かについて検討した。評価にあたっては Triethanolamine を比較対照物質として用い、その IC50 以上である場合を NI とする予測方法とした。その結果、厚生科学研究のデータからは施設内および施設間共に再現性が良好であるという結果を得た。また、追加データを加えた NI の予測能の検討では、低分子（分子量 180 未満）のアルコール、エステル、エーテル等を除外することにより、NI を予測できると思われた。

以上より、SIRC 細胞毒性試験は試験法の特性を理解して用いるならば、GHS の NI に分類される物質を予測できる試験法であると判断した。

7. SIRC 細胞毒性試験の第三者評価(要旨)(Annex 14)

SIRC細胞毒性試験(本試験)は、ウサギ角膜上皮由来細胞(SIRC細胞)に被験物質を暴露した後、72時間培養後のSIRC細胞の細胞生存率を評価指標として、眼の非刺激性を判定する方法である。本試験は、眼に対する非刺激性物質をスクリーニングする目的でウサギを用いた眼刺激性試験(Draize法)の代替法として、厚生労働科学研究の補助金を受けて開発された。JaCVAMは、本試験の有用性を評価するために、眼刺激性試験代替法評価委員会(本委員会)を組織して、本試験法の第三者評価を依頼した。評価は、主導施設(株式会社資生堂)が用意した Background Review Document (BRD) を主資料として行われた。

本バリデーション試験では、供試被験物質の物質区分、液体・固体の物性などにおいては十分な数の被験物質が用いられた。SIRC細胞毒性試験での非刺激性物質検出における正確性をGHS分類による眼刺激性分類と比較した場合、感度は75%、特異度は50%、一致度は65%であった。一方、被験物質の化学物質分類において、アルコール類およびエステル類は偽陰性率が高い区分として認められた。この特定の区分を除くと、感度は89%に、一致度は74%に改善された。試験法の信頼性については、施設内・間変動において良好な結果が得られ問題ないと判断された。

以上の結果から、我が国のGHSに準拠する化学物質に関わる法規制において、アルコール類およびエステル類などの特定の化学物質の特性や培養に適さない物理化学的性状を考慮した上で、化学物質の眼刺激性の段階的評価の1つとして、非刺激性物質を検出する目的のためにSIRC細胞毒性試験法を使用する事は可能であると判断された。

しかし、本試験の正確性と信頼性を厳密に評価するには、提案プロトコールに従い、3 施設以上の GLP 施設にて十分な数の被験物質をコード化して追加バリデーションを実施する事が望まれた。

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9. 英語の略名

略名	英語名称	日本語名称
AOI	Acute Ocular Irritation Index	急性眼刺激性指標
ATCC	American Type Culture Collection	
BCOP	Bovine Corneal Opacity and Permiability Test	牛角膜混濁および透過性試験
CV	Coefficient of Variation	変動係数
CVS	Crystal Violet Staining	クリスタルバイオレット染色
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals	化学物質の環境毒物学と毒物学の欧州センター
ECVAM	European Centre for the Validation of Alternative Methods	欧州代替法検証センター
GHS	Global Harmonized System	世界調和システム
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods	米国動物実験代替法関連官庁調整委員会
IC50	Half Maximal (50%) Inhibitory Concentration	50%阻害濃度
ICE	Isolated Chicken Eye test	摘出鶏眼試験
IUCLID	International Uniform ChemicaL Information Database	国際統一化学物質情報データベース
OECD	Organisation for Economic Co-operation and Development	経済協力開発機構
MAS	Maximal Average Draize Total Socre	最大平均評価点
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide	
NRU	Neutral Red Uptake	ニュートラルレッド取り込み
QA	Quality Assurance	品質保証
SD	Standard Deviation	標準偏差
SIDS	Screening Information Data Set	スクリーニング情報データセット
SIRC	Statens Seruminstitut Rubbit Cornea	

Annex 1　Draize 試験による化粧品、医薬部外品の原料の眼刺激性評価

(1) Draize 試験の公的認知と法規制への取り入れ

Draize 試験は、1981 年に OECD テストガイドラインに収載され、1987、 2002 年に動物愛護の観点から改正された(OECD, 2002)。多くの国々で規制に取り入れられてきており、本邦でも医薬部外品の規制において、Draize 試験が用いられている。化粧品・医薬部外品製造販売ガイドブック 2008(化粧品・医薬部外品製造販売ガイドブック検討会, 2006)には、以下のような試験法が記載されている。

表 1 化粧品・医薬部外品製造販売ガイドブック 2008 に掲載されている試験方法の例
－眼刺激性試験－

試験動物	原則として若齢成熟白色ウサギ
動物数	原則として1群3匹以上
用量	原則として 0.1mL(液体)又は 100mg(固体)
投与方法	片方の眼の下眼瞼を眼球より穏やかに引き離し, 結膜嚢内に投与し, 上下眼瞼を約 1 秒間穏やかに合わせる。他方の眼は未処置のまま残し, 無処置対照眼とする。眼刺激性を示す物質は点眼後に洗眼を行う。
観察	原則として 1、 24、 48、72 および 96 時間後に眼の観察を行う。持続性の角膜障害等が認められた場合には、その経過および可逆性の有無について観察を続ける。

(2) Draize 試験における観察とスコアリング

Draize 試験ではウサギの眼に 0.1mL の試料を投与し、角膜、虹彩、結膜の障害を肉眼観察しスコア化するが、スコアリングには、Draize の基準が用いられる。被験物質の眼刺激性の強さを表す指標としては、一般的に最大平均評価点(MAS)が用いられる。理論的な MAS の最高点は 110 点である。配点は、角膜 80 点、虹彩 10 点、結膜 20 点であり、ヒトでの障害の重要性から角膜の変化に重きが置かれている(金子, 1996)。

表 2 Draize 試験のスコアリング

Ⅰ 角膜	
A 不透明度:混濁の程度(もっとも混濁した領域を読み取る)	
不透明度なし	0
虹彩を明視できる程度の散在からび慢性の不透明化	1
虹彩の細部がわずかにぼやけて見える	2
虹彩の細部が観察できないが、瞳孔の大きさはかろうじて識別できる	3
虹彩が透視できない	4
B 角膜損傷域	
正常	0
0＜A＜1/4	1
1/4≦A＜1/2	2
1/2≦A＜3/4	3
3/4≦A	4
評点:A×B×5(最大値:80)	
Ⅱ 虹彩(A)	
正常	0
皺壁形成亢進、充血、腫脹、角膜周囲の充血(いずれか1つ、あるいは全て、若しくは組み合わせ)が見られるが、対光反射は認められる (緩除反応陽性)。	1
対光反射消失、出血、広範囲の破壊(いずれか1つ、あるいは全て)が見られる。	2
評点:A×5 (最大値:10)	
Ⅲ 結膜	
A 発赤(角膜および虹彩を除く瞼、球結膜)	
正常	0
充血亢進	1
広範囲かつ深紅色となり、血管の識別困難	2
全域の深紅色化	3
B 結膜浮腫	
正常	0
腫脹亢進(瞬瞼を含む)	1
眼瞼の部分的外反を伴う腫脹	2
腫脹を伴う 1/2 程度の眼瞼閉鎖	3
腫脹を伴う 1/2 以上の眼瞼閉鎖	4
C 分泌物	
正常	0
常量以上の分泌物(正常な動物の内眥に見られる少量は含まない)	1
眼瞼および眼瞼に接する被毛を湿潤	2
眼瞼および眼の周囲を相当範囲湿潤	3
評点:(A+B+C)×2 (最大値:20)	

(3)Draize 試験における評価基準

Draize 試験における評価基準としては、様々な基準が報告されている。Kay & Calandra(1962)法の評価基準を以下に示す。その他にも、Guillot ら(1982)の評価基準、DeSousa ら(1984)の評価基準などがある。

表 3 Kay & Calandra 法の評価基準

最大平均評価点	評価
0-0.5	無刺激性
0.5-2.5	実質的無刺激性
2.5-15	最軽度刺激性
15-25	軽度刺激性
25-50	中程度刺激性
50-80	強度刺激性
80-100	非常に強い刺激性
100-110	最強度刺激性

Ohno ら(1999)により Kay & Calandra 法の改変がなされた。これは、Kay & Calandra 法の基準のように細かく分類することは、Draize 試験のばらつきを考えると意義は乏しいとの考察に基づいている。そして、角膜に反応がほぼ認められない点数である MAS15 点を化粧品原料評価の判断基準としている。これは、ウサギの眼の刺激性物質に対する反応が、わずかな刺激を示す場合には結膜に反応が表れ、一定の刺激強度を持った物質については角膜に反応が表れてくるが、このうち一過性の弱い結膜刺激については許容可とする考え方である。なお、本基準は、後述する厚生科学研究による眼刺激性試験代替法の施設間バリデーションの開始前に提示され、代替法の解析に用いられた。

表 4 kay & Calandra 変法の評価基準(Ohno et al, 1999)

最大平均評価点	評価
0～15(以下)	わずかな眼刺激性
15～25	弱い眼刺激性
25～50	中程度の眼刺激性
50～110	強い眼刺激性

また、Ohno(2004)は化粧品原料の眼刺激性評価において、さらに安全性に留意した基準として先の MAS 15 点でなく、5 点を採用した化粧品原料評価の判断基準も示している。この基準は後述する厚生科学研究による眼刺激性試験代替法バリデーションの終了後に、バリデーション研究で得られた結果に基づき考案され、「代替法を組み込んだ化粧品の眼刺激性評価ガイダンス案」に取り入れられた。厚生科学研究のデータに基づき、MAS 5 点を予測するようにインビトロの判断基準を定めておけば、インビトロ試験を実際に用いた際に、陰性であれば陰性であれば眼刺激性はわずかであること、すなわち Draize 試験 MAS15 以下であることがさらに確実に示せるためと考えられる。

表 5 Ohno(2004)による評価基準

最大平均評価点	評価
0～5(以下)	わずかな眼刺激性
5～25	弱い眼刺激性
25～50	中程度の眼刺激性
50～110	強い眼刺激性

Annex 2 SIRC 細胞毒性試験による 68 種の化学物質の評価・プロトコール
(株)資生堂 リサーチセンター
2009 年 11 月 30 日作成　　(※ただし、試験計画書変更書の内容の一部を反映させた)
試験施設:横浜市金沢区福浦 2-12-1 (株)資生堂 リサーチセンター金沢八景
試験責任者:萩野 滋延

1.目的

本試験は、68 種の化学物質を用い、SIRC 細胞毒性試験が眼刺激性試験代替法として GHS の無刺激性物質(NI)とそれ以外(刺激性物質)を区別できるかどうかを調べることを目的とする。

2.試験法の原理

SIRC 細胞毒性試験は Crystal violet が生細胞の細胞膜に入り込んで染色する性質を利用した方法で生細胞のみを測定する。Crystal violet 染色法はほとんどの細胞に適用でき、得られる結果も比較的安定しているため、細胞毒性の簡易試験法として用いられている。また、操作が簡便で、標本の資料保管が可能であることは本試験法の優位性を示すものである。

3.材料

3.1.細胞

細胞はウサギ角膜由来の株化細胞である SIRC 細胞(Statens Seruminstitut rabbit corneal: ATCC No. CCL-60)を用いる。具体的には、本細胞は大日本製薬株式会社を通じて ATCC(American Type Culture Collection)より入手し、液体窒素中で凍結保存されたものを用いる。

3.2.材料(機材)

炭酸ガスインキュベーター(三洋電機バイオメディカ(株)製 MCO-17AIC)
クリーンベンチ(日立製 CCV1300E)
マイクロプレートリーダー(バイオ・ラッド ラボラトリーズ(株)製 Benchmark Plus™)
位相差顕微鏡(Nikon 製 ECLIPSE TS100)
オートクレーブ(TOMY 製 BS-325 および SS-320)
低速冷却遠心機(KUBOTA 製 5800)

3.3.材料(器具)

培養用プラスチックフラスコ(培養面積:75cm^2 または 175cm^2)
96 穴マイクロプレート
マルチチャンネルピペットおよびマイクロピペット
ディスペンサートレイ
遠心管(15mL、 50mL)
マイクロピペット用チップ(200μL、1000μL、5mL)
マイクロプレートシーリングテープ

3.4.材料(培養液および試薬)

Minimum Essential Medium (MEM)
Fetal Bovine Serum (FBS)
Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
(Antibiotic-Antimycotic x100)
L-Glutamine
Sodium bicarbonate
Phosphate-Buffered Saline(-) (PBS(-))

0.25w/v% Trypsin (1mmol/L EDTA・4Na)
Dimethyl sulfoxide (DMSO)
Ethanol (EtOH)
Crystal violet
Methanol
Sodium Dodecyl Sulfate (SDS)
Triethanolamine
なお、試薬のメーカー、ロットは表 1 の通りとする。

3.5.培養液

MEM を精製水(1L)に溶解させ、オートクレーブにて滅菌する。
使用時に、FBS を 10%濃度に、P/S/F を 1%濃度に、L-Glutamine を 200mM 濃度になるように添加し、さらに 7.5% Sodium bicarbonate 水溶液を培養液の色が薄赤色になるまで加える。

3.6.Crystal violet 溶液

Crystal violet をメタノールに溶解し、0.4%溶液を調製する。

3.7.被験物質

表 2 に示す物質を被験物質として用いる。

3.7.1.被験物質の調製

被験物質は培養液に 10000μg/mL の濃度に溶解または均一に懸濁させて被験物質液とする。被験物質を溶解または懸濁させる際に、ミキサー、加温機や超音波処理機を用いることができる。また、DMSO および Ethanol を溶媒として用い、培養液中に溶解または均一に懸濁させることができる。溶媒を用いる際、被験物質液中の DMSO および Ethanol の最高濃度は 10000μg/mL とする。最終的な被験物質の最高試験濃度は 5000μg/mL、溶媒の試験濃度は 5000μg/mL とする。なお、被験物質適用後に沈殿等が認められた場合、該当する濃度は均一に懸濁していなかったものとする。

3.7.2.被験物質液の希釈

被験物質液の濃度段階は公比 2 で 8 段階(100μL/well)とし、希釈液 1 濃度に対して 2 ウェルを設ける。

3.8.対照物質

3.8.1.陽性対照物質

陽性対照として、SDS を用いる。SDS の調製濃度は 1000μg/mL とする。

3.8.2.比較対照物質

比較対照として、Triethanolamine を用いる。Triethanolamine の調製濃度は 10000μg/mL とする。

3.8.3.陰性対照物質

陰性対照として、培養液、10000μg/mLDMSO 培養液溶液または 10000μg/mLEthanol 培養液溶液を用いる。これらは被験物質を溶解または懸濁させる際に用いた溶媒によって選択する。

4.方法

4.1.細胞の培養と継代

①10%牛胎児血清(FBS)を添加した MEM 培養液を用い、37℃、5%の CO_2 で培養する。培養

液にはAntibiotic-Antimycotic (GIBCO BRL)を1%の濃度になるように培養液中へ加えたものを用いる。なお、この時の抗生物質の濃度は Penicillin 100U/mL、Sreptmycin 100 μg/mL、Amphotericin B 250ng/mL である。

②SIRC 細胞の継代はまず培養フラスコから培養液を取り除き、さらに Trypsin inhibiter となる血清を充分取り除くため、PBS(-)10mL で細胞表面を 2 回洗浄する。

③PBS(-)を除去した後、0.25%Trypsin 液 (1.5-2mL)を細胞表面の全体に行き渡るよう加える (2-10 秒程度)。

④0.25%Trypsin 液を除去した後、細胞を剥離させるために 37℃中で 2～3 分間インキュベートし、フラスコの細胞接着面裏から軽くタップし剥離させる。剥離後、適量の MEM(10%FBS)を加えた後、十分なピペッティングにより単細胞化させ均等な細胞浮遊液を調製する。血球計算板にて細胞数を計測し、培養液にて 6～8x10^5cells/mL に調製する。1mL の細胞(6～8x10^5 cells)を 15～30mL の MEM(10%FBS)に加え継代する。

4.2.細胞浮遊液の調製

①SIRC 細胞の培養フラスコから培養液を取り除き、さらに Trypsin inhibitor となる血清を充分取り除くため、PBS(-)10mL で細胞表面を 2 回洗浄する。

②PBS(-)を除去した後、0.25%Trypsin 液 (1.5-2mL)を細胞表面の全体に行き渡るよう加える (2-10 秒程度)。

③0.25%Trypsin 液を除去した後、細胞を剥離させるために 37℃中で 2～3 分間インキュベートする。

④フラスコの細胞接着面裏から軽くタップし剥離させる。

⑤剥離後、適量の培養液を加えた後、十分なピペッティングにより単細胞化させ均等な細胞浮遊液を調製する。

⑥血球計算板にて細胞数を計測し、培養液にて 2x10^5cells/mL に調製する。

4.3.被験物質の適用

①PBS(-)、陰性対照物質、並びに被験物質、陽性対照物質、比較対照物質の希釈系列(100μL/well)を図 1 に示すように 96 穴マイクロプレート内に作製する。(参照)

②2x10^5cells/mL の細胞浮遊液を 0.1mL、図 2 に示すウェルに添加する。

③被験物質が揮発性し周囲のウェルへ影響を与える可能性を考慮し、ウェルを覆うマイクロプレートシーリングテープを貼付する。なお、被験物質が他のウェルに影響を与えた場合には、希釈して再試験することができる。

④添加した 96 穴マイクロプレートは細胞を培養床に均一に沈着・接着させるために、そのままクリーンベンチ内で静置(室温、20 分間)し、その後、CO_2 インキュベータ中に移す。

⑤約 72 時間、37℃、5% CO_2 条件下で培養する。

4.4.Crystal violet 染色

①培養期間終了後、96 穴マイクロプレートを静かに反転し被験物質を含む培養液を捨てる。

②PBS(-)を 200μL 添加し優しく撹拌した後、反転させ PBS(-)を捨てる。これを2回繰り返す。

③96 穴マイクロプレートの各ウェルに Crystal violet methanol 溶液を 100μL 分注し、30 分間染色する。

④染色期間が終了後、96 穴マイクロプレートを静かに反転させ Crystal violet 溶液を捨て、じゅうぶん水洗する。ペーパータオル上にプレートを伏せ、水分を吸い取らせる。

⑤じゅうぶんに風乾した後、マイクロプレートリーダーを用いて各ウェルの吸光度(588nm)を測定する。

4.5.IC50 の算出

被験物質を含まない陰性対照ウェルの細胞生存率を 100%とした場合における各ウェルの細胞生存率を吸光度から算出する。細胞生存率 50%を示す被験物質濃度(IC50)の算出にあた

っては、細胞生存率 50%をはさむ 2 濃度とその濃度における細胞生存率から式 LogIC50=[(50-y1)logx2-(50-y2)logx1]/(y2-y1) を用いて算出する。(※記号は、被験物質濃度 x1(低濃度側)、 x2(高濃度側)におけるそれぞれの細胞生存率を y1、 y2 で示す。Log は常用対数である。)

被験物質の最高濃度である 5000μg/mL で細胞生存率が 50%以下にならない場合は IC50>5000μg/mL とする。また、試験した最低濃度である 39.1μg/mL で細胞生存率が 50%未満の場合は、IC50<39.1μg/mL とする。

なお、表計算ソフト(Excel)において、細胞生存率を算出する段階以降で小数点以下2桁目を四捨五入する。

4.6.評価

比較対照物質としてTriethanolamineを用い、被験物質の眼刺激性を予測し、評価する。被験物質の IC50 が Triethanolamine の IC50 以上を陰性、Triethanolamine の IC50 未満を陽性と判定する。試験は2回を繰り返して行い、その結果に基づき評価する。2回の評価結果が異なった場合には同様に 3 回目を実施し、2 回の同じ評価結果を採用し、その結果に基づき評価する。

4.7.品質基準

試験の精度管理を以下の 5 項目で行う。全ての項で基準を満たすことを試験成立の要件とする。

①陰性対照から得られる吸光度の絶対値は, 各ウェルに播種した $1x10^4$ 個の細胞が 72 時間のアッセイ期間中に正常な増加を示しているか否かを表している。したがって、96 穴マイクロプレートの左右に設定した陰性対照の平均吸光度が0.4を上回ることを試験の合格基準とする。

②陽性対照(SDS)の IC50 値が設定した範囲に収まることを試験成立の条件とする。その範囲は、厚生科学研究で得られた SDS の平均 IC50±3SD(99%信頼区間)である 77.7～258.7μg/mL を合格基準とする。

③体系的に試験精度を見極めるために、96 穴マイクロプレートの左右に陰性対照を設定し、両者の吸光度が同様であることを確認する。左右の陰性対照の平均吸光度が全体の平均吸光度の 15%以内(平均値±15%)に収まることを試験の合格基準とする。

④比較対照(Triethanolamine)の IC50 値が設定した範囲に収まることを試験成立の条件とする。その範囲は、1000μg/mL 以上 5000μg/mL 未満とする。

⑤実施された2回の試験結果が同様であることを確認するために、2試験間の誤差を確認することが必要である。したがって、2 試験間での陽性対照(SDS)の IC50 値が±2 倍以内に収まることを合格基準とする。

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表 1 試薬のメーカー、ロット等

Reagent or Medium	Manufacturer	Code	Model number or CAS	Lot
MEM (Minimum Essential Medium)	Nissui	Code#:	05900	603901
Fetal Bovine Serum	JRH Bioscience	Cat#:	12603C-500ML	6M0030
Penicillin-Streptomycin-Glutamine(x100)	GIBCO/BRL	REF#:	15240-062	546123
L-Glutamine (200mM)	GIBCO/BRL	REF#:	25030-081	624236
Sodium bicarbonate	Wako	CAS#:	191-01305	1892
Phosphate-Buffered Saline (PBS(-))	Nissui	Code#:	05913	167903
0.25w/v% Trypsin (1mmol/L EDTA・4Na)	Wako	Cat#:	209-16941	WTB9071
Dimethyl sulfoxide (DMSO)	Kanto	CAS #	67-68-5	107U1458
Ethanol (EtOH)	Wako	CAS #	64-17-5	KWK2614
Crystal violet	Wako	CAS#	548-62-9	WKF0614
Methanol	Wako	CAS #	67-56-1	ALF0566
Sodium Dodecyl Sulfate	Wako	CAS #	151-21-3	TCG8194
Triethanolamine	Kanto	CAS#	102-71-6	810W1077

Nissui: NISSUI PHARMACEUTICAL CO., LTD

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: KANTO CHEMICAL, CO., INC.

表 2 被験物質

No.	CAS	Substance	Alias	Manufacturer	Lot
1	609-14-3	Ethyl-2-methyl acetoacetate		Sigma-Aldrich	00619PC
2	6484-52-2	Ammonium nitrate		Sigma-Aldrich	09223AJ
3	29911-27-1	Butyl Dipropasol Solvent	Di(propylene glycol) propyl ether	Sigma-Aldrich	06127HJ
4	542-76-7	3-Chloropropionitrile		Sigma-Aldrich	17504LA
5	96-41-3	Cyclopentanol		Sigma-Aldrich	S23317-088
6	1119-62-6	3,3-Dithiodipropionic acid		Sigma-Aldrich	04619LB
7	101-86-0	Hexyl cinnamic aldehyde		Sigma-Aldrich	13102MO
8	137-16-6	N-Lauroylsarcosine sodium salt		Sigma-Aldrich	058K0069
9	12427-38-2	Maneb		Fluka	SZE9030X
10	105-30-6	2-Methyl-1-pentanol		Sigma-Aldrich	02929JJ
11	1569-01-3	Propasol Solvent P	Propylene glycol propyl ether	Sigma-Aldrich	03616HJ
12	2004-03-7	6-Methyl purine		Sigma-Aldrich	049K1156
13	96568-04-6	2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	Sigma-Aldrich	09620MU
14	9002-93-1	Triton X-100		Sigma-Aldrich	118K0160
15	29590-42-9	iso-Octyl acrylate	Isooctyl acrylate	Sigma-Aldrich	10428CH
16	5392-28-9	tetra-Aminopyrimidine sulfate	2,4,5,6-Tetraaminopyrimidine sulfate	Sigma-Aldrich	15022HH
17	446-35-5	2,4-Difluoronitrobenzene		Sigma-Aldrich	MKAA4323
18	598-65-2	n,n-Dimethylguanidine sulfate	1，1－Dimethylguanidine sulfate salt	Sigma-Aldrich	S42370-327
19	1462-55-1	2-(n-Dodecylthio)ethanol	Dodecyl 2-hydroxyethyl sulfide	Sigma-Aldrich	出荷伝票番号 833977017
20	75-26-3	iso-Propyl bromide	2-Bromopropane	Sigma-Aldrich	08331AE
21	108-83-8	Di-iso-butyl ketone	2,6-Dimethyl-4-heptane	Sigma-Aldrich	S26421-416
22	25103-09-7	iso-Octylthioglycolate	Isooctyl mercaptoacetate	Sigma-Aldrich	出荷伝票番号 833977018
23	625-69-4	2,4-Pentanediol		Sigma-Aldrich	02714CJ
24	3970-62-5	2,2-Dimethyl-3-pentanol		Sigma-Aldrich	06520KA
25	14075-53-7	Potassium tetrafluoroborate		Sigma-Aldrich	08216PE
26	623-39-2	3-Methoxy-1,2-propanediol		Sigma-Aldrich	10402CU
27	108-88-3	Toluene		Sigma-Aldrich	KWG6293
28	52-51-7	2-Bromo-2-Nitropropane-1,3-Diol		fluoro chem	F3542A
29	8001-54-5	Benzalkonium chloride		TCI	GC01
30	131-56-6	Benzophenone-1		Wako	ALM0931
31	131-55-5	Benzophenone-2		Wako	TSF1031
32	111-76-2	Butoxyethanol	2-Butoxyethanol	Wako	ALP5483
33	107-88-0	Butylene glycol	1, 3-Butanediol	Wako	TSQ5034
34	112-02-7	Cetrimonium chloride		Wako	WKF1369
35	36653-82-4	Cetyl alcohol		Wako	WKL2169
36	18472-51-0	Chlorhexidine digluconate 20% solution		Wako	TSQ4561
37	120-32-1	Chlorophene		Wako	TCK0988
38	88-04-0	Chloroxylenol		Wako	TSN6941
39	103-23-1	Diethylhexyl adipate	Octyl adipate	Wako	PEN5136
40	6938-94-9	Diisopropyl adipate		Wako	WKJ4099
41	577-11-7	Dioctyl sodium sulfosuccinate		Alfa aesar	K30S031
42	29806-73-3	Ethylhexyl palmitate	Octyl palmitate	Wako	TSM0246
43	107-41-5	Hexylene glycol	2-Methyl-2,4-pentanediol	Wako	PEN6553
44	25339-09-7	Isocetyl stearate	Isohexadecyl stearate	Wako	TCK0946
45	110-27-0	Isopropyl Myristate		TCI	AGN01
46	142-91-6	Isopropyl Palmitate		Wako	SDK5401
47	120-40-1	Lauramide DEA		Wako	ALM0258

48	108-65-6	Methoxyisopropyl acetate	2-Methoxy-1-methylethyl acetate	Wako	PEQ4882
49	143-28-2	Oleyl alcohol		Wako	LTK3360
50	9004-99-3	PEG-40 stearate		Wako	TSG0625
51	60-12-8	Phenethyl alcohol	2-Phenylethanol	Wako	PEP5880
52	122-99-6	Phenoxyethanol		Wako	WKE1655
53	74563-64-7	Phytantriol		Wako	TCP0387
54	108-32-7	Propylene carbonate	4-Methyl-1,3-dioxolan-2-one	Wako	TSF0417
55	108-46-3	Resorcinol		Wako	WKF1256
56	8001-23-8	Safflower (Carthamus tinctorius) oil		Wako	5765J
57	8008-74-0	Sesame (Sesamum indicum) oil		Wako	4363J
58	4418-26-2	Sodium dehydroacetate		Wako	ALP5014
59	532-02-5	Sodium naphthalenesulfonate		Wako	LTF0381
60	822-16-2	Sodium stearate		Wako	ALN6945
61	1338-43-8	Sorbitan oleate	Sorbitan monooleate	MP Biomedicals	7272H
62	8007-43-0	Sorbitan sesquioleate		Wako	DPR1512
63	111-01-3	Squalane		Wako	PEJ4649
64	122-19-0	Stearalkonium chloride		Wako	ALN0903
65	139-96-8	TEA-Lauryl sulfate 40% solution		Wako	TSG0252
66	102-76-1	Triacetin	Glycerol triacetate	Wako	ALR3379
67	112-27-6	Triethylene glycol	3,6-Dioxa-1,8-octanediol	Wako	WKG5787
68	122-20-3	Triisopropanolamine		Wako	PEN1131

Sigma-Aldrich: Sigma-Aldrich Corp.

TCI: Tokyo Chemical Industry Co., Ltd.

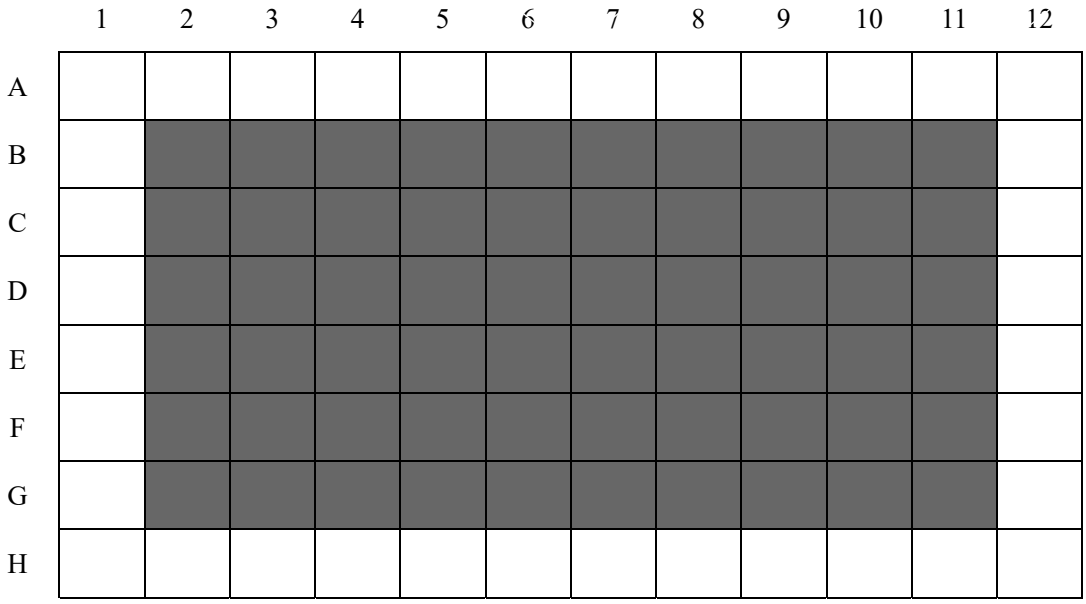
Wako: Wako Pure Chemical Industries, Ltd.

図 1 96ウェルマイクロプレートのレイアウト

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
C	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
D	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
E	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
F	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
G	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

PBS:PBS(-)を200μL、NC:培養液を100μL、S:被験物質の2倍希釈系列(100μL)、R:比較対照物質の2倍希釈系列(100μL)、P:陽性物質の2倍希釈系列(100μL)

図2 細胞浮遊液の添加



■:細胞浮遊液(100μL)

Annex 3　厚生科学研究バリデーション時のプロトコールと提案するプロトコールとの違い

バリデーション時のプロトコールと提案するプロトコールとの違いを示す。

表 1　バリデーション時のプロトコールと提案するプロトコールとの違い

項目	バリデーション時のプロトコール	提案するプロトコール
培養液	10%仔牛血清（CS）を添加した MEM 培養液を用いる。	10%牛胎児血清（FBS）を添加した MEM 培養液を用いる。
被験物質由来の汚染への対処	培養液および PBS(−)で調製する被験物質は、液体の場合無菌フィルターを用いて、固体の場合エタノール（添加後、蒸散）を用いて滅菌を行う。	培養液には適切な抗生物質を用いる。例えば、Antibiotic−Antimycotic（GIBCO BRL)または Penicillin Streptmycin（GIBCO BRL)を 1%の濃度になるように加える。
被験物質の調製	12 ページの図に従った被験物質の調製手順を細かく設定。	被験物質は培養液に 10000 μg/mL の濃度に溶解または均一に懸濁させて被験物質液とする。被験物質を溶解または懸濁させる際に、ミキサー、加温機や超音波処理機を用いることができる。また、DMSO および Ethanol を溶媒として用い、培養液中に溶解または均一に懸濁させることができる。溶媒を用いる際、被験物質液中の DMSO および Ethanol の最高濃度は 10000 μg/mL とする。最終的な被験物質の最高試験濃度は 5000 μg/mL、溶媒の試験濃度は 5000 μg/mL とする。なお、被験物質適用後に沈殿等が認められた場合、該当する濃度は均一に懸濁していなかったものとする。
予備試験	実施	実施しない*
被験物質の希釈系列	第一次バリデーションでは公比 2 を用いる。第二次バリデーションでは細胞生存率が 20〜80%の間に少なくとも 3 点が入る希釈系列とし、最小で公比 1.1 まで実施する。第三次バリデーションでは細胞生存率が 20〜80%の間に少なくとも 1 点が入る希釈系列とし、最小で公比 1.1 まで実施する。	最高試験濃度を 5000 μg/mL とし、公比 2 で希釈系列を作製のうえ、4段階以上の濃度を設ける。これより細かい公比を設けることができる。この時、少なくとも 1000〜5000 μg/mL の範囲の IC50 を求めることが可能な濃度段階の設定とする。
陽性対照を用いた試験成立の条件	記載なし	陽性対照として Sodium dodecyl sulfate（SDS）を用いる。標準的なプロトコールで試験された SDS の IC50 は、50〜250 μg/mL の範囲であり、これを試験成立の条件とする。
比較対照物質を用いた試験成立の条件	記載なし	GHS における NI を同定する比較対照物質として Triethanolamine を用いる。標準的なプロトコールで試験された Triethanolamine の IC50 は、1000〜5000 μg/mL の範囲であり、これを試験成立の条件とする。
陰性対照を用いた試験成立の条件	記載なし	陰性対照として、培養液、10000 μg/mL DMSO 培養液溶液または 10000 μg/mL Ethanol 培養液溶液を用いる。これらは被験

		物質を溶解または懸濁させる際に用いた溶媒によって選択する。標準的なプロトコールで試験された場合の吸光度は 0.4 を越えており、これを試験成立の条件とする。
洗浄の際の PBS(−)の量	0.2mL/ウェル	0.2～0.25mL/ウェル
マイクロプレートリーダーの測定波長	590nm 付近の吸光度を測定する。	588nm の吸光度を測定する。波長は 570nm～595nm の範囲内で設定することができる。
IC50 計算法	片対数グラフに濃度−反応曲線を作成し、陰性対照の 50%となる濃度を求める。または解析ソフトを用いる。	細胞生存率 50%をはさむ 2 濃度とその濃度における生存率から計算する。また、片対数グラフに濃度−反応曲線を作成し、陰性対照の 50%となる濃度を求めても良い。適切な解析ソフトがあればそれを用いても良い。
結果の評価	バリデーションにおける試験終了後に、MAS15 から回帰直線を用いて外挿した細胞毒性の IC50 値を基準に設定し評価した。	比較対照物質である Triethanolamine の IC50 との比較により、GHS で NI に分類される物質を予測する。なお、比較対象物質として triethanolamine を選定した理由については Appendix2 に示した。
試験の繰り返し	記載なし	試験は 2 回を繰り返して行い、その結果に基づき評価する。2 回の評価結果が異なった場合には同様に 3 回目を実施し、2 回の同じ評価結果を採用し、その結果に基づき評価する。
Neutral red 取り込み試験を実施した後のプレートの使用	第一次バリデーションでは、Neutral red 取り込み試験を実施した後のプレートを使用せずに試験を実施する。 第二次と第三次バリデーションにおいては Neutral red 取り込み試験を実施した後のプレートを用いて、Crystal violet 染色試験を実施する。	Neutral red 取り込み試験を実施した後のプレートを使用しない。

*:比較対照物質との比較により評価をするため、限定的な範囲の濃度設定で試験することになり、予備試験は必要ない。

Annex 4 トリエタノールアミンを比較対照物質とした理由

SIRC 細胞毒性試験において原体の NI を同定する比較対照物質として Triethanolamine を設定した。これは厚生科学研究のバリデーションにおいて原体の眼刺激性を評価する基準があらかじめ設定されていなかったためである。Triethanolamine は厚生科学研究のバリデーションにおける被験物質の一つであり、原体の Draize 試験結果で眼刺激性が無く、GHS 分類の NI の予測に適する細胞毒性を有し、試薬として購入でき、水溶性のために試験を実施する上で扱いやすい特性がある。さらに、選定にあたっては、厚生科学研究のバリデーションで用いられた各被験物質が比較対照として選ばれた時に in vitro と in vivo の対応がどのようになるのかを調べた。その結果、細胞毒性の IC50 値が求められなかった 6 種の被験物質を除いたうえで、最も重要な考慮点である偽陰性物質が比較的少なく、その物質のカテゴリー（低分子のアルコール）も明らかにでき、さらに最も一致率の高かった triethanolamine が選定された。（表 1 参照）

表 1 厚生科学研究・バリデーションデータにおいてそれぞれの被験物質を比較対照にした場合の in vitro、in vivo の一致率

被験物質	In vivo 評価	細胞毒性平均値 (ug/mL)	順位付けに利用した値	True Negative	False Negative	True Positive	False Positive	一致率(%)
Polyethylene glycol 400	NI	35300<	35300	1	0	27	6	82
Silicic anhydride	NI	14800<	14800	2	0	26	6	82
Glycerin	NI	11600	11600	3	0	25	6	82
Isotonic sodium chloride solution	NI	10000<	10000	4	1	24	5	82
Ethanol	1or2A	10000<	10000	4	1	24	5	82
Isopropyl myristate	NI	9330<	9330	5	1	24	4	85
Butanol*	1or2A	8880<	8880	5	2	23	4	82
Triethanolamine	NI	2090	2090	6	2	23	3	85
Lactic acid	1	1230	1230	6	3	22	3	82
Benzyl alcohol	1or2A	1190	1190	6	4	21	3	79
Polyoxyethylene sorbitan monooleate (20E.O.)	NI	963	963	7	4	21	2	82
Sodium salicylate	1or2A	952	952	7	5	20	2	79
Glycolic acid*	1or2A	868	868	7	6	19	2	76
Acetic acid*	1or2A	721	721	7	7	18	2	74
Diisopropanolamine*	1, 2Aor2B	699	699	7	8	17	2	71
2-Ethylhexyl p-dimethylamino benzoate	NI	474	474	8	8	17	1	74
Calcium thioglycolate	1	392	392	8	9	16	1	71
Acid red 92	1or2A	297	297	8	10	15	1	68
Sucrose fatty acid ester	1or2A	286	286	8	11	14	1	65
m-Phenylenediamine	1or2A	218	218	8	12	13	1	62
Methyl p-hydroxybenzoate	NI	207	207	9	12	13	0	65
Di (2-ethylhexyl) sodium sulfosuccinate*	1or2A	181	181	9	13	12	0	62
Sodium lauryl sulfate*	1or2A	168	168	9	14	11	0	59
Sodium hydrogenated tallow L-glutamate*	1or2A	140	140	9	15	10	0	56
Potassium laurate*	1or2A	120 (Data from 4 labs)	120	9	16	9	0	53
Chlorhexidine gluconate (20% solution)*	1or2A	67.6	67.6	9	17	8	0	50
Polyoxyethylene octylphenylether (10 E.O.)*	1or2A	38.4	38.4	9	18	7	0	47
Distearyldimethylammonium chloride	1	37.8	37.8	9	19	6	0	44
Benzalkonium chloride*	1or2A	19.0	19	9	20	5	0	41
Domiphen bromide*	1or2A	12.1	12.1	9	21	4	0	38
Monoethanolamine*	1or2A	9.62	9.62	9	22	3	0	35
Cetyltrimethylammonium bromide*	1or2A	2.59 (Data from 4labs)	2.59	9	23	2	0	32
Cetylpyridinium chloride*	1	1.67	1.67	9	24	1	0	29
Stearyltrimethylammonium chloride*	1	1.58	1.58	9	25	0	0	26

Annex 5 Data of triethanolamine and Sodium dodecyl sulfate

(1) Results of triethanolamine

IC50 (μg/mL)	Lab.	Year	Lot.
1540	B	1995	611E1858
1320	B	1995	611E1858
1850	C	1995	611E1858
1650	C	1995	611E1858
1910	D	1995	611E1858
2075	D	1995	611E1858
3200	E	1995	611E1858
4500	E	1995	611E1858
1580	A(Shiseido)	1995	611E1858
1300	A(Shiseido)	1995	611E1858
2164.2	A(Shiseido)	2009	810W1077
2000.5	A(Shiseido)	2009	810W1077
1675.7	A(Shiseido)	2009	810W1077
1757.2	A(Shiseido)	2009	810W1077
1656.6	A(Shiseido)	2009	810W1077
1940.1	A(Shiseido)	2009	810W1077
1709.2	A(Shiseido)	2009	810W1077
2228.9	A(Shiseido)	2009	810W1077
1558.9	A(Shiseido)	2009	810W1077
1868.2	A(Shiseido)	2009	810W1077
1669.9	A(Shiseido)	2009	810W1077
1932.9	A(Shiseido)	2009	810W1077
1945.1	A(Shiseido)	2009	810W1077
1424.0	A(Shiseido)	2009	810W1077
1666.2	A(Shiseido)	2009	810W1077
1526.8	A(Shiseido)	2009	810W1077
1501.7	A(Shiseido)	2009	810W1077
1763.3	A(Shiseido)	2009	810W1077
1773.9	A(Shiseido)	2009	810W1077
1614.6	A(Shiseido)	2009	810W1077
1435.9	A(Shiseido)	2009	810W1077
1500.2	A(Shiseido)	2009	810W1077
1525.0	A(Shiseido)	2009	810W1077
1820.5	A(Shiseido)	2009	810W1077
1349.7	A(Shiseido)	2009	810W1077
1786.8	A(Shiseido)	2009	810W1077
1664.1	A(Shiseido)	2009	810W1077
1338.9	A(Shiseido)	2009	810W1077
2145.3	A(Shiseido)	2009	810W1077
1861.3	A(Shiseido)	2009	810W1077
1770.2	A(Shiseido)	2009	810W1077
1611.9	A(Shiseido)	2009	810W1077
1550.9	A(Shiseido)	2009	810W1077
1408.8	A(Shiseido)	2009	810W1077
1260.3	A(Shiseido)	2009	810W1077
1267.2	A(Shiseido)	2009	810W1077
1695.5	A(Shiseido)	2009	810W1077
1495.1	A(Shiseido)	2009	810W1077
1339.4	A(Shiseido)	2009	810W1077
1218.0	A(Shiseido)	2009	810W1077
1484.0	A(Shiseido)	2009	810W1077
1468.0	A(Shiseido)	2009	810W1077
1531.6	A(Shiseido)	2009	810W1077
1222.7	A(Shiseido)	2009	810W1077
1737.8	A(Shiseido)	2009	810W1077
1662.5	A(Shiseido)	2009	810W1077
1706.2	A(Shiseido)	2009	810W1077
1436.5	A(Shiseido)	2009	810W1077
1446.6	A(Shiseido)	2009	810W1077
1471.9	A(Shiseido)	2009	810W1077
1545.5	A(Shiseido)	2009	810W1077
1584.5	A(Shiseido)	2009	810W1077
1413.8	A(Shiseido)	2009	810W1077
1439.4	A(Shiseido)	2009	810W1077
1622.5	A(Shiseido)	2009	810W1077
1621.0	A(Shiseido)	2009	810W1077
1464.9	A(Shiseido)	2009	810W1077
1857.2	A(Shiseido)	2009	810W1077
1403.1	A(Shiseido)	2009	810W1077
1713.5	A(Shiseido)	2009	810W1077
1513.6	A(Shiseido)	2009	810W1077
1631.5	A(Shiseido)	2009	810W1077
1825.7	A(Shiseido)	2009	810W1077
1685.9	A(Shiseido)	2009	810W1077
1769.7	A(Shiseido)	2009	810W1077
1642.3	A(Shiseido)	2009	810W1077

IC50 (μg/mL)	Lab.	Year	Lot.
1620.4	A(Shiseido)	2009	810W1077
1808.3	A(Shiseido)	2009	810W1077
1401.5	A(Shiseido)	2009	810W1077
1604.0	A(Shiseido)	2009	810W1077
1687.6	A(Shiseido)	2009	810W1077
1674.5	A(Shiseido)	2009	810W1077
1704.8	A(Shiseido)	2009	810W1077
1694.8	A(Shiseido)	2009	810W1077
1386.8	A(Shiseido)	2009	810W1077
1663.4	A(Shiseido)	2009	810W1077
1576.9	A(Shiseido)	2009	810W1077
1461.8	A(Shiseido)	2009	810W1077
1599.5	A(Shiseido)	2009	810W1077
1251.7	A(Shiseido)	2009	810W1077
1347.1	A(Shiseido)	2009	810W1077
1690.2	A(Shiseido)	2009	810W1077
1448.5	A(Shiseido)	2009	810W1077
1206.8	A(Shiseido)	2009	810W1077
1808.9	A(Shiseido)	2009	810W1077
1452.7	A(Shiseido)	2009	810W1077
1295.2	A(Shiseido)	2009	810W1077
1429.1	A(Shiseido)	2009	810W1077
1683.3	A(Shiseido)	2009	810W1077
1451.3	A(Shiseido)	2009	810W1077
1782.0	A(Shiseido)	2009	810W1077
1757.9	A(Shiseido)	2009	810W1077
1118.3	A(Shiseido)	2009	810W1077
1452.3	A(Shiseido)	2009	810W1077
1669.1	A(Shiseido)	2009	810W1077
1330.7	A(Shiseido)	2009	810W1077
1488.4	A(Shiseido)	2009	810W1077
1534.3	A(Shiseido)	2009	810W1077
2290.9	A(Shiseido)	2009	810W1077
1437.1	A(Shiseido)	2009	810W1077
1441.2	A(Shiseido)	2009	810W1077
1374.6	A(Shiseido)	2009	810W1077
1354.3	A(Shiseido)	2009	810W1077
1486.9	A(Shiseido)	2009	810W1077
1303.1	A(Shiseido)	2009	810W1077
1662.7	A(Shiseido)	2009	810W1077
1485.4	A(Shiseido)	2009	810W1077
1696.4	A(Shiseido)	2009	810W1077
1452.4	A(Shiseido)	2009	810W1077
1557.3	A(Shiseido)	2009	810W1077
1555.9	A(Shiseido)	2009	810W1077
1647.2	A(Shiseido)	2009	810W1077
1283.2	A(Shiseido)	2009	810W1077
1700.4	A(Shiseido)	2009	810W1077
1508.0	A(Shiseido)	2009	810W1077
2276.3	A(Shiseido)	2009	810W1077
1565.2	A(Shiseido)	2009	810W1077
1552.1	A(Shiseido)	2009	810W1077
1498.2	A(Shiseido)	2009	810W1077
1601.9	A(Shiseido)	2009	810W1077
1009.0	A(Shiseido)	2009	810W1077
1499.5	A(Shiseido)	2009	810W1077
1381.5	A(Shiseido)	2009	810W1077
1628.4	A(Shiseido)	2009	810W1077
1424.0	A(Shiseido)	2009	810W1077
1781.1	A(Shiseido)	2009	810W1077
1550.3	A(Shiseido)	2009	810W1077
1341.0	A(Shiseido)	2009	810W1077
1586.1	A(Shiseido)	2009	810W1077
1576.9	A(Shiseido)	2009	810W1077
1446.2	A(Shiseido)	2009	810W1077
1549.9	A(Shiseido)	2009	810W1077
1012.3	A(Shiseido)	2010	810W1077
1595.4	A(Shiseido)	2010	810W1077

The substances were obtained from Kanto Chemical CO., INC.

(2) Results of Sodium dodecyl sulfate

IC50 (μg/mL)	Lab.	Year	Manufaturer	Lot.
168	B	1994	Nikko Chemicals	2802
176	B	1994	Nikko Chemicals	2802
172	B	1994	Nikko Chemicals	2802
117	C	1994	Nikko Chemicals	2802
117	C	1994	Nikko Chemicals	2802
117	C	1994	Nikko Chemicals	2802
190	D	1994	Nikko Chemicals	2802
190	D	1994	Nikko Chemicals	2802
187	D	1994	Nikko Chemicals	2802
201	E	1994	Nikko Chemicals	2802
194	E	1994	Nikko Chemicals	2802
198	E	1994	Nikko Chemicals	2802
140	F	1994	Nikko Chemicals	2802
157	F	1994	Nikko Chemicals	2802
123	F	1994	Nikko Chemicals	2802
174	A(Shiseido)	1994	Nikko Chemicals	2802
189	A(Shiseido)	1994	Nikko Chemicals	2802
176	A(Shiseido)	1994	Nikko Chemicals	2802
102.2	A(Shiseido)	2009	Wako	TCG8149
87.2	A(Shiseido)	2009	Wako	TCG8149
91.1	A(Shiseido)	2009	Wako	TCG8149
91.0	A(Shiseido)	2009	Wako	TCG8149
90.5	A(Shiseido)	2009	Wako	TCG8149
90.5	A(Shiseido)	2009	Wako	TCG8149
103.1	A(Shiseido)	2009	Wako	TCG8149
101.7	A(Shiseido)	2009	Wako	TCG8149
90.6	A(Shiseido)	2009	Wako	TCG8149
95.1	A(Shiseido)	2009	Wako	TCG8149
96.1	A(Shiseido)	2009	Wako	TCG8149
91.4	A(Shiseido)	2009	Wako	TCG8149
92.4	A(Shiseido)	2009	Wako	TCG8149
96.2	A(Shiseido)	2009	Wako	TCG8149
90.3	A(Shiseido)	2009	Wako	TCG8149
98.8	A(Shiseido)	2009	Wako	TCG8149
101.7	A(Shiseido)	2009	Wako	TCG8149
108.0	A(Shiseido)	2009	Wako	TCG8149
104.2	A(Shiseido)	2009	Wako	TCG8149
92.7	A(Shiseido)	2009	Wako	TCG8149
100.2	A(Shiseido)	2009	Wako	TCG8149
97.2	A(Shiseido)	2009	Wako	TCG8149
103.5	A(Shiseido)	2009	Wako	TCG8149
113.7	A(Shiseido)	2009	Wako	TCG8149
107.2	A(Shiseido)	2009	Wako	TCG8149
93.5	A(Shiseido)	2009	Wako	TCG8149
85.9	A(Shiseido)	2009	Wako	TCG8149
91.8	A(Shiseido)	2009	Wako	TCG8149
91.2	A(Shiseido)	2009	Wako	TCG8149
92.1	A(Shiseido)	2009	Wako	TCG8149
96.9	A(Shiseido)	2009	Wako	TCG8149
91.9	A(Shiseido)	2009	Wako	TCG8149
96.0	A(Shiseido)	2009	Wako	TCG8149
86.3	A(Shiseido)	2009	Wako	TCG8149
93.4	A(Shiseido)	2009	Wako	TCG8149
95.2	A(Shiseido)	2009	Wako	TCG8149
91.8	A(Shiseido)	2009	Wako	TCG8149
95.5	A(Shiseido)	2009	Wako	TCG8149
93.3	A(Shiseido)	2009	Wako	TCG8149
96.0	A(Shiseido)	2009	Wako	TCG8149
94.0	A(Shiseido)	2009	Wako	TCG8149
90.7	A(Shiseido)	2009	Wako	TCG8149
89.9	A(Shiseido)	2009	Wako	TCG8149
90.8	A(Shiseido)	2009	Wako	TCG8149
94.4	A(Shiseido)	2009	Wako	TCG8149
96.6	A(Shiseido)	2009	Wako	TCG8149
90.0	A(Shiseido)	2009	Wako	TCG8149
92.0	A(Shiseido)	2009	Wako	TCG8149
91.5	A(Shiseido)	2009	Wako	TCG8149
91.5	A(Shiseido)	2009	Wako	TCG8149
90.7	A(Shiseido)	2009	Wako	TCG8149
92.2	A(Shiseido)	2009	Wako	TCG8149
89.1	A(Shiseido)	2009	Wako	TCG8149
93.0	A(Shiseido)	2009	Wako	TCG8149
98.7	A(Shiseido)	2009	Wako	TCG8149
93.6	A(Shiseido)	2009	Wako	TCG8149
93.6	A(Shiseido)	2009	Wako	TCG8149
96.5	A(Shiseido)	2009	Wako	TCG8149
100.6	A(Shiseido)	2009	Wako	TCG8149
91.3	A(Shiseido)	2009	Wako	TCG8149
93.8	A(Shiseido)	2009	Wako	TCG8149
89.1	A(Shiseido)	2009	Wako	TCG8149
96.6	A(Shiseido)	2009	Wako	TCG8149
92.8	A(Shiseido)	2009	Wako	TCG8149
94.4	A(Shiseido)	2009	Wako	TCG8149
91.4	A(Shiseido)	2009	Wako	TCG8149

IC50 (μg/mL)	Lab.	Year	Manufacturer	Lot.
90.8	A(Shiseido)	2009	Wako	TCG8149
89.1	A(Shiseido)	2009	Wako	TCG8149
91.8	A(Shiseido)	2009	Wako	TCG8149
93.2	A(Shiseido)	2009	Wako	TCG8149
95.1	A(Shiseido)	2009	Wako	TCG8149
92.5	A(Shiseido)	2009	Wako	TCG8149
93.1	A(Shiseido)	2009	Wako	TCG8149
92.4	A(Shiseido)	2009	Wako	TCG8149
96.5	A(Shiseido)	2009	Wako	TCG8149
89.6	A(Shiseido)	2009	Wako	TCG8149
89.4	A(Shiseido)	2009	Wako	TCG8149
86.0	A(Shiseido)	2009	Wako	TCG8149
94.8	A(Shiseido)	2009	Wako	TCG8149
96.7	A(Shiseido)	2009	Wako	TCG8149
89.7	A(Shiseido)	2009	Wako	TCG8149
88.1	A(Shiseido)	2009	Wako	TCG8149
91.5	A(Shiseido)	2009	Wako	TCG8149
91.3	A(Shiseido)	2009	Wako	TCG8149
86.0	A(Shiseido)	2009	Wako	TCG8149
91.4	A(Shiseido)	2009	Wako	TCG8149
91.5	A(Shiseido)	2009	Wako	TCG8149
89.1	A(Shiseido)	2009	Wako	TCG8149
90.6	A(Shiseido)	2009	Wako	TCG8149
89.0	A(Shiseido)	2009	Wako	TCG8149
91.0	A(Shiseido)	2009	Wako	TCG8149
96.4	A(Shiseido)	2009	Wako	TCG8149
93.0	A(Shiseido)	2009	Wako	TCG8149
90.4	A(Shiseido)	2009	Wako	TCG8149
92.8	A(Shiseido)	2009	Wako	TCG8149
95.3	A(Shiseido)	2009	Wako	TCG8149
87.1	A(Shiseido)	2009	Wako	TCG8149
90.4	A(Shiseido)	2009	Wako	TCG8149
113.6	A(Shiseido)	2009	Wako	TCG8149
92.4	A(Shiseido)	2009	Wako	TCG8149
91.1	A(Shiseido)	2009	Wako	TCG8149
94.3	A(Shiseido)	2009	Wako	TCG8149
88.2	A(Shiseido)	2009	Wako	TCG8149
93.9	A(Shiseido)	2009	Wako	TCG8149
92.9	A(Shiseido)	2009	Wako	TCG8149
91.7	A(Shiseido)	2009	Wako	TCG8149
91.1	A(Shiseido)	2009	Wako	TCG8149
92.5	A(Shiseido)	2009	Wako	TCG8149
90.1	A(Shiseido)	2009	Wako	TCG8149
89.7	A(Shiseido)	2009	Wako	TCG8149
94.3	A(Shiseido)	2009	Wako	TCG8149
91.0	A(Shiseido)	2009	Wako	TCG8149
92.9	A(Shiseido)	2009	Wako	TCG8149
92.7	A(Shiseido)	2009	Wako	TCG8149
93.6	A(Shiseido)	2009	Wako	TCG8149
109.2	A(Shiseido)	2009	Wako	TCG8149
91.3	A(Shiseido)	2009	Wako	TCG8149
92.2	A(Shiseido)	2009	Wako	TCG8149
93.5	A(Shiseido)	2009	Wako	TCG8149
87.2	A(Shiseido)	2009	Wako	TCG8149
101.6	A(Shiseido)	2009	Wako	TCG8149
89.7	A(Shiseido)	2009	Wako	TCG8149
91.5	A(Shiseido)	2009	Wako	TCG8149
93.8	A(Shiseido)	2009	Wako	TCG8149
91.8	A(Shiseido)	2009	Wako	TCG8149
93.7	A(Shiseido)	2009	Wako	TCG8149
93.5	A(Shiseido)	2009	Wako	TCG8149
92.1	A(Shiseido)	2009	Wako	TCG8149
95.2	A(Shiseido)	2009	Wako	TCG8149
91.0	A(Shiseido)	2009	Wako	TCG8149
92.6	A(Shiseido)	2009	Wako	TCG8149
91.9	A(Shiseido)	2009	Wako	TCG8149
90.7	A(Shiseido)	2010	Wako	TCG8149
95.1	A(Shiseido)	2010	Wako	TCG8149

Nikko Chemicals: Nikko Chemicals CO., LTD.
Wako: Wako Pure Chemical Industries, Ltd.

Annex 6 マイクロプレートシーリングテープの影響

SIRC 細胞毒性試験に対するマイクロプレートシーリングテープ使用の影響について検討した。テープの有無による細胞の生育状態を確認するために，常法に従い細胞を均一に播種した 96 穴マイクロプレートの半分のみにテープを貼付し、72 時間培養した後に染色および吸光度の測定を行った。試験を 2 回実施した結果、細胞の生育状況を示す陰性対照物質の吸光度に差はなく、比較対照物質および陽性対照物質の IC50 はいずれも同程度の値を示した. したがって、マイクロプレートシーリングテープの貼付が細胞毒性試験の評価に与える影響は少ないと判断した。

Table 1 Effect of microplate sealing tape in the SIRC cytotoxicity test

	Marker	No.1	No.2	Average
Without microplate sealing tape	OD of negative control	0.684	0.685	0.6845
	IC50（μg/mL）of trietanolamine	1805.2	1619.3	1712.25
	IC50（μg/mL）of SDS	90.2	89.4	89.8
With microplate sealing tape	OD of negative control	0.638	0.701	0.6695
	IC50（μg/mL）of trietanolamine	1413.3	1035.1	1224.2
	IC50（μg/mL）of SDS	86.8	90.2	88.5

Annex 7 References of the *in vivo* data of the 41 substances

No	Substance	References
28	2-Bromo-2-Nitropropane-1,3-Diol	JACT 3(3):139-155,1984. JEPT 4(4):47-61, 1980.
29	Benzalkonium chloride	JACT 8(4):589-625, 1989.
30	Benzophenone-1	JACT 2(5):35-77, 1983.
31	Benzophenone-2	JACT 2(5):79-84, 1983.
32	Butoxyethanol	JACT 15(6):462-526, 1996.
33	Butylene glycol	Hifu 26(5):1065-1074, 1984.
34	Cetrimonium chloride	IJT 16(S3):195-220,1997.
35	Cetyl alcohol	JACT 7(3):359-413, 1988.
36	Chlorhexidine digluconate (20% Solution)	JACT 12(3):201-23, 1993.
37	Chlorophene	IJT 23(S1):1-27 2004.
38	Chloroxylenol	JACT 4(5):147-69, 1985.
39	Diethylhexyl adipate	JACT 3(3):101-30, 1984.
40	Diisopropyl adipate	JACT 3(3):101-30, 1984.
41	Dioctyl sodium sulfosuccinate	IJT 17(S4):1-20, 1998.
42	Ethylhexyl palmitate	JACT 1(2):13-35, 1982.
43	Hexylene glycol	JACT 4(5):223-48, 1985.
44	Isocetyl stearate	JACT 4(5):107-46, 1985.
45	Isopropyl Myristate	JACT 1(4):55-80, 1982.
46	Isopropyl Palmitate	JACT 1(2):13-35, 1982.
47	Lauramide DEA	JACT 5(5):415-54, 1986.
48	Methoxyisopropyl acetate	IJT 27(S2), 2008.
49	Oleyl alcohol	JACT 4(5):1-29, 1985.
50	PEG-40 stearate	JACT 2(7):17-60, 1983.
51	Phenethyl alcohol	JACT 9(2):165-83, 1990.
52	Phenoxyethanol	JACT 9(2):259-77, 1990.
53	Phytantriol	IJT 26(Suppl. 1):107-117, 2007.
54	Propylene carbonate	JACT 6(1):23-51, 1987.
55	Resorcinol	JACT 5(3):167-203, 1986.
56	Safflower (Carthamus tinctorius) oil	JACT 4(5):171-97, 1985.
57	Sesame (Sesamum indicum) oil	JACT 12(3):261-77, 1993.
58	Sodium dehydroacetate	JACT 4(3):123-159, 1985.
59	Sodium naphthalenesulfonate	IJT 22(Suppl. 2:37-44,)2003.
60	Sodium stearate	JACT 1(2):143-77, 1982.
61	Sorbitan oleate	JACT 4(3):65-121, 1985.
62	Sorbitan sesquioleate	JACT 4(3):65-121, 1985.
63	Squalane	JACT 1(2):37-56, 1982.
64	Stearalkonium chloride	JACT 1(2):57-69, 1982.
65	TEA-Lauryl sulfate （40% Solution）	JACT 1(4):143-67, 1982.
66	Triacetin	IJT 22(S2):1-10, 2003.
67	Triethylene glycol	IJT 25(5):121-138,2006.
68	Triisopropanolamine	JACT 6(1):53-76, 1987.

IJT: International Journal of Toxicology, JACT: Journal of the American College of Toxicology
JEPT: Journal of Environmental Pathology & Toxicology

Annex 8　品質基準に適合しなかったケースについて

品質基準に適合しなかったケースとして、以下の 3 被験物質を計画書に基づき再試験を実施した. 3-Chloropropionitrile および 2,4-Difluoronitrobenzene については、揮発による周囲のウェルへの影響（細胞毒性）が認められため、開始濃度を下げて再試験を実施した。また、Cyclopentanol については、陽性対照の値（SDS： 63.2μg/mL）が基準値を下回ったため再試験を実施した。

Table 1 The reasons of the retesting

No	Substance	Number of test at each concentration			The reasons
		5000μg/mL	500μg/mL	50μg/mL	
4	3-Chloropropionitrile	Acceptance :1 Rejection:1	Acceptance :2 Rejection: 1		Effect of the volatile substance
5	Cyclopentanol	Acceptance :2 Rejection:1			Abnormal value of the positive control
17	2,4-Difluoronitrobenzene	Rejection:2	Rejection:2	Acceptance :2	Effect of the volatile substance

Annex 9 厚生科学研究データと追加データの比較

厚生科学研究におけるデータ[研究 1]と追加試験のデータ[研究 2]を比較し、同様な評価が可能か否かを確認した。両者で試験した物質は Isopropyl myristate、Triethanolamine、Polyoxyethylene octylphenylether (10 E.O.)(別名 Triton X-100)、Sodium dodecyl sulfate、Benzalkonium chloride、Di(2-ethylhexyl) sodium sulfosuccinate (別名 Dioctyl sodium sulfosuccinate) の 6 被験物質であった。Isopropyl myristate、Triethanolamine は同一メーカーでロットが異なり、残りの 4 被験物質はメーカーが異なっていた。GHS で NI が 2 被験物質、それ以外が 4 被験物質であった。研究 1 および研究 2 の IC50 値、並びに Triethanolamine を比較対照とした時の眼刺激性の予測結果を下表に示す。

比較の結果、陰性(NI)あるいは陽性(NI 以外)かの評価上の相違は両者間に認められなかった。6 被験物質における IC50 値の順位については、研究 2 の Triton X-100 と Benzalkonium chloride ともに IC50 値が 39.1ug/mL 未満であり、研究 1 の数値との同等性を詳細に確認することが出来なかったが、評価上の差は認められなかった. 。したがって、いずれの被験物質においても評価上の差異は認められず、両者の結果を用いて同様の評価が可能と判断した。

Table 1 The comparison between the Japanese validation study data and the additional data

Substance	MAS	GHS	The Japanese validation study data (IC50;μg/mL)									The additional data (IC50;ug/mL)
			Lab.A	Lab.B	Lab.C	Lab.D	Lab.E	Lab.F	Lab.G	Lab.H	Lab.I	Lab.A
Isopropyl myristate	0	NI	10000< (N)	10000< (N)		6000< (NE)			10000< (N)	10000< (N)	10000< (N)	4303< (N)
Triethanolamine	8	NI	1440 (N)	1430 (N)					1750 (N)	1993 (N)	3850 (N)	1579 (N)
Triton X-100*	41.3≦	1or2A	26.7 (P)	38.0 (P)	23.3 (P)	32.3 (NE)	51.0 (NE)	59.5 (NE)				<39.1 (P)
Sodium dodecyl sulfate*	15.0≦	1or2A	182 (P)	172 (P)	117 (P)	190 (NE)	198 (NE)	149 (NE)				94 (P)
Di(2-ethylhexyl) sodium sulfosuccinate*	57.0≦	1or2A	210 (P)	182 (P)					181 (P)	156 (P)	175 (P)	54 (P)
Benzalkonium chloride*	78.0≦	1or2A	16.2 (P)	25.2 (P)	13.2 (P)	15.5 (NE)	29.0 (NE)	15.0 (NE)				<39.1 (P)

P:Positive, N:Negative, NE:Could not be evaluated

Blank column: Not tested

The additional data of Triethanolamine and Sodium dodecyl sulfate are the mean of 134 tests.

Annex 10 追加ﾃﾞｰﾀと学会で報告されているデータの比較

今回追加した試験結果について、以前に2008年日本動物実験代替法学会第21回大会で報告されているSIRC試験データ(JSAAEデータ)との比較を行った。JSAAEデータでは、SIRC細胞の最終濃度が$1.5x10^5$個/mLであり、厚生科学研究や追加試験での$1x10^5$個/mLとは異なっていた。また、被験物質によっては溶媒の選択が異なり、さらに、GHSのNIの同定を目的とした研究ではないため、同一プレート上での Triethanolamine の試験を設定していなかった。そのため、得られたIC50は1000μg/mL未満を陽性、5000μg/mL以上を陰性とし、1000～5000μg/mLをEquivocalとした。

両者の対応を確認した結果、41 被験物質のうち 37 被験物質の評価が一致し、Equivocal は、Phenethyl alcohol および Triacetin の 2 被験物質であった。一方、一致しなかった被験物質はSorbitan oleateおよびSorbitan sesquioleateの2被験物質であった。

Table 1 The comparison between the additional data and the previous data reported at the 21th annual meeting of the JSAAE

No	Substance	*In vivo* Classification	Additional data			JSAAE data		
			Medium	IC50 (μg/mL)	Evaluation	Medium	IC50 (μg/mL±SD)	Evaluation
28	2-Bromo-2-Nitropropane-1,3-Diol	P	Medium	<39.1	P	Medium	6.42±0.85	P
29	Benzalkonium chloride	P	DMSO/Medium	<39.1	P	DMSO /Medium	3.47±0.47	P
30	Benzophenone-1	P	DMSO/Medium	72.7	P	DMSO /Medium	29.3±8.0	P
31	Benzophenone-2	P	DMSO/Medium	62.7	P	DMSO/Medium	53.4±6.4	P
32	Butoxyethanol	P	Medium	2187.2	N			
33	Butylene glycol	N	Medium	5000<	N	Medium	10000<	N
34	Cetrimonium chloride	P	Medium	<39.1	P	Medium	0.56±0.16	P
35	Cetyl alcohol	N	DMSO/Medium	<39.1	P	DMSO /Medium	25.1±12.1	P
36	Chlorhexidine digluconate (20% Solution)	P	DMSO/Medium	<7.82 【<39.1】	P	DMSO /Medium	7.92±3.92 【39.6±19.6】	P
37	Chlorophene	P	DMSO/Medium	<39.1	P	DMSO /Medium	25.6±9.1	P
38	Chloroxylenol	P	DMSO/Medium	75.4	P			
39	Diethylhexyl adipate	N	EtOH/Medium	5000<	N	Medium	Could not be tested	
40	Diisopropyl adipate	N	DMSO/Medium	353.0	P	DMSO /Medium	633±16	P
41	Dioctyl sodium sulfosuccinate	P	DMSO/Medium	54.4	P	DMSO/Medium	81.3±4.8	P
42	Ethylhexyl palmitate	N	EtOH/Medium	5000<	N	Medium	10000<	N
43	Hexylene glycol	P	Medium	5000<	N	Medium	7500±600	N
44	Isocetyl stearate	N	EtOH/Medium	5000<	N	Medium	Could not be tested	
45	Isopropyl Myristate	N	EtOH/Medium	>4294.5	N	Medium	Could not be tested	
46	Isopropyl Palmitate	N	EtOH/Medium	5000<	N	Medium	Could not be tested	
47	Lauramide DEA	P	DMSO/Medium	<39.1	P	DMSO /Medium	18.3±4.1	P
48	Methoxyisopropyl acetate	P	Medium	3323.5	N			
49	Oleyl alcohol	N	EtOH/Medium	<39.1	P	Ethanol /Medium	41.9±13.3	P
50	PEG-40 stearate	N	Medium	269.1	P	Medium	230±79	P
51	Phenethyl alcohol	P	DMSO/Medium	688.0	P	Medium	1830±1360	E
52	Phenoxyethanol	P	DMSO/Medium	1195.6	P			
53	Phytantriol	P	DMSO/Medium	<46.1	P	DMSO /Medium	37.2±11.8	P
54	Propylene carbonate	N	Medium	5000<	N	Medium	6050±490	N
55	Resorcinol	P	Medium	394.5	P			
56	Safflower (Carthamus tinctorius) oil	N	DMSO/Medium	2215.1	N	Medium	Could not be tested	
57	Sesame (Sesamum indicum) oil	N	DMSO/Medium	5000<	N	Medium	Could not be tested	
58	Sodium dehydroacetate	N	Medium	919.9	P	Medium	860±224	P
59	Sodium naphthalenesulfonate	P	DMSO/Medium	980.2	P			
60	Sodium stearate	N	Medium	266.1	P	Medium	56.5±8.2	P
61	Sorbitan oleate	N	DMSO/Medium	825.2	P	Medium	5170±1560	N
62	Sorbitan sesquioleate	N	DMSO/Medium	1178.6	P	Medium	10000<	N
63	Squalane	N	DMSO/Medium	5000<	N	Medium	Could not be tested	
64	Stearalkonium chloride	P	EtOH/Medium	<39.1	P	Ethanol /Medium	2.66±0.56	P
65	TEA-Lauryl sulfate 【40% Solution】	P	Medium	95.2 【238.0】	P	Medium	117±3 【290±4】	P
66	Triacetin	N	Medium	1476.4	P	Medium	1780±720	E
67	Triethylene glycol	N	Medium	5000<	N	Medium	10000<	N
68	Triisopropanolamine	P	Medium	729.8	P			

P: Positive, N:Negative, E: Equivocal

NE: It could not be evaluated.

【 】: The data was obtained from diluted agent.

〔 〕: The precipitation was appear at the concentration of 10000μg/mL in the culture of 72hr. The maximal concentrations without the precipitation were 5000ug/mL and 2500ug/mL in No31 and No38, respectively.

Blank column: Not selected, because of no *in vivo* data at 10% concentration

2 試験間で結果が一致しなかった 2 被験物質(Sorbitan oleate および Sorbitan sesquioleate)について、その原因は細胞数の差および溶媒の有無が関係していると考えられた。なお、これらの 2 被験物質は培養液に溶解せずに、懸濁させて適用した物質であった。

この原因を究明するために、同一条件下で溶媒の有無による差異(A と C の差)および同一条件下で細胞数による差異(A と B の差)を確認する 2 つの追加検討を実施した。その結果、溶媒の有無による試験結果は明らかに異なり、溶媒を用いた場合に毒性の増強が確認された。一方、細胞数の違いでは 2 被験物質で結果が異なり、Sorbitan oleate の試験結果はほぼ同等であったが、Sorbitan sesquioleate では明らかな差が確認された。

この結果より、非水溶性の被験物質は適切な溶媒を用いて溶解または均一に懸濁させることが適正な細胞毒性試験を行う上で必要があり、また細胞数も一定数に規定することが重要と考えられた。Sorbitan oleate と Sorbitan sesquioleate の評価についてはいずれも偽陽性であると判断した。

Table 2 Effect of cell concentration and/or medium in the SIRC cytotoxicity test of sorbitan oleate and sorbitan sesquioleate

Test condition	A		B		C	
Final cell concentration	$1x10^5$ cells/mL		$1.5x10^5$ cells/mL		$1x10^5$ cells/mL	
Medium	Medium		Medium		Medium	
Solvent	DMSO		DMSO		-	
Sorbitan oleate	IC_{50}=825.3 (1413.6) 【96.2】	P	IC_{50}=1490.7 (1753.0) 【90.2】	P	IC_{50}=1722.1 (1600.8) 【92.5】	N
Sorbitan sesquioleate	IC_{50}=1178.6 (1747.3) 【92.5】	P	IC_{50}=3476.4 (1774.0) 【89.8】	N	IC_{50}=4460.0 (1640.9) 【89.0】	N

(n=1:Test condition B and C)

( ): IC50 of triethanolamine (μg/mL)
【 】: IC50 of SDS (μg/mL)

Annex 11 被験物質の適用範囲の限定

SIRC 細胞毒性試験による化学物質・原体の眼刺激性の予測に関し、被験物質の適用範囲を限定して検討した。被験物質の適用範囲から除外した物質群は、アルコール、エステルおよびエーテルで、なおかつ分子量 180 未満の低分子とした。厚生科学研究における被験物質のうち除外条件に該当する被験物質は 3 種であった。

Table 1 The three preclusive substances in the Japanese validation study

Substance	CAS	MW	MAS	*In vivo* classification	*In vitro* classification
2-Ethylhexyl p-dimethylamino benzoate	21245-02-3	277.4	0.0	N	P
Isopropyl myristate	110-27-0	270.5	0.0	N	N
Isotonic sodium chloride solution	7647-14-5	58.4	0.0	N	N
Silicic anhydride	7631-86-9	60.1	2.7	N	N
Polyethylene glycol 400	25322-68-3	360～400	4.0	N	N
Glycerin	56-81-5	92.1	4.7	N	N
Polyoxyethylene sorbitan monooleate (20E.O.)	9005-65-6	-	4.7	N	P
Triethanolamine	102-71-6	149.2	8.0	N	N
Methyl p-hydroxybenzoate	99-76-3	152.2	8.7	N	P
Sucrose fatty acid ester	-	-	28.3	P	P
Benzyl alcohol	100-51-6	108.1	31.0	P	P
Ethanol	64-17-5	64.1	32.7	P	N
Acid red 92	18472-87-2	829.6	71.0	P	P
Calcium thioglycolate	814-71-1	130.2	79.7	P	P
m-Phenylenediamine	108-45-2	108.1	80.7	P	
Sodium salicylate	54-21-7	160.1	83.7	P	P
Distearyldimethylammonium chloride	107-64-2	586.5	96.3	P	P
Lactic acid	50-21-5	90.1	102.7	P	P
Sodium dodecyl sulfate*	151-21-3	288.4	15.0≦	P	P
Diisopropanolamine*	110-97-4	133.2	23.0≦	P	P
Monoethanolamine*	141-43-5	61.1	23.3≦	P	P
Glycolic acid*	79-14-1	76.1	25.0≦	P	P
Sodium hydrogenated tallow L-glutamate*	68187-34-8	-	26.7≦	P	P
Chlorhexidine gluconate (20% solution)*	18472-51-0	897.8	28.3≦	P	P
Butanol*	71-36-3	74.1	34.0≦	P	N
Potassium laurate*	10124-65-9	238.4	38.0≦	P	P
Polyoxyethylene octylphenylether (10 E.O.)*	9002-93-1	324.4	41.3≦	P	P
Di (2-ethylhexyl) sodium sulfosuccinate*	577-11-7	488.5	57.0≦	P	P
Acetic acid*	64-19-7	60.1	68.0≦	P	P
Cetyltrimethylammonium bromide*	57-09-0	364.5	76.7≦	P	P
Benzalkonium chloride*	8001-54-5	283.9	78.0≦	P	P
Stearyltrimethylammonium chloride*	112-03-8	348.1	91.3≦	P	P
Cetylpyridinium chloride*	123-03-5	340.0	94.7≦	P	P
Domiphen bromide*	538-71-6	414.5	96.3≦	P	P

厚生科学研究および追加実験において除外する被験物質は 14 種であった。

Table 2 The 14 preclusive substances

Substance	Class	Molecular Weight	Result
Benzyl alcohol	Alcohols	108.1	True negative
Butanol	Alcohols	74.1	False negative
Butoxyethanol	Alcohols	118.2	False negative
Butyl Dipropasol Solvent	Ethers	176.3	False negative
Cyclopentanol	Alcohols	86.1	False negative
2,2-Dimethyl-3-pentanol	Alcohols	116.2	False positive
Ethanol	Alcohols	46.1	False negative
Ethyl-2-methyl acetoacetate	Esters	144.2	False negative
Methoxyisopropyl acetate	Esters	132.2	False negative
Methyl p-hydroxybenzoate	Esters	152.2	False positive
2-Methyl-1-pentanol	Alcohols	102.2	False negative
Phenethyl alcohol	Alcohols	122.2	Ture positve
Phenoxyethanol	Alcohols	138.2	False negative
Propasol Solvent P	Ethers	118.2	False negative

Annex 12　偽陰性となった Hexylene glycol について

Hexylene glycol は SIRC 細胞毒性試験で偽陰性を示した。一方、構造の類似している Butylene glycol はインビボおよびインビトロ共に陰性であった。一般的に両親媒性の有機溶媒（代表例はエタノール、アセトン）の原体は眼刺激性（GHS; 1、2A または 2B）が認められるが、Hexylene glycol は Butylene glycol（陰性物質）などに比較して炭素数が 2 個多いことにより両親媒性の傾向が強まり、結果的に Ethanol と同じような挙動（偽陰性）を示すと推察された。下表に Butylene glycol と Hexylene glycol の溶解性の違いを示す。

Table 1 The differences of butylene glycol and hexylene glycol

	Butylene glycol	Hexylene glycol
Predicted GHS evaluation	NI	1, 2A or 2B
SIRC cytotoxicity test result	Negative	Negative
Molecular weight	90.1	118.2
Solubility		
Water	Soluble	Soluble
Alcohol	Soluble	Soluble
Ether	Insoluble	Soluble
Acetone	Soluble	-
Benzene	Insoluble	-
Carbon tetrachloride	Insoluble	-
Aliphatic hydrocarbons	Insoluble	Soluble
Aromatic hydrocarbons	-	Soluble
Fatty acids	-	Soluble

The data of solubility is taken from CIR final report（Journal of the American College of Toxicology, 4 (5), 223-248, 1985）.

Annex 13 SIRC 細胞毒性試験の結果が掲載されている論文を用いた偽陰性物質の探索

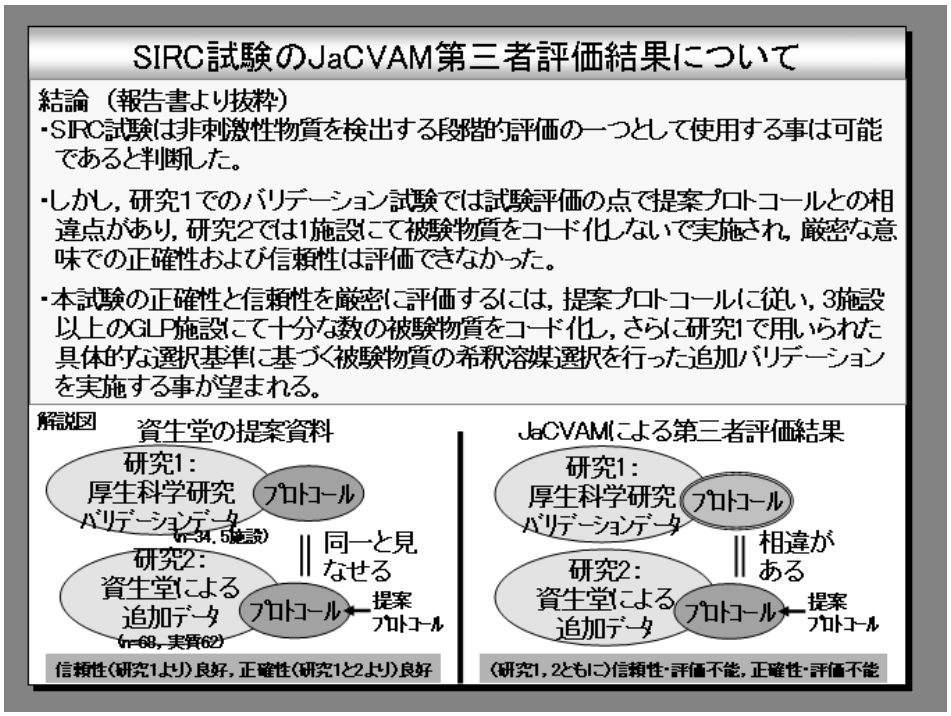
SIRC 細胞毒性試験による IC50 が掲載されている論文（Kitagaki et al., 2006）において、ICCVAM Recommended Reference Substance List、ECETOC Technical Report No.48 に GHS による眼刺激性分類あるいはこれを算出可能な Draize 試験データが掲載されている被験物質について抜粋し、インビトロとインビボの対応性を確認した。判断基準は SIRC 細胞毒性試験結果が 1000 μg/mL 未満を陽性、陰性は 5000 μg/mL 以上を陰性とし、1000～5000 μg/mL の間は Equivocal と分類した。その結果、Acetone および 2-Propanol が偽陰性に分類され、いずれも分子量は 180 未満であった。したがって、分子量 180 未満のアルコールに加えケトンも同様に偽陰性を示す可能性が示唆された。

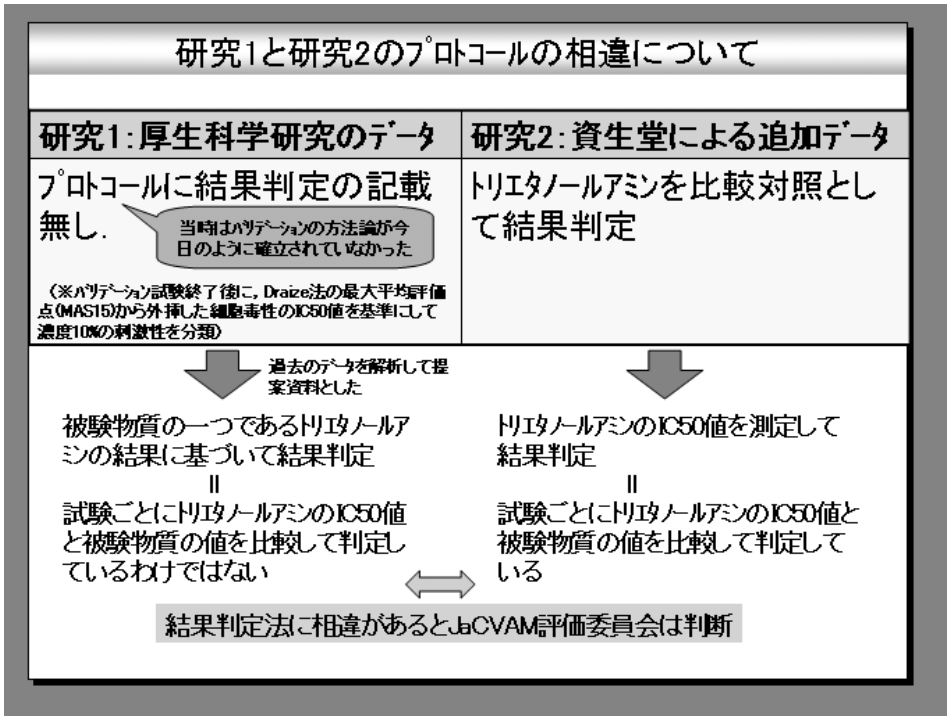
Table 1 The eye irritancy predicted by the SIRC cytotoxicity test

Substance	CAS	MW	Class	GHS	Reference of *in vivo* classification	IC50 Average（μg/mL）±SD	Evaluation
Styrene	100-42-5	104.2	Aromatics	NI	ECETOC Technical Report No.48	2068.8±1821.9	Equivocal
Ethyl acetate	141-78-6	88.1	Esters	NI	ECETOC Technical Report No.48	4575.7±1784.5	Equivocal
2-Propanol	67-63-0	60.1	Alcohols	2A	ECETOC Technical Report No.48	6534.9±581.1	False negative
Silver I nitrate	7761-88-8	169.9	Inorganic salts	1	ICCVAM Recommended Reference Substances List	2.1±0.2	True positive
1-Octanol	111-87-5	130.2	Alcohols	2A	ICCVAM Recommended Reference Substances List	328.1±243.9	True positive
Trichloroacetic acid	76-03-9	163.4	Acids	1	ICCVAM Recommended Reference Substances List	1989.4±123.6	Equivocal
Imidasol	288-32-4	68.1	Heterocyclic compounds	1	ICCVAM Recommended Reference Substances List	554.7±102.8	True positive
1-Hexanol	111-27-3	102.2	Alcohols	2A	ICCVAM Recommended Reference Substances List	643.4±15	True positive
2-Butoxyethanol	111-76-2	118.2	Alcohols	1	ICCVAM Recommended Reference Substances List	2549.8±1034.5	Equivocal
Sulfuric acid	7664-93-9	98.1	Acids	1	ICCVAM Recommended Reference Substances List	1454.6±785.5	Equivocal
Isobutanol	78-83-1	74.1	Alcohols	2A	ECETOC Technical Report No.48	2461.1±1329	Equivocal
Pyridine	110-86-1	58.1	Heterocyclic compounds	1	ICCVAM Recommended Reference Substances List	1762.5±951.8	Equivocal
Acetone	67-64-1	58.1	ketones	2A	ICCVAM Recommended Reference Substances List	7287.3±866.3	False negative
Potassium hydroxide	1310-58-3	56.1	Inorganic salts	1	ICCVAM Recommended Reference Substances List	752±434	True positive

The data of the SIRC cytotoxicity test is the same as that of Kitagaki et al.(2006).

Annex 14 SIRC 細胞毒性試験の JaCVAM 眼刺激性評価委員会による第三者評価





Study Plan

Version 1.1, 16 December 2011

Draft Study Plan for the validation of Statens Seruminstitut Rabbit Cornea (SIRC) cytotoxicity test as an alternative eye irritation test

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

INDEX

1. Aim of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non irritant and irritant in the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Finally the usefulness as alternative method for eye irritation test is examined.

2. Validation Management Team

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC cytotoxicity test as shown in Fig. 1 is established.

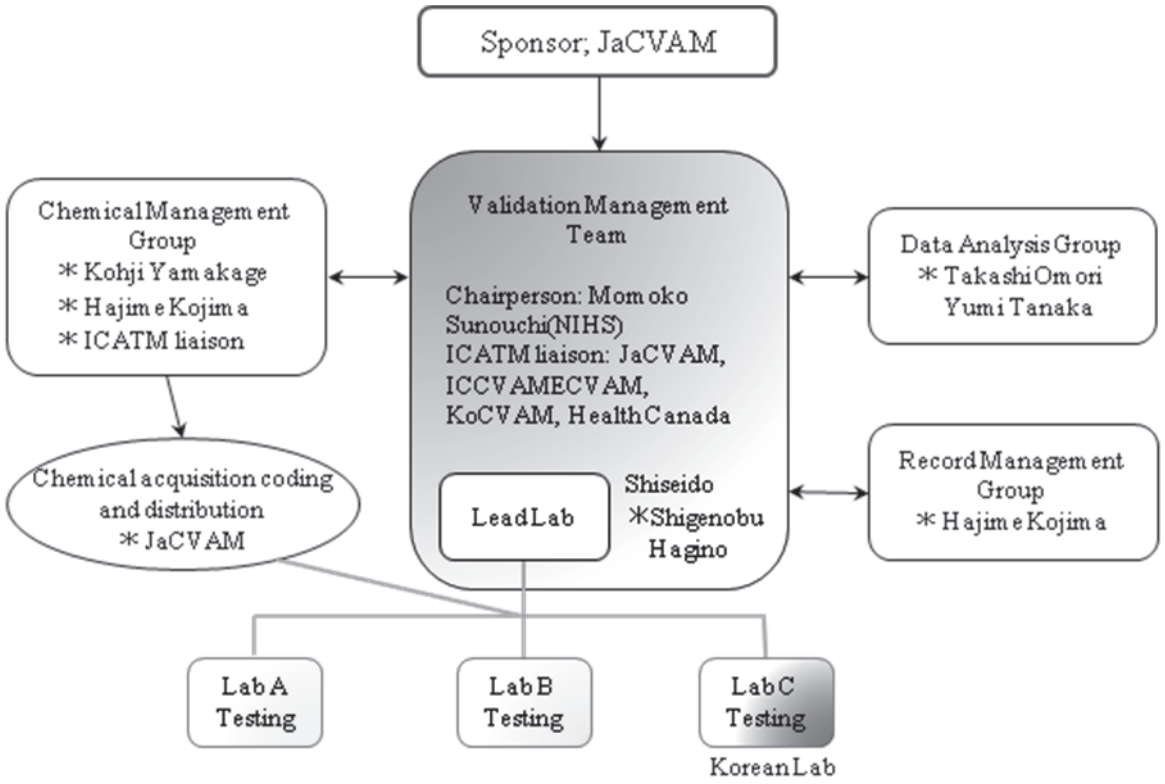


Fig.1 SIRC test Validation Management Team (VMT)

The SIRC cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports to the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control on the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate with participants and so on.

2-1. Chairperson

The chairperson is elected from among the members of the SIRC cytotoxicity test VMT. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test chemicals to be used in the validation study. The list of coded test chemicals is sent to the chemical distributor.

2-3. Data analysis group

The members of data analysis group are elected from among the members of the SIRC cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC cytotoxicity test VMT. They prepares protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from each laboratory in the validation study is also a observer of the SIRC cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the tested microplate can be stored. No other method can match it.

This SIRC cytotoxicity test validation consists of following three Phases.

1) Phase I for the technical transfer and training
   (within laboratory reproducibility)
2) Phase II for the validation using twenty coded substances
   (within laboratory reproducibility, between laboratory reproducibility)
3) Phase III for the validation using fourty coded substances
   (within laboratory reproducibility, between laboratory reproducibility)

3-1. Research laboratories

This validation study is work out by the participants of previous validation, with selection as necessary. Three laboratories are performed the SIRC cytotoxicity test with 60 chemicals within 80 chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Test chemicals

In this validation study, around 60 test chemicals were selected by the chairperson and chemical management group. All test chemicals are blinded, coded, rotated and distributed by JaCVAM until the end of March, 2012.

3-3. Study duration

Duration of this validation study is a year and a month from September 2011 to August 2012.

3-4. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group.

3-5. Quality assurance

All laboratories will work in the spirit of OECD GLP principle. After completion of experiments, all records will be submitted to the chairperson and record management group. They are checked by record management group.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC cytotoxicity test validation study is shown in Table 1.

Table 1. Schedule for SIRC cytotoxicity test validation study

Month	Activity
2011	
September	・ Selection of participating research laboratories ・ Establish the VMT ・ Election and approval of the chairperson and each group
October	・ Selection of test substances for Phase I study ・ Deliberation of draft study protocol
November	・ Deliberation, decision and read-through of draft study plan ・ Technical transfer by video-imaging
December 2012 January	・ Distribution of non-coded test substances, positive control and relative control substances, medium and fetal calf serum ・ Start of Phase I study
February	・ End of Phase I study (by early February) ・ VMT Meeting /Outline of Phase I study results ・ Deliberation and selection of test substances for Phase II study
March	・ Preparation, deliberation and decision of Phase I study report ・ Distribution of coded test substances for Phase II study
April	・ Start of Phase II study ・ VMT Meeting /Outline of study results
May	・ End of Phase II study ・ VMT Meeting /Outline of study results (by late May) ・ Deliberation and selection of test substances for phase III
June	・ Preparation, deliberation and decision of Phase II study report ・ Selection of test substances for phase III ・ Distribution of coded test substances for Phase III study
July	・ Start of Phase III study
August	・ End of Phase III study
September	・ VMT Meeting /Outline of study results ・ Preparation, deliberation and decision of the reports on Phase III study and the second validation of SIRC cytotoxicity test

Phase I study, Phase II study, Phase III study

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test As An Alternative Eye Irritation Test

Draft Study Plan: Phase II-A (Within And Between Laboratory Reproducibilities)

Version 1.51

August 20, 2012

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

INDEX

1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non -irritant and irritant. The in vivo standard for the assessment is based on the classification both of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA). Finally the usefulness of SIRC-CVS cytotoxicity test as an alternative method for eye irritation test is examined.

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-CVS cytotoxicity test as shown in Fig. 1 is established.

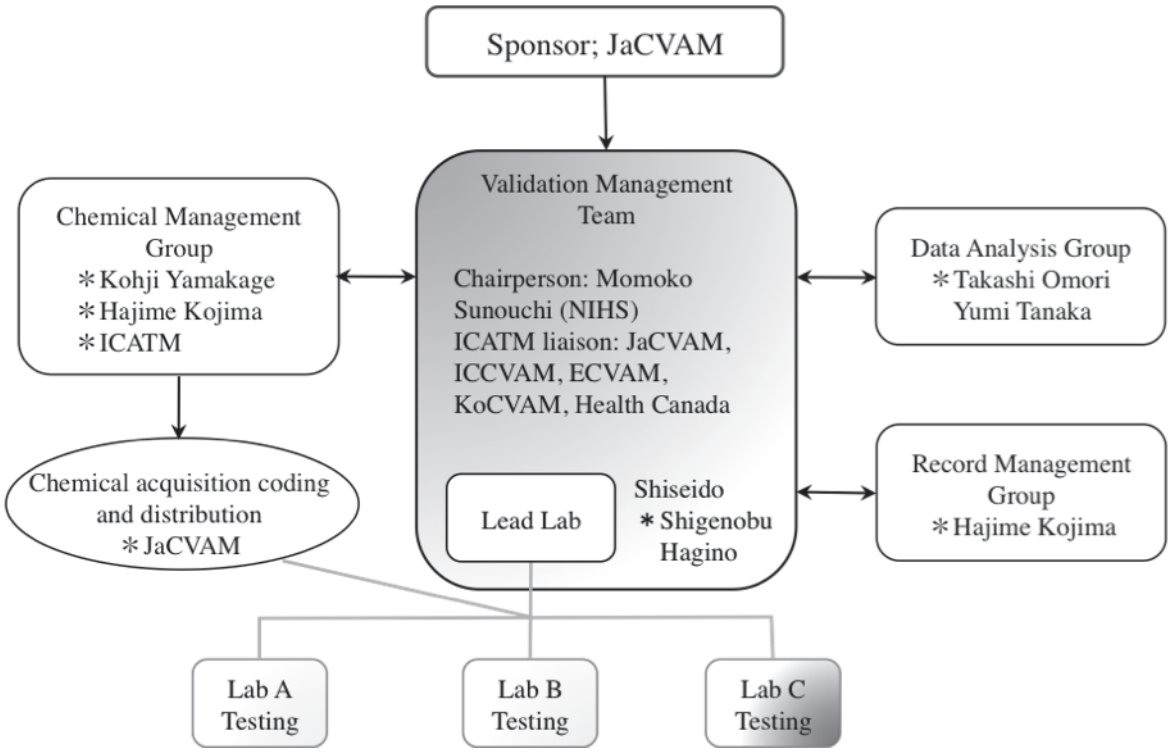


Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on.

2-1. Chairperson

The chairperson is elected from among the VMT members. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from each laboratory in the validation study is also an observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored micro plates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I for the technical transfer and training
   (Transferability)
2) **Phase II for the validation**
   (within laboratory reproducibility, between laboratory reproducibility**)**
3) Phase III for the validation
   (within laboratory reproducibility, between laboratory reproducibility)

3-1. Research laboratories

Three laboratories are performed the SIRC-CVS cytotoxicity test with sixty substances within eighty chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. All of the selected substances are commercially available. This is because they are selected from the substance list of the Eye Irritation Validation Study (EIVS) of ECVAM. All of the selected substances are commercially available. The selected substances have high quality in vivo data, especially individual animal data.

3-3. Test substances

The twenty more substances were selected for the phase II of the validation study at the meeting on February 22 and 23, 2012. The remaining substances will be selected before the next step. All of the test substances for phase II and phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study. are blinded, coded, rotated and distributed by JaCVAM. Three laboratories will test the same sixty substances. The twenty more substances were selected for the phase II test of SIRC-CVS validation study at the first VMT meeting on February 22 and 23, 2012. Five of them will be used for phase II-A and fifteen for phase II-B. The remaining substances will be selected before phase III test.

Three laboratories will test the same sixty substances.

GHS/EPA category of twenty substances for phase II test are GHS-1/EPA-I; 3 substances, GHS-2A/EPA-II; 3 substances, GHS-2B/EPA-II; 4 substances, GHS-Non/EPA-III; 5 substances and GHS-Non/EPA-IV; 5 substances.

Table 1. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
II-II-A	5	3	Within and between laboratory reproducibilities
II-II-B	15	3	
IIIIII	40	1	Between laboratory reproducibility

3-4. Study duration

Phase II-A validation test will be performed for about ten eleven weeks from the early July late June to the early September, 2012. (See Table 2)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The

eye irritation of the test substance is evaluated by using triethanolamine as a relative control in accordance with the protocol, Annex 1. ***Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.***

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 2.

Table 2. Schedule for Phase II-A of SIRC-CVS cytotoxicity test validation study

Month	Activity
2012	
June	・ Distribution of five test substances coded for phase II-A study
JulyJune	・ Distribution of the study plan for phase II-A-A and the revised protocol for SIRC-CVS validation phase II study ・ Distribution of five test substances coded for phase II-A study ・ Start of phase II-A study by mid July
July	・ Phase II-A study
August	
September	・ Provision of the data of phase II-A study to the data analysis group by early mid September ・ Data Analysis
October	・ Japanese VMT and the laboratory's meeting /Outline of study results on phase II-A ・ Report to VMT members /Outline of study results on phase II-A ・ End of phase II-A study ・ Distribution of the study plan and fifteen test substances for phase II-B study
November	・ Start of phase II-B study by early November・ Provision of the data of phase II-B test to the data analysis group by late November
December	
2013	
January	・ Provision of the data of phase II-B study to the data analysis group by mid January ・・ Data Analysis
February	・ Japanese VMT and the laboratory's meeting /Outline of study results on phase II-B ・ International VMT meeting /Outline of Phase II study results ・ Selection of forty substances for Phase III study ・ Submit the report of phase II in SIRC-CVS cytotoxicity test validation study ・ Preparation and deliberation of Phase II study report

March	・ Distribution of the substances for phase III validation study

7. List of abbriviations and acronyms

ATCC American Type Culture Collection
DMSO Dimethyl Sulfoxide
EPA United States Environmental Protection Agency
FBS Fetal Bovine Serum
GHS Globally Harmonized System of Classification and Labelling of Chemicals
IC_{50} 50% Inhibitory Concentration
JaCVAM Japanese Center for the Validation of Alternative Methods
MEM Minimum Essential Medium
NI Non Irritant
OD Optical density
PBS(-) Phosphate-Buffered Saline (-)
SDS Sodium Dodecyl Sulfate
SIRC cell Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS Statens Seruminstitut Rabbit Cornea-Crystal Violet Staining

8. About the revision of this study plan

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test
As An Alternative Eye Irritation Test

Study Plan

Version 1.53

For Phase II-B (Within And Between Laboratory Reproducibility)

October 25, 2012

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

INDEX

1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non-irritant and irritant. The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-CVS cytotoxicity test as shown in Fig. 1 is established.

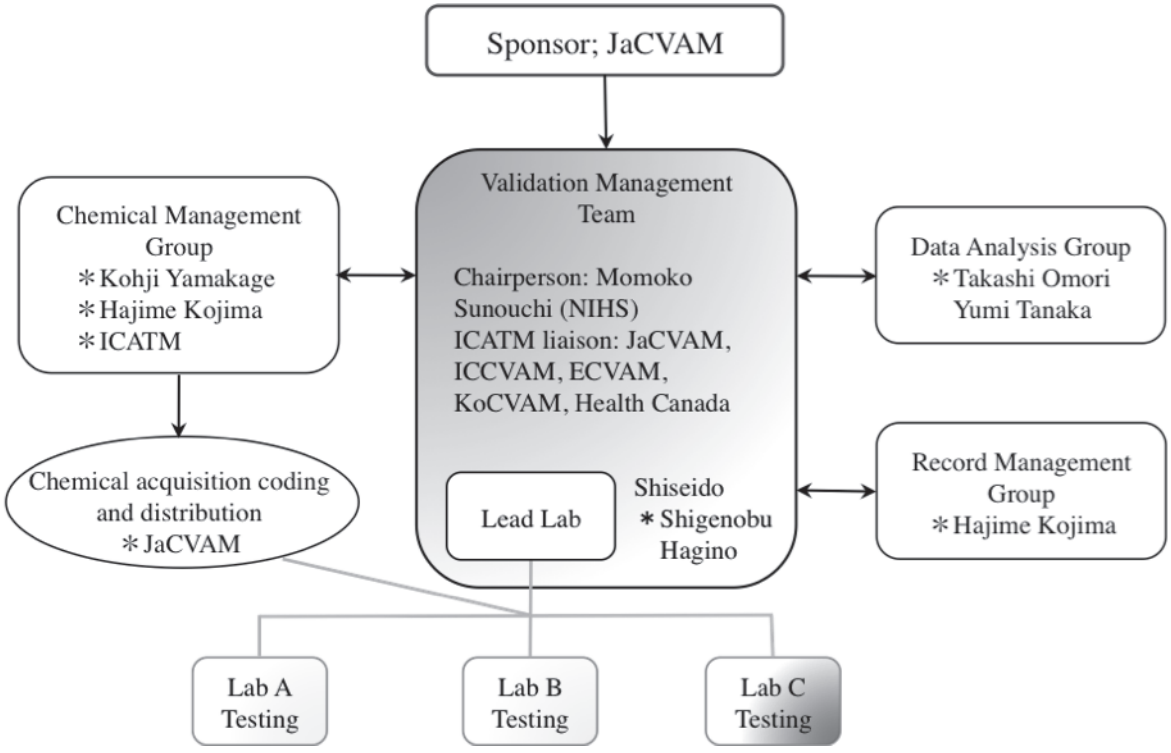


Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of

the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on.

2-1. Chairperson

The chairperson is elected from among the VMT members. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from of the laboratories participating in the validation study is also an

observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol (version 2.12) for SIRC-CVS cytotoxicity test. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored microplates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I for the technical transfer and training (Transferability)

The study for Phase I was ended.

2) Phase II for the validation

(within laboratory reproducibility, between laboratory reproducibility**)**

The study for Phase II-A was ended.

The study for Phase II-B will be started by late October 2012.

3) Phase III for the validation

(between laboratory reproducibility)

3-1. Research laboratories

Three laboratories perform the SIRC-CVS cytotoxicity tests with sixty substances within eighty chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. This is because they are selected from the substance list of the Eye Irritation Validation Study (EIVS)

of ECVAM. All of the selected substances are commercially available.

3-3. Test substances

The twenty more substances were selected for the phase II of the validation study at the meeting on February 22 and 23, 2012. The remaining substances will be selected before the next step. All of the substances for phase II and phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study.

Three laboratories will test the same sixty substances.

Table 1. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
II-A	5	3	**Within and between laboratory reproducibility**
II-B	**15**	**3**	
III	40	1	Between laboratory reproducibility

3-4. Study duration

Phase II-B validation test will be performed for about eleven weeks from the late October 2012 to the mid January 2013. (See Table 2)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The eye irritations of the test substances are evaluated by using triethanolamine as a relative control in accordance with the protocol version 2.12, Annex 1. Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 2.

Table 2. Schedule for Phase II-B of SIRC-CVS cytotoxicity test validation study

Month	Activity
2012	
June	・ Distribution of five test substances coded for phase II-A study
July	・ Distribution of the study plan for phase II-A and the revised protocol for SIRC-CVS validation phase II study ・ Start of phase II-A study by mid July
August	
September	・ Provision of the data of phase II-A study to the data analysis group by mid September ・ Data Analysis
October	・ Japanese VMT and the laboratory's meeting on the 16th October Outline of study results on phase II-A ・ End of phase II-A validation study ・ Distribution of the study plan and fifteen test substances for phase II-B study ・ Start of phase II-B study by late October
November	
December	
2013	
January	・ Provision of the data of phase II-B study to the data analysis group by the 15th January ・ Data Analysis
February	・ Japanese VMT and the laboratory's meeting at Kyoto on the 15th February Outline of study results on the phase II-B and on the whole phase II ・ International VMT/he laboratory's meeting at Kyoto on the 16th February Outline of study results on the phase II-B and on the whole phase II Selection of forty test substances for phase III study ・ Submit the report of phase II in SIRC-CVS cytotoxicity test validation study ・ Preparation and deliberation of phase II study report
March	・ Distribution of the test substances for phase III validation study

7. List of abbriviations and acronyms

ECVAM ; European Center for the Alternative Methods
EPA ; United States Environmental Protection Agency
GHS ; Globally Harmonized System of Classification and Labelling of Chemicals
GLP ; Good Laboratory Practice
IC50 ; IC50% Inhibitory Concentration
ICCVAM ; The Interagency Coordinating Committee on the Validation of Alternative Methods
ICATM ; The International Cooperation on Alternative Test Method
JaCVAM ; Japan Center for the Alternative Methods
KoCVAM ; Korean Center for the Alternative Methods
JaCVAM ; Japanese Center for the Validation of Alternative Methods
SIRC cell ; Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS ; Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining
VMT ; Validation Management Team

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test

As An Alternative Eye Irritation Test

Study Plan

Version 1.56

Phase III Study　(For Predictability)

March 26, 2013

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

INDEX

1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells to discriminate between non-irritant and irritant. The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-Crystal Violet Staining (CVS) cytotoxicity test as shown in Fig. 1 is established.

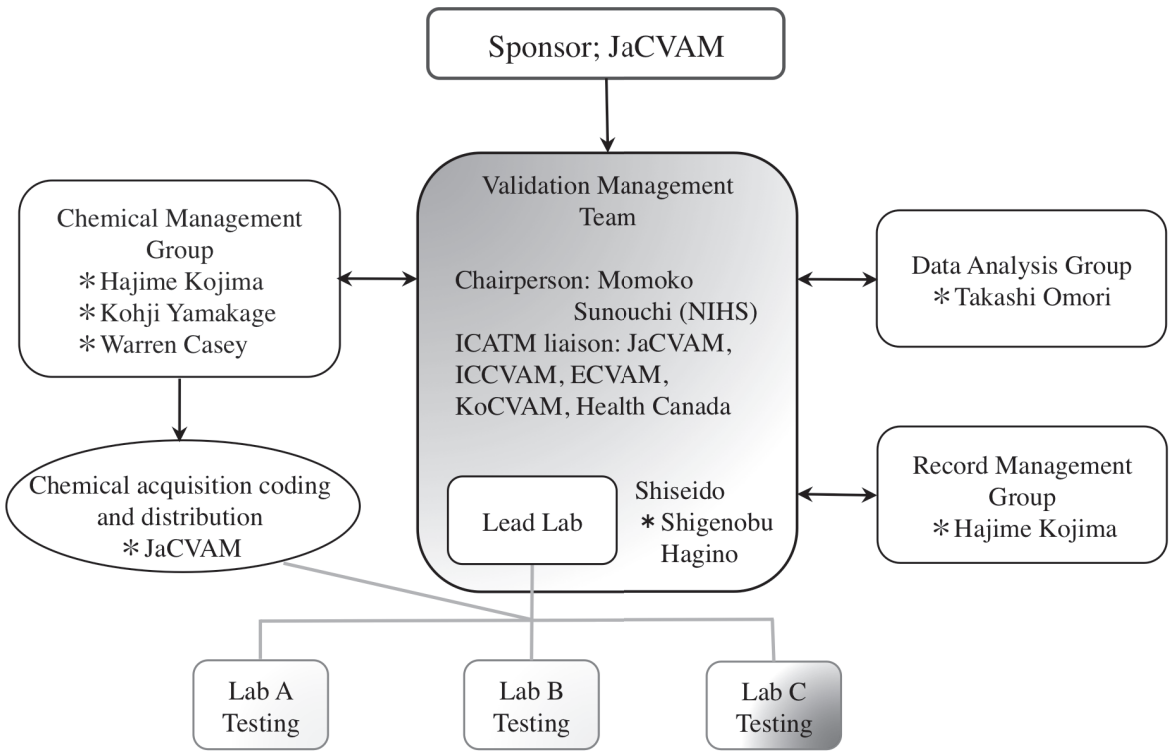


Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on. The VMT members are shown at Table 1.

Table 1. Members of SIRC-CVS Validation Management Team (VMT)

Name	Organization	Action
Momoko Sunouchi	JaCVAM, NIHS Japan	Chairperson
Hajime Kojima	JaCVAM, NIHS Japan	JaCVAM,Chemical Management
Warren Casey	ICCVAM, NIH USA	NICEATM,Chemical Management
Michael Oelgeschlaeger	ECVAM, Federal Institute Risk Assessment, DEU	
Takashi Omori	Doshisha University, Japan	Data Analysis
Kohji Yamakage	FOOD AND DRUG SAFTY CENTER, Hatano Researh Institute,Japan	Chemical Management
Shigenobu Hagino	Shiseido Research Center,Japan	Lead laboratory
KoCVAM		
Health Canada		

2-1. Chairperson

The chairperson is elected from among the VMT members. She prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from of the laboratories participating in the validation study is also an observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out

experiments according to the study protocol (version 2.13E) for SIRC-CVS cytotoxicity test. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored microplates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I study (For transferability)

The phase I study by the protocol version 1.6E was ended.

2) Phase II study (For within- and -between laboratory reproducibility)

The phase II-A study by the protocol version 2.09E was ended.

The phase II-B study by the protocol version 2.12E was ended.

3) Phase III study (For predictability)

The phase III study is performed by the protocol version 2.13E.

3-1. Research laboratories

Three laboratories perform the SIRC-CVS cytotoxicity tests with forty substances each.

Laboratory Name

1) Bozo Research Center Inc.

2) Nihon Kolmar Co., Ltd

3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. This is because they are selected based on the substance list of the Eye Irritation Validation Study (EIVS) of ECVAM and others. All of the selected substances are commercially available.

3-3. Test substances

The use of one hundred substances in total was determined for the phase III of the validation study at the VMT meeting on 16th February 2013. These substances were selected by the chemical management group and approved by the VMT members. All of the substances for phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study.

Each of three laboratories will test the forty substances and ten of the forty will be in common (Table 2).

Table 2. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
III	**100 (coded) in total 40; each laboratory 10; in common 30; different**	**1**	**Predictability**

3-4. Study duration

Phase III validation test will be performed for about twelve weeks from the beginning April 2013 to the end June 2013. (See Table 3)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The eye irritations of the test substances are evaluated by using triethanolamine as a relative control in accordance with the protocol version 2.13, Annex 1. Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 3.

Table 3. Schedule for Phase III of SIRC-CVS cytotoxicity test validation study

Month	Activity
2013	
March -	・ Distribution of the medium by early in March ・ Distribution of forty test substances coded from mid-March to early in April
April - June	・ Start of phase III study by mid-April ・ Provision of the data of phase III study to the data analysis group by the end of June
July – September	・ Data Analysis ・ VMT-FTF meetings will be held at the end of September, 2013.
October	
November	
December	・ Submission of the report on the validation study of SIRC-CVS cytotoxicity test as an alternative eye irritation test to JaCVAM steering committee by late December

7. List of abbreviations and acronyms

ECVAM; European Center for the Alternative Methods
EPA; United States Environmental Protection Agency
FTF; Face To-Face
GHS; Globally Harmonized System of Classification and Labelling of Chemicals
GLP; Good Laboratory Practice
IC50; IC50% Inhibitory Concentration
ICATM; The International Cooperation on Alternative Test Method
ICCVAM; Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM; Japanese Center for the Validation of Alternative Methods
KoCVAM; Korean Center for the Validation of Alternative Methods
SIRC cell; Statens Seruminstitut Rabbit Corneal cpell
SIRC-CVS; Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining
VMT; Validation Management Team

Protocol for SIRC-CVS cytotoxicity test
Version 3.8

May 24, 2016

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Contents

1 Purpose

The Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining (SIRC-CVS) test method has been designed to be used in a bottom up approach[1–3] for distinguishing between ocular non-irritants (NI) and ocular irritants (I) by calculating the half maximal inhibitory concentration (IC_{50}) of a chemical substance in Statens Seruminstitut rabbit corneal cells (SIRC) as a measure of cytotoxicity. The results are then used to predict whether the chemical substance is a non-irritant or an irritant per the UN Globally Harmonized System of Classification for Labelling of Chemicals (GHS).

2 The principle of SIRC cytotoxicity test

Cytotoxicity is considered a useful index for evaluating the eye irritation potency of chemical substances. The reason is that corneal epithelium cells are well suited for cytotoxicity tests, because corneal damage has a significant impact on total eye irritation.[4] Cytotoxicity tests are useful for identifying ocular non-irritants that have almost no effect on the cornea. The Statens Seruminstitut rabbit corneal cell line used in this test is derived from rabbit corneas. We chose an application time of 72 hours, because in vivo data from previous research projects[5] has shown that, in general, maximal eye irritation caused by chemicals other than acids or alkalis typically occurs within 72 hours of ocular instillation.

In the SIRC cytotoxicity test, crystal violet, which penetrates via a cell membrane treated with methanol and stains biological macromolecules, is used as a means of measuring viable cells. This technique is suitable for many types of cultured cells and produces highly consistent results.[5–9] A relative control is also used to help ensure consistency.[10, 11] Not only is the test procedure simple and easy to perform, the tested microplate can be stored and used to verify the test results at any time. In this respect, the SIRC-CVS cytotoxicity test is unique among tests used to measure cytotoxicity.

The single greatest disadvantage of this test method is that test chemicals must be dissolved or uniformly suspended in a liquid medium.

3 Materials

3.1 Cell line

The Statens Seruminstitut rabbit corneal cell line used in this test is derived from rabbit corneas and obtained from the American Type Culture Collection (ATCC No. CCL-60). It is also suitable for storage frozen in liquid nitrogen. Prior to performing the test, the cells should be checked to ensure the absence of mycoplasma using a test such as the Venor GeM Mycoplasma Detection Kit (Minerva Biolabs GmbH, 11-1025). The cells are to undergo no more than 35 passages from their purchased stock. (e.g., if the cell culture starts at passage number 435 and is passaged every four days, it should be disposed of after passage number 470.) Quality control is to be performed as described in section 4.6.

3.2 Equipment

- CO2 incubator, such as the MCO-17AIC from Sanyo Electric Co., Ltd
- Clean bench, such as the CCV1300E from Hitachi, Ltd

- Microplate reader, such as the Benchmark Plus™ from Bio-Rad Laboratories
- Inverse phase contrast microscope, such as the Eclipse TS100 from Nikon
- Autoclave, such as the BS-325 or SS-320 from Tomy Seiko Co., Ltd
- Centrifuge, such as the 5800 from Kubota Corporation
- Water bath
- Electronic chemical balance
- Ultrasonic bath sonicator
- Vortex mixer
- Magnetic stirrer
- Hemocytometer or cell counter, such as the 03-303-5 from Erma Inc.

3.3 Instruments

- 25-cm2 and 75-cm2 tissue culture flasks, such as the 353108 and 353136 from BD Falcon
- 96-well flat bottom tissue culture microtiter plates, such as the 353072 from BD Falcon
- Storage plates, such as the AB-0765 0.8-mL Storage plate from Thermo Scientific
- Multichannel pipettes, micropipettes
- Dispenser trays
- Tubes
- 1.5-mL cryotubes
- 15-mL and 50-mL centrifuge tubes
- 200-µL, 100-µL, and 5-mL tips for micropipettes
- Microplate sealing tape
- Paper towels, such as the 61000 Kim towel™ from Nippon Paper Crecia Co., Ltd
- Wrapping film, such as the Saran Wrap

3.4 Culture medium and reagents

- Minimum Essential Medium (MEM)
- Fetal Bovine Serum (FBS)
  The fetal bovine serum is to be inactivated before use. Inactivate by placing in a water bath at 56°C for 30 minutes. After cooling, store the serum in 56-mL or 28-mL tubes. The serum is stored at −70 or −20°C.
- Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
  (Antibiotic-Antimycotic 100×, GIBCO BRL)

- Modified PBS, comprising phosphate-buffered saline without calcium or magnesium
- 0.25% (w/v) Trypsin (1 mmol/L EDTA·4Na)
- Dimethyl Sulfoxide (DMSO, CAS Number 67-68-5)
  Measured per either weight or volume.
- Ethanol (CAS Number 64-17-5)
  Measured per either weight or volume.
- Crystal Violet (CAS Number 548-62-9)
- Methanol (CAS Number 67-56-1)
- Sodium Dodecyl Sulfate (SDS, CAS Number 151-21-3)
- Triethanolamine (CAS Number 102-71-6)
  Purity of 98% or higher.
- Hydrochloric Acid (CAS Number 7647-01-0)
- Sodium Hydroxide (CAS Number 1310-73-2)

Typical specifications and manufacturers for reagents are shown in Table 1.

3.5 Medium

The medium used in this test (the Medium) comprises MEM supplemented with10% inactivated FBS and about 1% antibiotic (P/S/F solution). For example, 500 mL of MEM is supplemented with 56 mL of FBS and 5.6 mL P/S/F. At this time, the concentrations of the antibiotics are 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of Amphotericin B.

3.6 Crystal violet solution

A 0.4% crystal violet solution is prepared using methanol.

3.7 Test chemicals

3.7.1 Determining solubility or suspensibility of test chemicals in the Medium

Confirm in advance the solubility or suspensibility of each test chemical in the Medium, using the procedure shown in Fig. 1. First, determine whether the test chemical can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL (1% w/v). Use a vortex mixer, water bath, or sonicator as necessary. If the test chemical cannot be dissolved or uniformly suspended in the Medium, the next step is to determine whether the test chemical is more easily dissolved in DMSO or ethanol. Next, dissolve or uniformly suspend the test substance in the more suitable solvent at a concentration of 10,000 μg/mL and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL. If not, dissolve or uniformly suspend the test substance in the more suitable solvent at a concentration of 5,000 μg/mL (0.5% w/v) and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL. If not, the test

substance is considered to be outside the applicability domain of the test. These judgments can all be performed by visually confirming the absence or presence of precipitate in the solution.

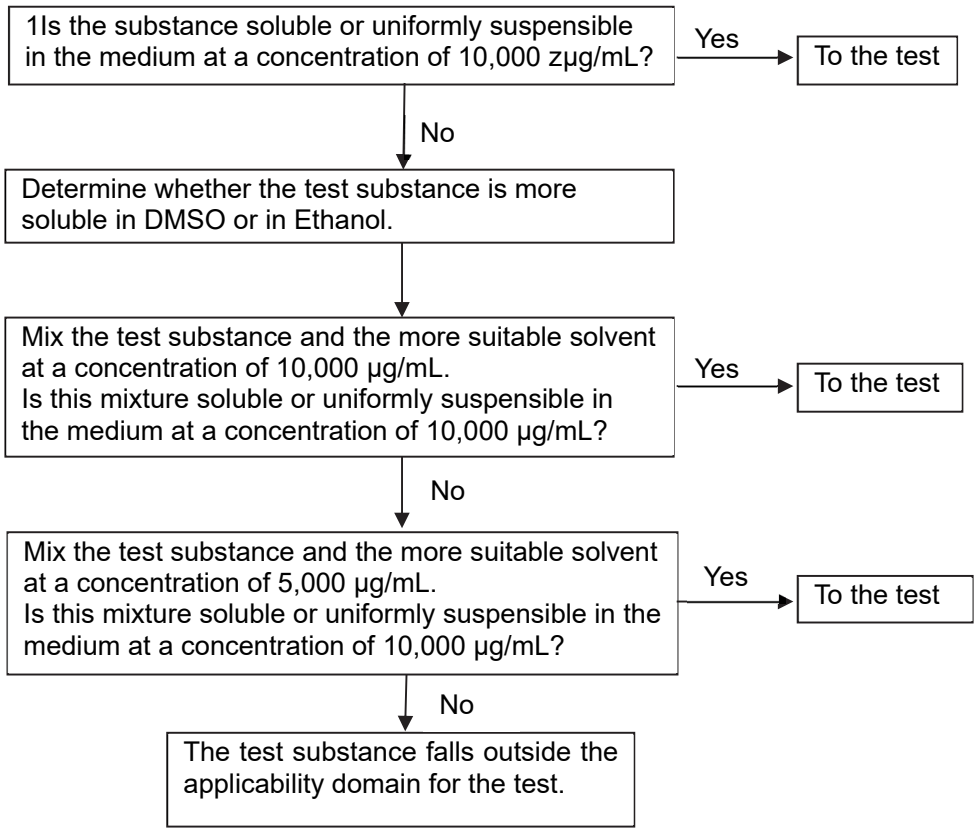

Figure 1: Determining solubility or suspensibility of test chemicals in the Medium

3.7.2 Preparing test chemicals

After determining an appropriate concentration for each test chemical per the procedure described in section 3.7.1. When the maximal concentration of a stock test chemical dilution series is 10,000 μg/mL, once the test chemical dilution series in the microplate is mixed with the Medium containing the SIRC cells, as described in section 4.3, the final maximal concentration is halved to 5,000 μg/mL (0.5% w/v). When either DMSO or ethanol is used as a solvent, the final maximal concentration is 5,000 μg/mL (0.5% w/v).

When the maximal concentration of a stock test chemical dilution series is 5,000 μg/mL, the final maximal concentration in the microplate is 2,500 μg/mL (0.25 w/v%) for the test chemical dilution series and 5,000 μg/mL (0.5% w/v) for the solvents. If precipitation is observed in a well at any time after mixing the test chemical solution and the cells, especially after the 72-hr incubation period, the test data must be rejected.

3.7.3 Preparing test chemical dilution series

Prepare in duplicate on the microplate an eight-well, two-fold serial dilution for each test chemical, as shown in Fig. 2: Layout of 96-well microplate.

3.8 Reference substances

3.8.1 Positive control

Use a solution of SDS at a final concentration of 1,000 μg/mL in the Medium as the positive control.

3.8.2 Relative control

Use a solution of triethanolamine at a final concentration of 10,000 μg/mL in the Medium as the relative control.

3.8.3 Negative control

Use the Medium, a DMSO-medium solution at a final concentration 10,000 μg/mL, or an ethanol-medium solution at a final concentration of 10,000 μg/mL as the negative control. The negative control should match the solvent used to dissolve or uniformly suspend the test chemical.

4 Test procedure

4.1 Passaging SIRC cells

Cell culture

1. Culture SIRC cells in MEM supplemented with 10% FBS and 1% P/S/F (the Medium) at 37°C in a humidified incubator at 5% CO2 in air. The concentrations of the antibiotics are 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of Amphotericin B.
2. Remove the Medium from the culture flask, then rinse the SIRC cells twice with 10 mL of modified PBS to remove the serum, which is a trypsin inhibiter.
3. Remove the modified PBS, then add and ensure that all the cells in the culture flask are exposed to 1.5 to 2.0 mL of 0.25% trypsin solution.
4. Remove the 0.25% trypsin solution, then incubate the cells as is for two or three minutes at 37°C. Detach the cells from the inside surface of the flask by tapping. Collect the cells in an appropriate volume of MEM (10% FBS). Count the cells and prepare a cell suspension at a density of 6 to 8 $\times 10^5$ cells in 15 to 30 mL of medium. Use this culture to passage the cells.

Freezing and preserving cells

1. Prepare a mixture of medium and 10% DMSO for freezing and preserving cells. Commercially available cell preservation solution such as Cellbanker 1or 2 (Juji Field, Inc.) may be used, and the solution may be either a serum type or non-serum type.

2. Add a solution at a density of 1×10^6 cells/mL to a stock tube and slowly lower the temperature until frozen. For example, cool the stock tube for 5 minutes in ice, 50 minutes at about −20°C, and 12 hours at about −70°C before placing it in liquid nitrogen. Commercially available freezing vessels such as Bicell (Nihon Freezer Co., Ltd) may be used to hold the tube.
3. The tube containing the cells is then preserved in liquid nitrogen.

Thawing of frozen cells

1. Immerse the stock tube in hot water at a temperature of 37°C to thaw the frozen cells.
2. Add 10 mL of the Medium to the cell suspension and centrifuge at 1,000 rpm for 5 minutes.
3. Remove the supernatant, then add the Medium to prepare the cell suspension. Passage the preserved cells at least once to confirm appropriate growth.

4.2 Preparing a cell suspension

1. Remove the Medium from the culture flask, then rinse the SIRC cells twice with 10 mL of modified PBS to remove the serum, which is a trypsin inhibiter.
2. Remove the modified PBS, then add and ensure that all the cells in the culture flask are exposed to 1.5 to 2.0 mL of 0.25% trypsin solution.
3. Remove the 0.25% trypsin solution, then incubate the cells as is for two or three minutes at 37°C.
4. Detach the cells from the inside surface of the flask by tapping.
5. Collect the cells in an appropriate volume of MEM (10% FBS) with a pipette.
6. Count the cells and prepare a cell suspension at a density of 2×10^5 cells/mL.

4.3 Exposing the cells to a test chemical

1. Prepare 100 µL of modified PBS and the negative control as well as 100 µL of the serial dilutions of the test chemical, positive control, and relative control in a 96 well microplate, as shown in Fig. 1.
2. Add 100 µL of the 2×10^5 cells/mL cell suspension to the wells, as shown in Fig. 2.
3. Seal the microplate to prevent contamination from volatile test chemicals. Wrapping film may be used for this purpose. The six measurements described in steps (1)–(6) of section 4.6 Quality Control are to be used to verify that there is no contamination of other wells by volatile test chemicals. The criterion for toxic effect is the same as that for quality control. If contamination is found, the test is to be redone at a lower concentration.
4. After mixing the test chemical and the cell suspension, allow to stand for 20 minutes on a clean bench. Once the cells adhere to the bottom of the wells, the microplate is moved to the incubator.
5. Incubate for about 72 hours at 37°C and 5% CO_2 in air.

4.4 Crystal violet staining

1. After incubation, remove the Medium containing the test chemicals by gently but quickly turning the microplate upside down.
2. Add 200 µL of modified PBS and shake gently to rinse the cells, then remove the modified PBS by gently but quickly turning the microplate upside down. Repeat this procedure twice.
3. Add 100 µL of crystal violet methanol solution to each well and allow to stand for 30 minutes.
4. After the staining, remove the crystal violet methanol solution by gently but quickly turning the microplate upside down. Wash the cells thoroughly with tap water and blotted away any residual water with a paper towel.
5. After drying, measure the optical absorbance at 588 nm with an automatic microplate reader. Any nearby wavelength for which equivalency can be demonstrated is suitable for measurements.

4.5 Calculating IC_{50}

Absorbance in the negative control wells, which contain no test chemical, minus the absorbance of the blank is considered to be 100%, and the percentage of absorbance for the mean of two wells is calculated on this basis. Cell viability is a percentage calculated by dividing the mean absorbance of two wells at the same concentration minus the absorbance of a blank well by the mean absorbance of all negative control wells minus the absorbance of a blank well.

IC_{50} is the concentration at which the growth of cells was inhibited to 50% of the control and calculated as follows using two concentrations around the predicted concentration of 50% cell viability.

$\text{Log } IC_{50} = [(50 - y1)\log x2 - (50 - y2)\log x1]/(y2 - y1)$,

where x1 is low concentration, x2 is high concentration, y1 is cell viability at low concentration, y2 is cell viability at high concentration, and log means the common logarithm.

If cell viability is greater than 50% at maximal concentration of 5,000 µg/mL, the result for that test chemical is $IC_{50} > 5{,}000$ µg/mL. Also, if the cell viability is less than 50% at a minimal concentration of 39.1 µg/mL, the result for that test chemical is $IC_{50} < 39.1$ µg/mL. IC_{50} at other maximal and minimal concentrations of test chemicals are expressed in the same manner.

If multiple concentrations of a test chemical yield a 50% cell viability, use the lowest value of IC_{50}.

In the Excel spreadsheet, cell viability is rounded to the nearest tenth.

4.6 Quality control

Quality control of the SIRC cytotoxicity test is performed by taking six measurements, which must satisfy the following criteria. Failure to satisfy the criteria means that the test substance must be retested. In particular, if a volatile test chemical fails to satisfy the criteria, it must be retested at a lower concentration.

1. The absolute OD obtained from the negative control is an index of the normal proliferation of SIRC cells seeded at a concentration of 2×10^4 cells/well and incubated for 72 hours. The mean

OD of the negative control (right and left wells) must be greater than 0.4 for the test data to be considered valid.

2. Sodium dodecyl sulfate (SDS) is used as a positive control. The IC_{50} of SDS should be between 77.7 and 258.7 μg/mL when tested using the standard protocol. This criterion must be satisfied for the test data to be considered valid.

3. Triethanolamine is used as a relative control (See Annex 1.). The IC_{50} of triethanolamine should be between 1,000 and 2,500 μg/mL when tested using the standard protocol (See Annex 2). This criterion must be satisfied for the test data to be considered valid.

4. Any discrepancy between the two dilution series of the test chemical is to be reviewed. The IC_{50} of both the first series and the second series must be within 20% of the mean IC_{50} of the two dilution series together. This criterion must be satisfied for the test data to be considered valid. The minimum value for IC_{50} is 39.1 μg/mL and the maximum value is 5000 μg/mL. IC_{50} at other maximal and minimal concentrations of test chemicals are expressed in the same manner. These values of IC_{50} are only used for quality control calculations.

5. The difference between left and right wells of the negative control should be reviewed to confirm systematic quality. The mean OD of the left side and the mean OD of the right side should be within 15% of the mean OD of both sides combined. This criterion must be satisfied for the test data to be considered valid.

6. The two test results adopted for making a prediction must be checked for equality. The higher of the two IC_{50} values of the two positive controls (SDS) must be no more than twice as large as the lower of the two values. (The higher value ÷ the lower value ≦ 2)

4.7 Evaluation

Eye irritation potency of the test chemical is predicted using triethanolamine as a relative control (See Annex 1.). Triethanolamine is classified No Category under GHS, and using this as a reference, a test chemical is identified as negative (No Category) when the IC_{50} is higher than or equal to that of triethanolamine and is identified as positive (Category 1 or 2) when the IC_{50} is lower than that of triethanolamine. The test is performed twice. If the results of the two tests are different, a third test is performed and the data of the two tests with the same result are adopted for evaluating. If discrepancies between three results must be reviewed, the test is repeated three times.

5 References

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6 List of abbreviations and acronyms

°C	degrees Centigrade
ATCC	American Type Culture Collection
DMSO	Dimethyl Sulfoxide
EPA	United States Environmental Protection Agency
FBS	Fetal Bovine Serum
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
IC_{50}	half maximal inhibitory concentration
I	Irritant
JaCVAM	Japanese Center for the Validation of Alternative Methods
MEM	Minimum Essential Medium
NI	Non Irritant
OD	Optical density
Modified PBS	Phosphate-Buffered Saline without calcium or magnesium
SDS	Sodium Dodecyl Sulfate
SIRC cells	Statens Seruminstitut Rabbit Corneal cells
SIRC-CVS	Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining

7 Revision history

(1) The revision of ver.1 – ver.1.71 that is the same as ver.1.71j and ver.1.71e, is shown by the green character in ver. 2.13. The protocol of the ver.1 was used for evaluating 68 chemicals at Shiseido Research Center in 2009-2010 and was subjected to the peer review by JaCVAM.

(2) The revision after ver.1.71 is shown by the blue character in ver. 2.13.

(3) The revision from ver.2.07 to ver.2.08:

In　4.7.(4), “The difference between two dilution series of the substance should be confirmed. The IC50s of the first series and the second series should be within + 20% of the mean IC50 of two series （the mean + 20%）, respectively.” was changed to “The difference between two dilution series of the substance should be confirmed. The IC50s of the first series and the second series should be within + 20% of the mean IC50 of two series(the mean of the first IC50 and the second IC50), respectively.”

(4) The revision form ver.2.08 to ver.2.09:

In 4.3.(3), “The five measurements (4.7.(1)-(5)) of the quality control should be used for checking wether the volatile substance has an effect on other wells. The criterion of the toxic effect is the same as that of the quality control. When the volatile substance has an effect on other wells, the retest should be performed using dilution.”

(5) The revision from ver.2.09 to ver.2.11:

“SIRC cytotoxicity test” was changed to “SIRC-CVS cytotoxicity test”. “Table 1” of 3.7.1. was changed to “Figure 1”.

(6) The revision from ver.2.11 to ver.2.12:

The version was added to title. SIRC-CVS was added to list of abbriviations and acronyms of 6. “Figure 1. Layout of 96 well microplate“ was changed to “Figure 2. Layout of 96 well microplate”. “Figure 2. Addition of cell suspension” was changed to “Figure 3. Addition of cell suspension”

(7)The version from ver.2.12 to ver.2.13:

In 3.7.2., “Futhermore, the data of the well with precipitation or so on at anytime after the mix of the substance and the cell should be reject for unsuitable suspension” was changed to “

Furthermore, the data of the well with precipitation or so on at anytime after the mix of the substance and the cell, especially after 72 hr incubation, should be rejected for unsuitable suspension”.

The address of the author, “2-12-1, Fukuura, Kanazawa-ku, Yokohama-shi, 236-8643 Japan” was changed to “2-2-1, Hayabuchi, Tsuzuki-ku, Yokohama-shi, 224-8558, Japan”.

(8)The revision from ver.2.12 from ver.3.1:

The version and the date were renewed.

In 1., “and United States Environmental Protection Agency (EPA)” was deleted.

In 3.1, “（e.g. When the cell culture starts at passage number of 435 and is maintained by two passages per one week, it should be used within passage number of 458）” was added.

In 3.4., ”The manufacturer and so on of the reagent is shown in the Table 1.” was changed to “The example of the manufacturer and so on of the reagent is shown in the Table 1.”.

In 3.7.1., “The solubility of the substance in the medium should be confirmed in advance.” was changed to “The solubility of each substance in the medium should be confirmed in advance, using the procedure shown in Fig. 1.”.

In figure 1, “Select the solvent on the basis of the examination of the solubility in DMSO or Ethanol.” was changed to “Examine which solvent is more soluble, DMSO or Ethanol，and select appropriate solvent.

In 4.6., “For EPA standard, the test substance is judged as negative (Category 4) when the IC50 is higher than that of triethanolamine, and is judged as positive (Category 1-3) when the IC50 is lower than or equal to that of triethanolamine （see table 3 and 4 of annex 1）.” was deleted.

In table 1., “The manufacturer and so on of the reagent” was changed to “The example of the manufacturer and so on of the reagent” . Also, lot numbers were deleted from remarks.

In annex 1, “The same examination that the in vivo evaluation is performed by EPA classification is shown in table 2. The in vivo results are discriminated between category 4 and others (category 1-3) of the EPA standard. Triethanolamine is classified as category 3. Therefore, when the IC50 of future test substance is higher than that of triethanolamine, it should be evaluated as a non irritant. If the IC50 of the test substance is lower than or equal to that of triethanolamine, it should be evaluated as an irritant.” was deleted.

Table 2 and Table 4 of annex 1 were deleted and table number was moved up.

(8)The revision from ver.3.1 to ver.3.2:

In 4.7. (4), “Treatment of IC50 expressed with inequality sign is performed in the same manner.” was added.

(9)The revision from ver.3.2 to ver.3.3:

In “1.Purpose”, “as a bottom up approach（Scott et al., 2010）” was added.

In “2. The principle of SIRC cytotoxicity test”, ”The relative control was additionally used to obtain the invariable results (Ohno, 2004).“ was added.

In triethanolamine of “3.4. Culture medium and reagent”, “It should be used that of Purity>=98.0%.” was added.

In 3.7.2., overlap with 3.7.1 was removed. “The test substance is solved or uniformly suspended with medium at a concentration of 10,000 μg/mL (1% w/v). It is solved or uniformly suspended using vortex mixer, waterbath and sonicator when it finds necessary. DMSO or ethanol is used for solving or suspending if needed. The concentration of DMSO or ethanol is 10,000μg/mL (1% w/v) in the initial substance solution. The solvent selection is medium, DMSO in medium and ethanol in medium in order. In addition, the concentration of the substance is decreased to 5,000 μg/mL (0.5% w/v) for suspending when it finds necessary. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test.”was deleted. And, “The test substance is solved or uniformly suspended at appropriate concentration by the procedure described in 3.7.1.” was added.

In “4.6.Evaluation”, “on the basis of the in vivo data by Ohno et al. “ was deleted.

In “5.References” and the text, the reference numbers were added. The format of reference was changed. The references of the following paper were added.

Ohno, Y., ATLA, 32, Supplement 1, 643-655, 2004.

Scott, L. et al., Toxicol. In Vitro, 24(1), 1-9 (2010).

The reference of the following paper was deleted.

Ohno, Y. et al., In Vitro Toxicol.,7, 89 (1994)

Table 2 of annex 1 and the related sentences were deleted.

In reference of annex 1, the format of references was changed.

(10)The revision from ver.3.3 to ver.3.4:

In 2., “The application time of 72hr is set with consideration of time (within 72hr) from ocular instillation to maximal eye irritation for general chemicals except acid or alkali, on the basis of in vivo data at the previous research project5).” was added.

In 2., “The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet.” was changed to “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane and stains biological macromolecules.”

In 2., “On the other hand, the disadvantage of this method is to be confined to test substances which are solved or uniformly suspended in the medium.” was added.

In 4.5., “If the multiple concentrations showing 50% cell viability were obtained from one substance, the lowest IC50 should be adopted.” was added.

(11)The revision from ver.3.4 to ver.3.5:

In 2., “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane and stains biological macromolecules” was changed to “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane treated with methanol and stains biological macromolecules.”.

In 3.1., “The cells should be used during 3 months after the start of cultivation” was changed to “The cells should be used within 35 passages from their purchased stock”.

In 3.4., the explanation of Phosphate-Buffered Saline（-）,“Calcium and magnesium are removed from PBS” was added.

4.4 was reinstated from mistaken deletion in ver.3.4.

In (1) of 4.7., “seeded at the concentration of 1x104 cells/well” was changed to “seeded at the concentration of 2x104 cells/well”.

(12)The revision from ver 3.5 to ver.3.6:

In 4.5., “Treatment of IC50 expressed with inequality sign is performed in the same manner.” was added.

The order of 4.6.Evaluation and 4.7. Quality control was changed to that of 4.6. Quality control and 4.7. Evaluation.

In (4) of 4.6., “The treatment to IC50 expressed with equality sign are only used at the calculation for quality control.” was added.

(13) The revision from ver.3.6 to ver.3.7:

In 4.7, “ of GHS standard” was revised to “of UN GHS classification system”.

In 3.7.1., “and has no test.” was revised to “and is not judged as testable.”

(14) The revision form ver.3.7 to ver 3.8

In 3.8, many minor revisions for readability were made by a native-English speaker. (see Word file)

Table 1. Typical reagents and their manufacturers

Reagent or Medium	Manufacturer	Catalog number		Notes
MEM (Minimum Essential Medium)	GIBCO	Code No.	11095	
Fetal Bovine Serum	GIBCO	REF No.	26140-079	
Penicillin-Streptomycin-Amphotericin (100x)	GIBCO/BRL	REF No.	15240-062	
Phosphate-Buffered Saline (modified PBS)	Nissui	Code No.	05913	
0.25% (w/v) Trypsin (1mmol/L EDTA·4Na)	Wako	Cat No.	209-16941	
Dimethyl sulfoxide (DMSO)	Kanto	Cat No.	2950-1B	
Ethanol	Wako	Cat No.	057-00456	
Crystal violet	Wako	Cat No.	031-04852	
Methanol	Wako	Cat No.	131-01826	
Sodium Dodecyl Sulfate	Wako	Cat No.	191-07145	
Triethanolamine	Kanto	Cat No.	40268-00	

Products of the same specification from the specified manufacturer may be used for Minimum Essential Medium, Fetal Bovine Serum, Penicillin-Streptomycin-Amphotericin, Sodium Dodecyl Sulfate, and Triethanolamine. Equivalent products from other manufacturers are acceptable for other reagents.

Nissui: Nissui Pharmaceutical Co., Ltd

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: Kanto Chemical, Co., Inc.

Figure 2: Layout of the 96-well microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
C	PBS	NC	S1	S2	S3	S4	S5	S6	S7	S8	NC	PBS
D	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
E	PBS	NC	R1	R2	R3	R4	R5	R6	R7	R8	NC	PBS
F	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
G	PBS	NC	P1	P2	P3	P4	P5	P6	P7	P8	NC	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

PBS: 200 µL of modified PBS

NC: The Medium, a DMSO-Medium solution at a concentration of 10,000 µg/mL, or an ethanol-Medium solution at a concentration of 10,000 µg/mL

S: Eight-well, two-fold serial dilution of the test chemical (100 µL per well)

R: Eight-well, two-fold serial dilution of the relative control (100 µL per well)

P: Eight-well, two-fold serial dilution of the positive control (100 µL per well).

Serial dilution of the test chemical is made using the Medium, a 10,000-µg/mL concentration of DMSO-Medium solution, or a 10,000-µg/mL concentration of ethanol-Medium solution. Serial dilution of the positive and relative controls are made using the Medium.

Figure 3. Addition of cell suspension

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Cell suspension (100 µL)

Annex 1 Rational for using triethanolamine as a reference control

Triethanolamine was selected as a relative control substance of the SIRC cytotoxicity test for distinguishing between ocular non-irritants (NI) and irritants (I) per the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). It is one of the substances used in the previous validation study that was performed by a Research Grant for Health Sciences, MHW and Japanese Cosmetic Industry Association, and reported by Ohno et al.[1] and Tani et al.[2] Also, it is readily available commercially, is soluble in the Medium, and has been studied extensively, with plenty of useful cytotoxicity and in vivo data available for evaluating eye irritation potency.

In selecting the relative control substance, accuracy and other characteristics for distinguishing between NI and I were checked for every available substance from previous validations. As a result of this comparative study, triethanolamine was selected as a relative control substance because of a relatively low instance of false negatives, a high accuracy except for substances which for which cytotoxicity could not be quantified (10000 < etc.), and clarity of categorization (for example, alcohol) for substances yielding false negative, as shown in table 1. Identification of substances likely to result in false negatives is important from the perspective of minimizing the risk of eye irritation.

References

1) Ohno, Y. et al., Toxicology in Vitro 13, 73-98 (1999)

2) Tani, N. et al., Toxicology in Vitro 13, 175-187(1999)

Table 1 The correlative evaluation on the basis of the previous validation study data for selecting relative control substance
- GHS classification -

Key: TN: true negative, FN: false negative, TP: true positive, FP: false positive

Substances	GHS classification based on In vivo testing[NB1]	SIRC cytotoxicity: IC_{50} (μg/mL)[NB2]	Ranking	TN	FN	TP	FP	Acc (%)
Polyethylene glycol 400	NI	35300 <	35300	1	0	27	6	82
Silicic anhydride	NI	14800 <	14800	2	0	26	6	82
Glycerin	NI	11600	11600	3	0	25	6	82
Isotonic sodium chloride solution	NI	10000 <	10000	4	1	24	5	82
Ethanol	1 or 2A	10000 <	10000	4	1	24	5	82
Isopropyl myristate	NI	9330 <	9330	5	1	24	4	85
Butanol*	1 or 2A	8880 <	8880	5	2	23	4	82
Triethanolamine	NI	2090	2090	6	2	23	3	85
Lactic acid	1	1230	1230	6	3	22	3	82
Benzyl alcohol	1 or 2A	1190	1190	6	4	21	3	79
Polyoxyethylene sorbitan monooleate (20E.O.)	NI	963	963	7	4	21	2	82
Sodium salicylate	1 or 2A	952	952	7	5	20	2	79
Glycolic acid*	1 or 2A	868	868	7	6	19	2	76
Acetic acid*	1 or 2A	721	721	7	7	18	2	74
Diisopropanolamine*	1, 2A, or2B	699	699	7	8	17	2	71
2-Ethylhexyl p-dimethylamino benzoate	NI	474	474	8	8	17	1	74
Calcium thioglycolate	1	392	392	8	9	16	1	71
Acid red 92	1 or 2A	297	297	8	10	15	1	68

Sucrose fatty acid ester	1 or 2A	286	286	8	11	14	1	65
m-Phenylenediamine	1 or 2A	218	218	8	12	13	1	62
Methyl p-hydroxybenzoate	NI	207	207	9	12	13	0	65
Di (2-ethylhexyl) sodium sulfosuccinate*	1 or 2A	181	181	9	13	12	0	62
Sodium lauryl sulfate*	1 or 2A	168	168	9	14	11	0	59
Sodium hydrogenated tallow L-glutamate*	1 or 2A	140	140	9	15	10	0	56
Potassium laurate*	1 or 2A	120 (Data from 4 labs)	120	9	16	9	0	53
Chlorhexidine gluconate (20% solution)*	1 or 2A	67.6	67.6	9	17	8	0	50
Polyoxyethylene octylphenylether (10 E.O.)*	1 or 2A	38.4	38.4	9	18	7	0	47
Distearyldimethylammonium chloride	1	37.8	37.8	9	19	6	0	44
Benzalkonium chloride*	1 or 2A	19.0	19	9	20	5	0	41
Domiphen bromide*	1 or 2A	12.1	12.1	9	21	4	0	38
Monoethanolamine*	1 or 2A	9.62	9.62	9	22	3	0	35
Cetyltrimethylammonium bromide*	1 or 2A	2.59 (Data from 4 labs)	2.59	9	23	2	0	32
Cetylpyridinium chloride*	1	1.67	1.67	9	24	1	0	29
Stearyltrimethylammonium chloride*	1	1.58	1.58	9	25	0	0	26

NB1 The Draize eye test results do not always discriminate between GHS Category 1 and Category 2 when data was recorded on day 21. Data was recorded on day 14.

NB2 Data for the SIRC cytotoxicity test are the mean IC_{50} (ug/mL) from five or more laboratories, except for one part.

* The in vivo results for “as is” applications were predicted from data taken with 10% concentrations.

Annex 2 The basis for the set IC50 range of triethanolamine as a reference control

The IC_{50} range of triethanolamine, 1,000–2,500 µg/mL, is based on the mean +2 standard deviations from the data (n=144).

Data sheet format

1) Raw data
2) Run1
3) Run2
4) Run3
5) Summary

SIRC validation study datasheet

SIRC-CVS:TEA for protocol ver. 3.3	Laboratory **ABC.inc**	Study director Mai Endo

Test substance	AAA2015
Code of Test Substance	03
Relative control substance	Triethanolamine
Positive control substance	Sodium Dodecyl Sulfate
Negative control substance	DMSO

Total nums of runs 2	**Run1** 1	**Run2** 2	**Run3**
Cell seeding day(yyyy/mm/dd)	2013/05/27	2015/05/31	
Name of experimenter	Takashi Omori	Yumi Takaka	
Passage No. at the time of purchase	1	1	
Passage No. after thawing	4	4	
Passage No. at the time of assay	6	7	
Test substance conc.(%)	1.000	1.000	
Relative control substance conc.(%)	1.000	1.000	
Positive control substance conc.(%)	0.100	0.100	

Run1 1

0.056	0.070	0.094	0.109	0.073	0.086	0.086	0.205	0.050	0.060	0.069	0.127
0.109	0.551				0.543	0.511	0.422	0.586	0.501	0.592	0.128
0.074	0.594				0.439	0.556	0.562	0.452	0.522	0.547	0.143
0.072	0.555	0.066	0.080	0.336	0.526	0.584	0.489	0.448	0.568	0.527	0.072
0.095	0.602	0.064	0.092	0.356	0.414	0.435	0.437	0.422	0.594	0.526	0.092
0.072	0.514	0.059	0.100	0.076	0.050	0.051	0.114	0.429	0.530	0.512	0.116
0.066	0.509	0.066	0.071	0.110	0.063	0.063	0.105	0.561	0.536	0.524	0.116
0.068	0.124	0.092	0.098	0.110	0.056	0.092	0.068	0.136	0.116	0.099	0.052
Test substance			Rlative control substance			Positive control substance			Negative control substance		

Run2 2

0.093	0.084	0.09	0.066	0.053	0.056	0.054	0.061	0.062	0.051	0.134	0.092
0.056	0.533	0.539	0.522	0.588	0.537	0.536	0.593	0.575	0.582	0.453	0.065
0.057	0.526	0.461	0.451	0.578	0.556	0.591	0.582	0.415	0.402	0.481	0.064
0.079	0.581	0.065	0.096	0.461	0.534	0.546	0.525	0.51	0.529	0.498	0.066
0.071	0.468	0.078	0.0621	0.222	0.683	0.548	0.508	0.597	0.505	0.539	0.056
0.067	0.579	0.054	0.056	0.055	0.060	0.064	0.255	0.356	0.653	0.513	0.068
0.091	0.585	0.044	0.043	0.051	0.072	0.063	0.153	0.347	0.579	0.418	0.067
0.078	0.11	0.076	0.086	0.066	0.056	0.108	0.052	0.122	0.068	0.099	0.072
Test substance			Rlative control substance			Positive control substance			Negative control substance		

Run3

0.055	0.054	0.056	0.058	0.059	0.055	0.054	0.053	0.057	0.062	0.053	0.052
0.051	1.065	0.341	0.623	0.427	0.318	0.635	0.778	0.878	0.901	1.129	0.051
0.054	1.088	0.257	0.584	0.466	0.343	0.638	0.797	0.837	0.808	1.154	0.052
0.05	0.998	0.061	0.08	0.598	0.988	1.091	1.129	1.1	1.115	1.164	0.051
0.052	1.015	0.068	0.078	0.715	0.872	1.029	1.056	1.079	1.176	1.065	0.052
0.055	1.047	0.134	0.056	0.052	1.169	1.152	1.183	1.182	1.152	1.141	0.053
0.055	1.032	0.141	0.058	0.052	1.281	1.174	1.184	1.146	1.092	1.116	0.054
0.055	0.056	0.053	0.054	0.054	0.054	0.053	0.054	0.052	0.05	0.052	0.054
Test substance			Rlative control substance			Positive control substance			Negative control substance		

Comment:

aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Sub_exp.1	1										
0.056	0.07	0.094	0.109	0.073	0.086	0.086	0.205	0.05	0.06	0.069	0.127
0.109	0.551	0	0	0	0.543	0.511	0.422	0.586	0.501	0.592	0.128
0.074	0.594	0	0	0	0.439	0.556	0.562	0.452	0.522	0.547	0.143
0.072	0.555	0.066	0.08	0.336	0.526	0.584	0.489	0.448	0.568	0.527	0.072
0.095	0.602	0.064	0.092	0.356	0.414	0.435	0.437	0.422	0.594	0.526	0.092
0.072	0.514	0.059	0.1	0.076	0.05	0.051	0.114	0.429	0.53	0.512	0.116
0.066	0.509	0.066	0.071	0.11	0.063	0.063	0.105	0.561	0.536	0.524	0.116
0.068	0.124	0.092	0.098	0.11	0.056	0.092	0.068	0.136	0.116	0.099	0.052

(a) Blank	0.093
(b) Mean OD of negative controls (left side)	0.554
(c) Mean OD of negative controls (right side)	0.538
(d) Mean OD of both negative controls	0.546
(e) Negative control (l) - Blank [(b) - (a)]	0.461
(f) Negative control (r) - Blank [(c) - (a)]	0.445
(g) Standard deviation of OD for negative controls	0.034
(h) 15% mean of negative controls [(d) * 0.15]	0.082
(i) Mean OD of negative controls+15% [(d) + (h)]	0.628
(j) Mean OD of negative controls -15% [(d) - (h)]	0.464

Test substance AAA2015

Prepared solution (%) 1.0 | Common ratio 2 | Final maximum conc. (μg/mL) 5000

Conc.	Data1	Data2	Mean	Cell Viability (%)
5000	0	0	0	-20.5
2500	0	0	0	-20.5
1250	0	0	0	-20.5
625	0.543	0.439	0.491	87.8
312.5	0.511	0.556	0.534	97.2
156.3	0.422	0.562	0.492	88.1
78.1	0.586	0.452	0.519	94.0
39.1	0.501	0.522	0.512	92.4

Conc.	Cell Viability (%)
Low 625	87.8
High 1250	-20.5

IC50 Not estimated μg/mL

Data1	Data2	Mean
Not est	Not est	Not estimated

Relative contorol substance

Prepared solution (%) 1.0 | Common ratio 2 | Final maximum conc. (μg/mL) 5000

Conc.	Data1	Data2	Mean	Cell Viability (%)
5000	0.066	0.064	0.065	-6.2
2500	0.08	0.092	0.086	-1.6
1250	0.336	0.356	0.346	55.8
625	0.526	0.414	0.47	83.2
312.5	0.584	0.435	0.51	91.9
156.3	0.489	0.437	0.463	81.7
78.1	0.448	0.422	0.435	75.5
39.1	0.568	0.594	0.581	107.7

Conc.	Cell Viability (%)
Low 1250.0	55.8
High 2500.0	-1.6

IC50 1,340.7 μg/mL

Positive contorol substance

Prepared solution (%) 0.1 | Common ratio 2 | Final maximum conc. (μg/mL) 500

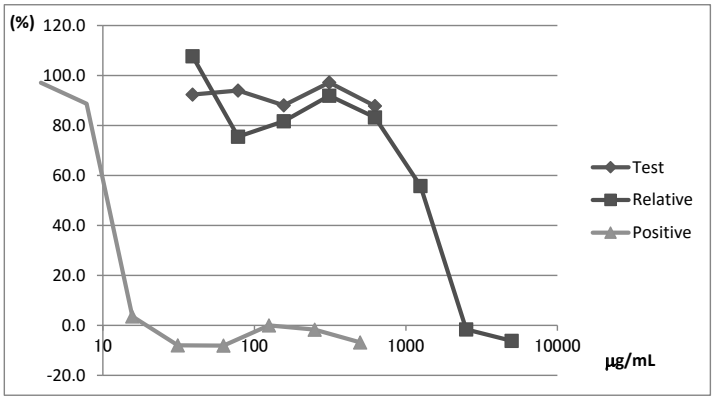
Conc.	Data1	Data2	Mean	Cell Viability (%)
500	0.059	0.066	0.063	-6.8
250	0.1	0.071	0.086	-1.7
125	0.076	0.11	0.093	0.0
62.5	0.05	0.063	0.057	-8.1
31.25	0.051	0.063	0.057	-8.0
15.63	0.114	0.105	0.11	3.6
7.8	0.429	0.561	0.495	88.7
3.9	0.53	0.536	0.533	97.1

Conc.	Cell Viability (%)
Low 7.8	88.7
High 15.6	3.6

IC50 10.7 μg/mL

QC check		Judgement
(1)	0.546	OK
(2)	10.7	Retest
(3)	1,340.7	OK
(4)M	Not estin	Retest
(4)1	Not estin	(Retest)
(4)2	Not estin	(Retest)
(5)M	0.546	OK
(5)L	0.554	(OK)
(5)R	0.538	(OK)

Conc.	Test	Relativ	Positive
5000	#N/A	#N/A	#N/A
2500	#N/A	#N/A	#N/A
1250	#N/A	#N/A	#N/A
625	87.8	#N/A	#N/A
312.5	97.2	#N/A	#N/A
156.3	88.1	#N/A	#N/A
78.1	94.0	#N/A	#N/A
39.1	92.4	#N/A	#N/A
500.0	#N/A	#N/A	-6.8
250.0	#N/A	#N/A	-1.7
125.0	#N/A	#N/A	0.0
62.5	#N/A	#N/A	-8.1
31.3	#N/A	#N/A	-8.0
15.6	#N/A	#N/A	3.6
7.8	#N/A	#N/A	88.7
3.9	#N/A	#N/A	97.1
5000	#N/A	-6.2	#N/A
2500	#N/A	-1.6	#N/A
1250	#N/A	55.8	#N/A
625	#N/A	83.2	#N/A
313	#N/A	91.9	#N/A
156	#N/A	81.7	#N/A
78	#N/A	75.5	#N/A
39	#N/A	107.7	#N/A



Sub_exp.2	2										
0.093	0.084	0.09	0.066	0.053	0.056	0.054	0.061	0.062	0.051	0.134	0.092
0.056	0.533	0.539	0.522	0.588	0.537	0.536	0.593	0.575	0.582	0.453	0.065
0.057	0.526	0.461	0.451	0.578	0.556	0.591	0.582	0.415	0.402	0.481	0.064
0.079	0.581	0.065	0.096	0.461	0.534	0.546	0.525	0.51	0.529	0.498	0.066
0.071	0.468	0.078	0.062	0.222	0.683	0.548	0.508	0.597	0.505	0.539	0.056
0.067	0.579	0.054	0.056	0.055	0.06	0.064	0.255	0.356	0.653	0.513	0.068
0.091	0.585	0.044	0.043	0.051	0.072	0.063	0.153	0.347	0.579	0.418	0.067
0.078	0.11	0.076	0.086	0.066	0.056	0.108	0.052	0.122	0.068	0.099	0.072

(a) Blank	0.075
(b) Mean OD of negative controls (left side)	0.545
(c) Mean OD of negative controls (right side)	0.484
(d) Mean OD of both negative controls	0.515
(e) Negative control (l) - Blank [(b) - (a)]	0.47
(f) Negative control (r) - Blank [(c) - (a)]	0.409
(g) Standard deviation of OD for negative controls	0.053
(h) 15% mean of negative controls [(d) * 0.15]	0.077
(i) Mean OD of negative controls+15% [(d) + (h)]	0.592
(j) Mean OD of negative controls -15% [(d) - (h)]	0.437

Test substance AAA2015

Prepared solution (%) 1.0 | Common ratio 2 | Final maximum conc. (μg/mL) 5000

Conc.	Data1	Data2	Mean	Cell Viability (%)
5000	0.539	0.461	0.5	96.7
2500	0.522	0.451	0.487	93.6
1250	0.588	0.578	0.583	115.6
625	0.537	0.556	0.547	107.3
312.5	0.536	0.591	0.563	111.1
156.3	0.593	0.582	0.588	116.6
78.1	0.575	0.415	0.495	95.6
39.1	0.582	0.402	0.492	94.9

	Conc.	Cell Viability (%)
Low	0.0	#N/A
High	0.0	#N/A

IC50 >5000 μg/mL

Data1	Data2	Mean
5,000	5,000	5,000

Relative contorol substance

Prepared solution (%) 1.0 | Common ratio 2 | Final maximum conc. (μg/mL) 5000

Conc.	Data1	Data2	Mean	Cell Viability (%)
5000	0.065	0.078	0.072	-0.8
2500	0.096	0.062	0.079	0.9
1250	0.461	0.222	0.342	60.6
625	0.534	0.683	0.609	121.4
312.5	0.546	0.548	0.547	107.4
156.3	0.525	0.508	0.517	100.5
78.1	0.51	0.597	0.554	108.9
39.1	0.529	0.505	0.517	100.6

	Conc.	Cell Viability (%)
Low	1250.0	60.6
High	2500.0	0.9

IC50 1,413.7 μg/mL

Positive contorol substance

Prepared solution (%) 0.1 | Common ratio 2 | Final maximum conc. (μg/mL) 500

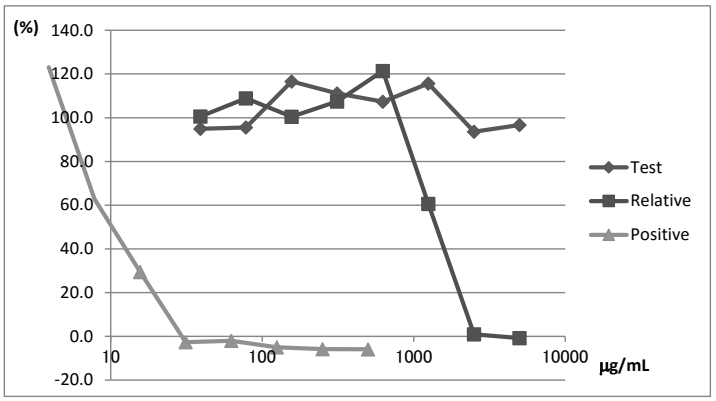
Conc.	Data1	Data2	Mean	Cell Viability (%)
500	0.054	0.044	0.049	-5.9
250	0.056	0.043	0.05	-5.8
125	0.055	0.051	0.053	-5.0
62.5	0.06	0.072	0.066	-2.0
31.25	0.064	0.063	0.064	-2.6
15.63	0.255	0.153	0.204	29.4
7.8	0.356	0.347	0.352	62.9
3.9	0.653	0.579	0.616	123.1

	Conc.	Cell Viability (%)
Low	7.8	62.9
High	15.6	29.4

IC50 10.2 μg/mL

QC check		Judgement
(1)	0.515	OK
(2)	10.2	Retest
(3)	1,413.7	OK
(4)M	5,000.0	OK
(4)1	5,000.0	(OK)
(4)2	5,000.0	(OK)
(5)M	0.515	OK
(5)L	0.545	(OK)
(5)R	0.484	(OK)

Conc.	Test	Relativ	Positive
5000	96.7	#N/A	#N/A
2500	93.6	#N/A	#N/A
1250	115.6	#N/A	#N/A
625	107.3	#N/A	#N/A
312.5	111.1	#N/A	#N/A
156.3	116.6	#N/A	#N/A
78.1	95.6	#N/A	#N/A
39.1	94.9	#N/A	#N/A
500.0	#N/A	#N/A	-5.9
250.0	#N/A	#N/A	-5.8
125.0	#N/A	#N/A	-5.0
62.5	#N/A	#N/A	-2.0
31.3	#N/A	#N/A	-2.6
15.6	#N/A	#N/A	29.4
7.8	#N/A	#N/A	62.9
3.9	#N/A	#N/A	123.1
5000	#N/A	-0.8	#N/A
2500	#N/A	0.9	#N/A
1250	#N/A	60.6	#N/A
625	#N/A	121.4	#N/A
313	#N/A	107.4	#N/A
156	#N/A	100.5	#N/A
78	#N/A	108.9	#N/A
39	#N/A	100.6	#N/A



Sub_exp.3	0									
0.055	0.054	0.056	0.058	0.059	0.055	0.054	0.053	0.057	0.062	0.053	0.052
0.051	1.065	0.341	0.623	0.427	0.318	0.635	0.778	0.878	0.901	1.129	0.051
0.054	1.088	0.257	0.584	0.466	0.343	0.638	0.797	0.837	0.808	1.154	0.052
0.05	0.998	0.061	0.08	0.598	0.988	1.091	1.129	1.1	1.115	1.164	0.051
0.052	1.015	0.068	0.078	0.715	0.872	1.029	1.056	1.079	1.176	1.065	0.052
0.055	1.047	0.134	0.056	0.052	1.169	1.152	1.183	1.182	1.152	1.141	0.053
0.055	1.032	0.141	0.058	0.052	1.281	1.174	1.184	1.146	1.092	1.116	0.054
0.055	0.056	0.053	0.054	0.054	0.054	0.053	0.054	0.052	0.05	0.052	0.054

(a) Blank	0.054
(b) Mean OD of negative controls (left side)	1.041
(c) Mean OD of negative controls (right side)	1.128
(d) Mean OD of both negative controls	1.085
(e) Negative control (l) - Blank [(b) - (a)]	0.987
(f) Negative control (r) - Blank [(c) - (a)]	1.074
(g) Standard deviation of OD for negative controls	0.056
(h) 15% mean of negative controls [(d) * 0.15]	0.163
(i) Mean OD of negative controls+15% [(d) + (h)]	1.247
(j) Mean OD of negative controls -15% [(d) - (h)]	0.922

Test substance AAA2015

Prepared solution (%) 0.0 | Common ratio 2 | Final maximum conc. (μg/mL) 0

Conc.	Data1	Data2	Mean	Cell Viability (%)
0	0.341	0.257	0.299	23.8
0	0.623	0.584	0.604	53.3
0	0.427	0.466	0.447	38.1
0	0.318	0.343	0.331	26.8
0	0.635	0.638	0.637	56.5
0	0.778	0.797	0.788	71.2
0.0	0.878	0.837	0.858	78.0
0.0	0.901	0.808	0.855	77.7

	Conc.	Cell Viability (%)
Low	0.0	23.8
High	0.0	23.8
IC50	#NUM!	μg/mL

Data1	Data2	Mean
#NUM!	#NUM!	#NUM!

Relative contorol substance

Prepared solution (%) 0.0 | Common ratio 2 | Final maximum conc. (μg/mL) 0

Conc.	Data1	Data2	Mean	Cell Viability (%)
0	0.061	0.068	0.065	1.0
0	0.08	0.078	0.079	2.4
0	0.598	0.715	0.657	58.5
0	0.988	0.872	0.93	85.0
0	1.091	1.029	1.06	97.6
0	1.129	1.056	1.093	100.8
0.0	1.1	1.079	1.09	100.5
0.0	1.115	1.176	1.146	105.9

	Conc.	Cell Viability (%)
Low	0.0	1.0
High	0.0	1.0
IC50	#NUM!	μg/mL

Positive contorol substance

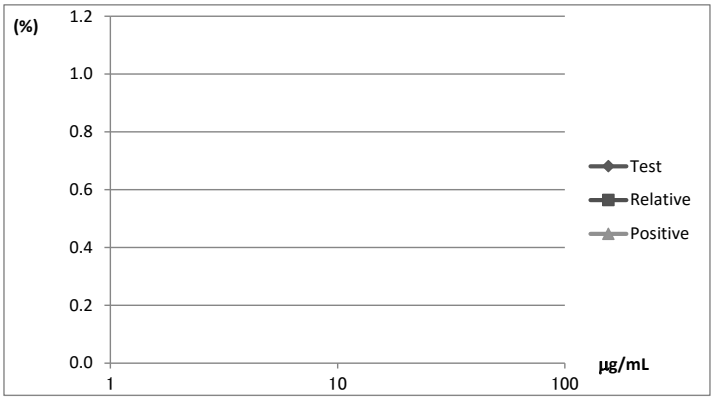
Prepared solution (%) 0.0 | Common ratio 2 | Final maximum conc. (μg/mL) 0

Conc.	Data1	Data2	Mean	Cell Viability (%)
0	0.134	0.141	0.138	8.1
0	0.056	0.058	0.057	0.3
0	0.052	0.052	0.052	-0.2
0	1.169	1.281	1.225	113.6
0	1.152	1.174	1.163	107.6
0	1.183	1.184	1.184	109.6
0.0	1.182	1.146	1.164	107.7
0.0	1.152	1.092	1.122	103.6

	Conc.	Cell Viability (%)
Low	0.0	8.1
High	0.0	8.1
IC50	#NUM!	μg/mL

QC check		Judgement
(1)	1.085	OK
(2)	#####	#NUM!
(3)	#NUM!	#NUM!
(4)M	#NUM!	#NUM!
(4)1	#NUM!	#NUM!
(4)2	#NUM!	#NUM!
(5)M	1.085	OK
(5)L	1.041	(OK)
(5)R	1.128	(OK)

Conc.	Test	Relativ	Positive
0	23.8	#N/A	#N/A
0	53.3	#N/A	#N/A
0	38.1	#N/A	#N/A
0	26.8	#N/A	#N/A
0	56.5	#N/A	#N/A
0	71.2	#N/A	#N/A
0.0	78.0	#N/A	#N/A
0.0	77.7	#N/A	#N/A
0.0	#N/A	#N/A	8.1
0.0	#N/A	#N/A	0.3
0.0	#N/A	#N/A	-0.2
0.0	#N/A	#N/A	113.6
0.0	#N/A	#N/A	107.6
0.0	#N/A	#N/A	109.6
0.0	#N/A	#N/A	107.7
0.0	#N/A	#N/A	103.6
0	#N/A	1.0	#N/A
0	#N/A	2.4	#N/A
0	#N/A	58.5	#N/A
0	#N/A	85.0	#N/A
0	#N/A	97.6	#N/A
0	#N/A	100.8	#N/A
0	#N/A	100.5	#N/A
0	#N/A	105.9	#N/A



SIRC-CVS:TEA for protocol ver. 3.3

Laboratory	ABC.inc
Test substance	AAA2015
Code of test substance	03
Relative control substance	Triethanolamine
Positive control substance	Sodium Dodecyl Sulfate
Negative control substance	DMSO

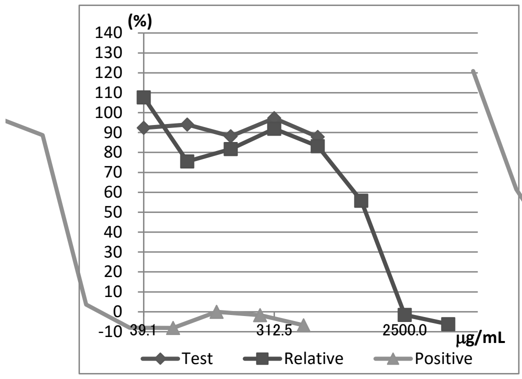
	IC50 (μg/mL)			Judgement	Final judgement
	Test substance	Relative control substance	Positive control substance		
Run1	Not estimated	1340.7	10.7	Not decided	
Run2	>5000	1413.7	10.2	Negative	
Run3					

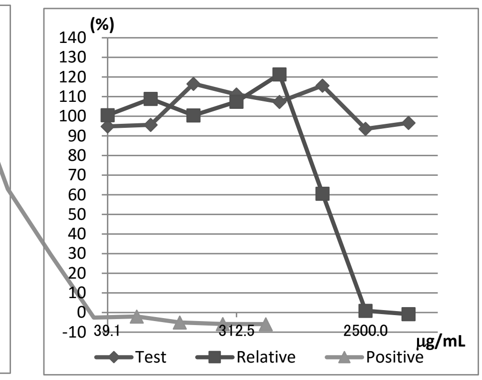
Total Quality Check for Cells and Assay

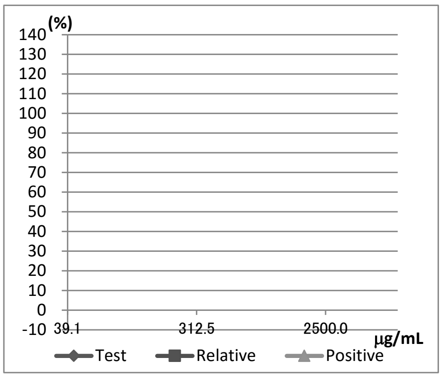
	(1)	(2)	(3)	(4)		(5)	
Run1	OK 0.546	Retest 10.7	OK 1340.7	Retest	Retest	OK 1.5%	-1.5%
Run2	OK 0.515	Retest 10.2	OK 1413.7	OK 0.0%	0.0%	OK 6.0%	-6.0%
Run3	OK 0.000					OK -4.0%	4.0%
Total	**OK**	**Retest**	**OK**	**Retest**		**OK**	

(6)	
10.2 μg/mL μg/mL	1.0
OK	

Some problems are fineded. Retest may be needed.







Run1

Cell seeding day(yyyy/mm/dd)	
2013/05/27	
Test substance	
Test substance conc. (%)	1.0
Common ratio	2
Final maximum conc. (μg/mL)	5000
Relative contorol substance	
Relative contorol substance conc. (%)	1.0
Common ratio	2
Final maximum conc. (μg/mL)	5000
Positive contorol substance	
Positive contorol substance conc. (%)	0.1
Common ratio	2
Final maximum conc. (μg/mL)	500

Run2

Cell seeding day(yyyy/mm/dd)	
2015/05/31	
Test substance	
Test substance conc. (%)	1.0
Common ratio	2
Final maximum conc. (μg/mL)	5000
Relative contorol substance	
Relative contorol substance conc. (%)	1.0
Common ratio	2
Final maximum conc. (μg/mL)	5000
Positive contorol substance	
Positive contorol substance conc. (%)	0.1
Common ratio	2
Final maximum conc. (μg/mL)	500

Run3

Cell seeding day(yyyy/mm/dd)	
0:00:00	
Test substance	
Test substance conc. (%)	0.0
Common ratio	2
Final maximum conc. (μg/mL)	0
Relative contorol substance	
Relative contorol substance conc. (%)	0.0
Common ratio	2
Final maximum conc. (μg/mL)	0
Positive contorol substance	
Positive contorol substance conc. (%)	0.0
Common ratio	2
Final maximum conc. (μg/mL)	0

Comment:

aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaab

Rational for the quality control acceptance ranges

(1) The acceptance range of the absolute OD from the negative control, >0.4 was obtained from the previous Shiseido's data. The results using >0.4 showed that the SIRC-CVS:TEA test was appropriate as an alternative method for eye irritation (JaCVAM, 2011).

Table 1. Mean OD of negative control in the Shiseido's data

No.	Mean OD of the negative control (12wells)
1	0.725
2	0.648
3	0.906
4	1.085
5	0.739
6	0.582
7	0.784
8	0.731
9	0.660
10	0.731
11	0.703
12	0.648
13	0.668
14	0.670
15	0.879
16	0.965
17	0.854
18	0.976
19	0.749
20	0.961
21	0.681
22	0.617
23	0.889
24	0.648
25	1.023
26	0.987
27	0.872
28	0.822
29	0.990
30	0.658
31	0.684
32	0.578
33	0.746
34	0.654
35	0.653
36	0.649
37	0.675
38	0.933
39	1.110
40	0.958
41	0.914
42	0.883
43	0.718
44	0.923
45	0.586
46	0.870
47	0.707
48	0.862
49	0.747
50	0.714
51	0.712
52	0.728
53	0.975
54	0.748
55	0.744
56	0.857
57	0.667
58	0.736
59	0.676
60	0.806
61	0.658
62	0.813
63	0.811
64	0.630

65	0.658
66	0.646
67	0.624
68	0.583
69	0.742
70	0.686
71	0.684
72	0.780
73	0.670
74	0.874
75	0.809
76	0.798
77	0.782
78	0.725
79	0.692
80	0.716
81	0.777
82	0.811
83	0.565
84	0.775
85	0.706
86	0.723
87	0.643
88	0.689
89	0.774
90	0.703
91	0.689
92	0.701
93	0.719
94	0.731
95	0.785
96	0.821
97	0.812
98	0.695
99	0.669
100	0.736
101	0.695
102	0.739
103	0.684
104	0.715
105	0.662
106	0.705
107	0.704
108	0.566
109	0.717
110	0.668
111	0.689
112	0.749
113	0.799
114	0.634
115	0.784
116	0.668
117	0.711
118	0.691
119	0.796
120	0.630
121	0.732
122	0.735
123	0.630
124	0.699
125	0.805
126	0.719
127	0.751
128	0.645
129	0.776
130	0.719
131	0.748
132	0.728
133	0.766
134	0.781
135	0.727
136	0.662
137	0.643
138	0.647
139	0.782
140	0.617
Average	0.745
Standard deviation	0.106

Table 2. Rejected mean OD of negative control in the Shiseido's data

No.	Mean OD of the negative control (12 wells)	The reason of rejection
1	0.351	The substance, 2,4-Difluoronitrobenzene affected the negative control wells.
2	0.320	The substance, 2,4-Difluoronitrobenzene affected the negative control wells.
3	0.378	The substance, 2,4-Difluoronitrobenzene affected the negative control wells.

(2) The acceptance range for IC50 of SDS, 77.7-258.7μg/mL was obtained from mean±3SD in the previous validation study data of MHW (Tani et al, 1999). That was confirmed by the previous Shiseido's data as shown in table 3 and 4.

Table 3. IC50 of SDS in the Shiseido's data

No	IC50 (μg/mL) of SDS
1	102.2
2	90.8
3	87.2
4	89.1
5	91.1
6	91.8
7	91.0
8	93.2
9	98.0
10	104.4
11	97.0
12	90.5
13	95.1
14	90.5
15	92.5
16	103.1
17	93.1
18	101.7
19	92.4
20	90.6
21	96.5
22	95.1
23	89.6
24	96.1
25	89.4
26	91.4
27	86.0
28	92.4
29	94.8
30	96.2
31	96.7
32	90.3
33	89.7
34	90.7
35	95.1
36	90.8
37	100.8
38	98.8
39	88.1
40	101.7
41	91.5
42	108.0
43	91.3
44	103.2
45	0.9

46	92.7
47	91.4
48	100.2
49	91.5
50	97.2
51	89.1
52	103.5
53	90.6
54	113.7
55	89.0
56	107.2
57	91.0
58	93.5
59	96.4
60	85.9
61	93.0
62	91.8
63	90.4
64	91.2
65	92.8
66	92.1
67	95.3
68	96.9
69	87.1
70	91.9
71	90.4
72	96.0
73	113.6
74	86.3
75	92.4
76	93.4
77	91.1
78	95.2
79	94.3
80	91.8
81	88.2
82	95.5
83	93.9
84	93.3
85	92.9
86	96.0
87	91.7
88	94.0
89	91.1
90	90.7
91	92.5
92	89.9
93	90.1
94	90.8
95	89.7
96	94.4
97	94.3
98	96.6
99	91.0
100	90.0
101	92.9
102	92.0
103	92.7
104	91.5
105	93.6
106	91.5
107	109.2
108	90.7
109	91.3
110	92.2
111	92.2
112	89.1
113	93.5
114	93.0
115	87.2
116	98.7
117	101.6
118	93.6
119	89.7

120	93.6
121	91.5
122	96.5
123	93.8
124	100.6
125	91.8
126	88.1
127	91.3
128	93.7
129	93.8
130	93.5
131	89.1
132	92.1
133	96.6
134	95.2
135	92.8
136	91.0
137	94.4
138	92.6
139	91.4
140	91.9
Average	93.1
Standard deviation	9.2

Table 4. Rejected IC50 of SDS in the Shiseido's data

No.	IC50 (μg/mL) of SDS	The reason of rejection
1	63.2	Deviation of data of SDS
2	37.0	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization
3	17.1	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.

(3) The acceptance range of IC50 of TEA, 1000-2500 μg/mL was obtained from the previous validation study data of MHW (Tani et al, 1999), the Shiseido's data and Phase I data of this validation.
The Shiseido's data was obtained using the acceptable range of 1000-5000 μg/mL on the basis of the validation study of MHW. The range was appropriate as shown in table 5 and 6. After the phase I study, the range was changed from 1000-5000 μg/mL to 1000-2500 μg/mL on the basis of the results as shown in table 7.

Table 5. IC50 of TEA in the Shiseido's data

No	IC50(μg/mL) of TEA
1	2164.2
2	1620.4
3	2000.5
4	1808.3
5	1675.7
6	1401.5
7	1757.2
8	1604.0
9	1044.4
10	1656.6
11	1687.6
12	1768.5
13	1940.1
14	1674.5
15	1709.2
16	1704.8
17	2228.9
18	1694.8
19	1558.9
20	1386.6
21	1868.2
22	1663.4
23	1669.9
24	1576.9
25	1932.9
26	1461.8
27	1945.1
28	1599.5
29	1424.0
30	1251.7
31	1666.2
32	1347.1
33	1012.3
34	1595.4
35	1526.8
36	1690.2
37	1501.7
38	1448.5
39	1763.3
40	1206.8
41	1773.9
42	1808.9
43	1614.6
44	1452.7
45	1435.9
46	1295.2

47	1500.2
48	1429.1
49	1525.0
50	1683.3
51	1820.5
52	1451.3
53	1349.7
54	1782.0
55	1786.8
56	1757.9
57	1664.1
58	1118.3
59	1338.9
60	1452.3
61	2145.3
62	1669.1
63	1861.3
64	1330.7
65	1770.2
66	1488.4
67	1611.9
68	1534.3
69	1550.9
70	2290.9
71	1408.8
72	1437.1
73	1260.3
74	1441.2
75	1267.2
76	1374.6
77	1695.5
78	1354.3
79	1495.1
80	1486.9
81	1339.4
82	1303.1
83	1218.0
84	1662.7
85	1484.0
86	1485.4
87	1468.0
88	1696.4
89	1531.6
90	1452.4
91	1222.7
92	1557.3
93	1737.8
94	1555.9
95	1662.5
96	1647.2
97	1706.2
98	1283.2
99	1436.5
100	1700.4
101	1446.6
102	1508.0
103	1471.9
104	2276.3
105	1545.5

106	1565.2
107	1584.5
108	1552.1
109	1413.8
110	1498.2
111	1439.4
112	1601.9
113	1622.5
114	1009.0
115	1621.0
116	1499.5
117	1464.9
118	1381.5
119	1857.2
120	1628.4
121	1403.1
122	1424.0
123	1446.3
124	1713.5
125	1781.1
126	1513.6
127	1550.3
128	1631.5
129	1341.0
130	1825.7
131	1586.1
132	1685.9
133	1576.9
134	1769.7
135	1446.2
136	1642.3
137	1549.9
Average	1575.0
Standard deviation	225.8

Table 6. Rejected IC50 of TEA in the Shiseido's data

No.	IC50 (μg/mL) of TEA	The reason of rejection
1	908.3	Deviation of data of TEA
2	603.4	The substance, 3-Chloropropionitrile affected the other wells by volatilization.
3	662.8	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.
4	654.0	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.
5	72.4	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.
6	127.3	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.

Table 7. IC50 of TEA in the three labs of the phase I

N	Mean (μg/mL)	SD
4	1382.8	33.3
4	1529.3	132.7
4	1280.8	61.34

(4) The acceptance range of the difference between two dilution series of the substance on the plate, within ±20% was obtained from the previous Shiseido's data as shown in table 8 and 9.

Table 8. IC50 from two dilution series of the substance on the plate

Maximal conc.(μg/mL)	IC50 (μg/mL) (1)	IC50 (μg/mL) (2)	Average IC50	Average*0.8	Average*1.2	Evaluation
5000	2979.9	2979.9	2978.4	2382.7	3574.1	Pass
5000	3442.3	3377.9	3408.2	2726.6	4089.9	Pass
5000	1879.7	2210.9	1999.0	1599.2	2398.8	Pass
5000	1491.3	1380.6	1439.6	1151.7	1727.5	Pass
5000	2377.2	3128.0	2729.0	2183.2	3274.8	Pass
5000	3627.8	3675.0	3646.0	2916.8	4375.2	Pass
500	47.3	47.0	47.2	37.7	56.6	Pass
500	50.1	50.1	50.1	40.1	60.1	Pass
5000	48.4	55.4	52.3	41.8	62.8	Pass
5000	2806.2	2576.5	2890.8	2312.7	3469.0	Pass
5000	2321.3	2490.2	2366.4	1893.2	2839.7	Pass
5000	3133.4	3351.4	3239.4	2591.5	3887.3	Pass
5000	1463.7	1408.5	1436.8	1149.5	1724.2	Pass
5000	1272.7	1355.7	1315.7	1052.5	1578.8	Pass
5000	44.1	54.1	49.1	39.3	58.9	Pass
5000	110.2	140.7	125.5	100.4	150.6	Pass
5000	56.9	49.2	53.3	42.7	64.0	Pass
5000	53.7	56.6	55.2	44.1	66.2	Pass
5000	1743.9	1569.5	1665.9	1332.7	1999.1	Pass
5000	1825.2	1613.4	1687.2	1349.8	2024.7	Pass
5000	3828.2	4046.7	3889.9	3111.9	4667.8	Pass
5000	3818.3	3812.7	3816.8	3053.4	4580.1	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	89.3	78.1	84.3	67.4	101.1	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	351.9	294.2	327.7	262.2	393.2	Pass
5000	100.2	95.0	97.7	78.2	117.3	Pass
5000	77.2	92.5	85.7	68.5	102.8	Pass
50	26.1	32.2	30.4	24.3	36.5	Pass
50	36.9	35.3	36.2	28.9	43.4	Pass
500	35.1	32.9	33.4	26.8	40.1	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	1363.4	1399.4	1381.1	1104.9	1657.4	Pass
5000	1043.2	962.5	1010.5	808.4	1212.6	Pass
5000	39.1	42.0	39.1	31.3	46.9	Pass
5000	150.7	190.3	169.6	135.7	203.6	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	392.6	406.4	399.6	319.7	479.5	Pass
5000	220.5	217.4	219.0	175.2	262.8	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	3132.5	3124.8	3126.7	2501.3	3752.0	Pass
5000	1456.4	1341.3	1399.5	1119.6	1679.4	Pass
5000	937.1	1020.8	976.2	781.0	1171.5	Pass
5000	4086.9	5000.0	4599.9	3679.9	5519.8	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	96.6	83.3	92.9	74.3	111.4	Pass
5000	79.4	73.1	75.9	60.8	91.1	Pass
5000	1868.0	2378.9	2099.4	1679.5	2519.3	Pass
5000	2268.6	2277.1	2275.0	1820.0	2730.0	Pass

5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	77.7	83.9	81.0	64.8	97.2	Pass
5000	75.6	65.8	69.7	55.7	83.6	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	402.1	340.5	371.6	297.3	446.0	Pass
5000	55.1	51.0	53.2	42.6	63.8	Pass
5000	53.0	57.5	55.5	44.4	66.6	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	3928.8	3271.3	3606.0	2884.8	4327.2	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	2186.8	2943.3	2482.3	1985.9	2978.8	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	274.0	305.1	289.3	231.4	347.1	Pass
5000	534.7	671.0	621.0	496.8	745.2	Pass
5000	755.5	751.4	753.9	603.1	904.7	Pass
5000	1032.0	859.7	969.6	775.7	1163.5	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	52.2	53.8	53.0	42.4	63.6	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	405.4	395.3	401.8	321.5	482.2	Pass
5000	412.6	340.8	386.7	309.3	464.0	Pass
5000	1789.8	1784.3	1787.0	1429.6	2144.4	Pass
5000	2664.1	2628.6	2645.0	2116.0	3174.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	832.6	819.9	827.1	661.7	992.6	Pass
5000	1081.7	943.4	1012.7	810.1	1215.2	Pass
5000	1295.1	1436.8	1347.5	1078.0	1617.0	Pass
5000	755.1	558.4	639.4	511.5	767.3	Pass
5000	749.9	820.1	785.4	628.3	942.5	Pass
5000	848.0	888.0	865.7	692.5	1038.8	Pass
5000	3116.6	3182.9	3142.8	2514.3	3771.4	Pass
5000	1281.0	1565.8	1441.1	1152.9	1729.3	Pass
5000	915.6	917.6	916.5	733.2	1099.8	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	39.1	39.1	39.1	31.3	46.9	Pass
5000	229.1	239.1	234.4	187.5	281.3	Pass
5000	239.8	243.5	241.6	193.3	290.0	Pass
5000	1481.9	1457.1	1470.1	1176.0	1764.1	Pass
5000	1409.0	1545.2	1481.1	1184.9	1777.3	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	5000.0	5000.0	5000.0	4000.0	6000.0	Pass
5000	815.0	872.4	845.1	676.1	1014.1	Pass

Table 9. Rejected IC50 from two dilution series of the substance on the plate

Maximal conc. (μg/mL)	IC50 (μg/mL) (1)	IC50 (μg/mL) (2)	Average IC50	Average*0.8	Average*1.2	Evaluation	The reason of rejection
500	46.9	32.6	40.8	32.6	49.0	Reject	The substance, 3-Chloropropionitrile affected the other wells by volatilization.
5000	104.8	75.4	97.7	78.2	117.3	Reject	The substance, 3-Chloropropionitrile affected the other wells by volatilization.
5000	148.8	90.6	98.1	78.5	117.7	Reject	Deviation of data
500	583.8	425.5	483.0	386.4	579.6	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization .
5000	39.1	54.2	39.1	31.3	46.9	Reject	Deviation of data
5000	58.5	39.1	49.5	39.6	59.4	Reject	Deviation of data
5000	385.5	243.1	333.9	267.1	400.7	Reject	Deviation of data
5000	2112.1	5000.0	4164.6	3331.7	4997.6	Reject	Deviation of data
5000	195.2	358.3	248.8	199.0	298.6	Reject	Deviation of data
5000	963.9	2022.5	1420.0	1136.0	1704.0	Reject	Deviation of data
5000	211.9	115.9	194.1	155.3	232.9	Reject	Deviation of data
5000	126.3	664.8	150.6	120.5	180.7	Reject	Deviation of data
5000	467.5	734.2	614.0	491.2	736.8	Reject	Deviation of data

(5) The acceptance range of the difference between left and right wells of the negative control, within ±15% was obtained from the previous Shiseido's data as shown in table 10 and 11.

Table 10. OD of left and right wells of the negative control on the plate

Mean OD of left wells	Mean OD of right wells	Mean OD of negative control	Mean*0.85	Mean*1.15	Evaluation
0.757	0.694	0.726	0.617	0.871	Pass
0.646	0.650	0.648	0.551	0.778	Pass
0.942	0.870	0.906	0.770	1.087	Pass
1.070	1.100	1.085	0.922	1.302	Pass
0.727	0.751	0.739	0.628	0.887	Pass
0.586	0.579	0.583	0.495	0.699	Pass
0.782	0.786	0.784	0.666	0.941	Pass
0.711	0.751	0.731	0.621	0.877	Pass
0.718	0.602	0.660	0.561	0.792	Pass
0.659	0.802	0.731	0.621	0.877	Pass
0.649	0.647	0.648	0.551	0.778	Pass
0.715	0.622	0.669	0.568	0.802	Pass
0.697	0.643	0.670	0.570	0.804	Pass
0.898	0.861	0.880	0.748	1.055	Pass

0.929	1.002	0.966	0.821	1.159	Pass
0.864	0.844	0.854	0.726	1.025	Pass
0.948	1.004	0.976	0.830	1.171	Pass
0.695	0.802	0.749	0.636	0.898	Pass
0.952	0.969	0.961	0.816	1.153	Pass
0.653	0.709	0.681	0.579	0.817	Pass
0.644	0.590	0.617	0.524	0.740	Pass
0.868	0.910	0.889	0.756	1.067	Pass
0.676	0.619	0.648	0.550	0.777	Pass
1.026	1.020	1.023	0.870	1.228	Pass
1.024	0.950	0.987	0.839	1.184	Pass
0.876	0.868	0.872	0.741	1.046	Pass
0.808	0.837	0.823	0.699	0.987	Pass
0.993	0.987	0.990	0.842	1.188	Pass
0.694	0.621	0.658	0.559	0.789	Pass
0.730	0.638	0.684	0.581	0.821	Pass
0.536	0.620	0.578	0.491	0.694	Pass
0.735	0.757	0.746	0.634	0.895	Pass
0.649	0.658	0.654	0.555	0.784	Pass
0.610	0.695	0.653	0.555	0.783	Pass
0.664	0.635	0.650	0.552	0.779	Pass
0.944	0.922	0.933	0.793	1.120	Pass
1.168	1.052	1.110	0.944	1.332	Pass
1.047	0.870	0.959	0.815	1.150	Pass
0.883	0.945	0.914	0.777	1.097	Pass
0.919	0.848	0.884	0.751	1.060	Pass
0.726	0.711	0.719	0.611	0.862	Pass
0.970	0.875	0.923	0.784	1.107	Pass
0.581	0.590	0.586	0.498	0.703	Pass
0.873	0.867	0.870	0.740	1.044	Pass
0.766	0.648	0.707	0.601	0.848	Pass
0.823	0.901	0.862	0.733	1.034	Pass
0.756	0.738	0.747	0.635	0.896	Pass
0.722	0.706	0.714	0.607	0.857	Pass
0.688	0.735	0.712	0.605	0.854	Pass
0.742	0.714	0.728	0.619	0.874	Pass
0.924	1.026	0.975	0.829	1.170	Pass
0.775	0.721	0.748	0.636	0.898	Pass
0.763	0.724	0.744	0.632	0.892	Pass
0.847	0.866	0.857	0.728	1.028	Pass
0.672	0.662	0.667	0.567	0.800	Pass
0.706	0.766	0.736	0.626	0.883	Pass
0.678	0.675	0.677	0.575	0.812	Pass
0.834	0.779	0.807	0.686	0.968	Pass
0.669	0.647	0.658	0.559	0.790	Pass
0.834	0.791	0.813	0.691	0.975	Pass
0.808	0.814	0.811	0.689	0.973	Pass
0.695	0.566	0.631	0.536	0.757	Pass
0.644	0.671	0.658	0.559	0.789	Pass
0.630	0.662	0.646	0.549	0.775	Pass
0.616	0.633	0.625	0.531	0.749	Pass
0.647	0.519	0.583	0.496	0.700	Pass
0.714	0.770	0.742	0.631	0.890	Pass
0.739	0.633	0.686	0.583	0.823	Pass
0.622	0.746	0.684	0.581	0.821	Pass
0.756	0.804	0.780	0.663	0.936	Pass
0.652	0.687	0.670	0.569	0.803	Pass
0.939	0.809	0.874	0.743	1.049	Pass
0.808	0.809	0.809	0.687	0.970	Pass
0.756	0.839	0.798	0.678	0.957	Pass
0.813	0.751	0.782	0.665	0.938	Pass
0.709	0.741	0.725	0.616	0.870	Pass
0.720	0.664	0.692	0.588	0.830	Pass
0.687	0.746	0.717	0.609	0.860	Pass
0.802	0.752	0.777	0.660	0.932	Pass
0.849	0.772	0.811	0.689	0.973	Pass
0.602	0.527	0.565	0.480	0.677	Pass
0.724	0.825	0.775	0.658	0.929	Pass
0.689	0.723	0.706	0.600	0.847	Pass
0.697	0.749	0.723	0.615	0.868	Pass
0.687	0.599	0.643	0.547	0.772	Pass
0.708	0.670	0.689	0.586	0.827	Pass
0.719	0.829	0.774	0.658	0.929	Pass
0.677	0.728	0.703	0.597	0.843	Pass

0.701	0.677	0.689	0.586	0.827	Pass
0.691	0.711	0.701	0.596	0.841	Pass
0.716	0.723	0.720	0.612	0.863	Pass
0.718	0.744	0.731	0.621	0.877	Pass
0.769	0.801	0.785	0.667	0.942	Pass
0.835	0.808	0.822	0.698	0.986	Pass
0.801	0.824	0.813	0.691	0.975	Pass
0.672	0.717	0.695	0.590	0.833	Pass
0.709	0.629	0.669	0.569	0.803	Pass
0.722	0.751	0.737	0.626	0.884	Pass
0.673	0.717	0.695	0.591	0.834	Pass
0.753	0.726	0.740	0.629	0.887	Pass
0.655	0.712	0.684	0.581	0.820	Pass
0.691	0.739	0.715	0.608	0.858	Pass
0.747	0.578	0.663	0.563	0.795	Pass
0.721	0.688	0.705	0.599	0.845	Pass
0.762	0.645	0.704	0.598	0.844	Pass
0.516	0.616	0.566	0.481	0.679	Pass
0.752	0.682	0.717	0.609	0.860	Pass
0.647	0.689	0.668	0.568	0.802	Pass
0.687	0.692	0.690	0.586	0.827	Pass
0.775	0.724	0.750	0.637	0.899	Pass
0.787	0.811	0.799	0.679	0.959	Pass
0.627	0.641	0.634	0.539	0.761	Pass
0.790	0.779	0.785	0.667	0.941	Pass
0.720	0.616	0.668	0.568	0.802	Pass
0.762	0.660	0.711	0.604	0.853	Pass
0.746	0.637	0.692	0.588	0.830	Pass
0.763	0.829	0.796	0.677	0.955	Pass
0.590	0.670	0.630	0.536	0.756	Pass
0.696	0.768	0.732	0.622	0.878	Pass
0.793	0.676	0.735	0.624	0.881	Pass
0.659	0.601	0.630	0.536	0.756	Pass
0.682	0.716	0.699	0.594	0.839	Pass
0.833	0.777	0.805	0.684	0.966	Pass
0.768	0.671	0.720	0.612	0.863	Pass
0.766	0.737	0.752	0.639	0.902	Pass
0.650	0.639	0.645	0.548	0.773	Pass
0.805	0.747	0.776	0.660	0.931	Pass
0.774	0.665	0.720	0.612	0.863	Pass
0.716	0.780	0.748	0.636	0.898	Pass
0.711	0.745	0.728	0.619	0.874	Pass
0.810	0.722	0.766	0.651	0.919	Pass
0.729	0.832	0.781	0.663	0.937	Pass
0.714	0.740	0.727	0.618	0.872	Pass
0.643	0.681	0.662	0.563	0.794	Pass
0.631	0.655	0.643	0.547	0.772	Pass
0.688	0.606	0.647	0.550	0.776	Pass
0.843	0.721	0.782	0.665	0.938	Pass
0.644	0.591	0.618	0.525	0.741	Pass

Table 11. Rejected OD of left and right wells of the negative control on the plate

OD of left wells	OD of right wells	Mean OD of negative control	Mean*0.85	Mean*1.15	Evaluation	The reason of rejection
0.474	0.932	0.703	0.598	0.808	Reject	The substance, 3-Chloropropionitrile affected the other wells by volatilization.
0.533	0.818	0.676	0.574	0.777	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilizaition.
0.256	0.446	0.351	0.298	0.404	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilizaition.
0.230	0.410	0.320	0.272	0.368	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilizaition.
0.116	0.639	0.378	0.321	0.434	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilizaition.

(6) The acceptance range between two test results of SDS, ≥2 was obtained from the previous Shiseido's data as shown in table 12 and 13.

Table 12. IC50 of two test results of SDS

IC50 (μg/mL) of SDS (1)	IC50 (μg/mL) of SDS (2)	High value/low value	Evaluation
102.2	90.8	1.13	Pass
87.2	89.1	1.02	Pass
91.1	91.8	1.01	Pass
91.0	93.2	1.02	Pass
104.4	97.0	1.08	Pass
90.5	95.1	1.05	Pass
90.5	92.5	1.02	Pass
103.1	93.1	1.11	Pass
101.7	92.4	1.10	Pass
90.6	96.5	1.07	Pass
95.1	89.6	1.06	Pass
96.1	89.4	1.07	Pass
91.4	86.0	1.06	Pass
92.4	94.8	1.03	Pass
96.2	96.7	1.01	Pass
90.3	89.7	1.01	Pass
90.7	95.1	1.05	Pass
90.8	100.8	1.11	Pass
98.8	88.1	1.12	Pass
101.7	91.5	1.11	Pass
108.0	91.3	1.18	Pass
104.2	86.0	1.21	Pass
92.7	91.4	1.01	Pass
100.2	91.5	1.10	Pass
97.2	89.1	1.09	Pass
103.5	90.6	1.14	Pass
113.7	89.0	1.28	Pass
107.2	91.0	1.18	Pass
93.5	96.4	1.03	Pass
85.9	93.0	1.08	Pass
91.8	90.4	1.02	Pass
91.2	92.8	1.02	Pass
92.1	95.3	1.03	Pass
96.9	87.1	1.11	Pass
91.9	90.4	1.02	Pass
96.0	113.6	1.18	Pass
86.3	92.4	1.07	Pass
93.4	91.1	1.03	Pass
95.2	94.3	1.01	Pass
91.8	88.2	1.04	Pass
95.5	93.9	1.02	Pass
93.3	92.9	1.00	Pass
96.0	91.7	1.05	Pass
94.0	91.1	1.03	Pass
90.7	92.5	1.02	Pass
89.9	90.1	1.00	Pass
90.8	89.7	1.01	Pass
94.4	94.3	1.00	Pass
96.6	91.0	1.06	Pass
90.0	92.9	1.03	Pass
92.0	92.7	1.01	Pass
91.5	93.6	1.02	Pass
91.5	109.2	1.19	Pass
90.7	91.3	1.01	Pass
92.2	92.2	1.00	Pass
89.1	93.5	1.05	Pass
93.0	87.2	1.07	Pass
98.7	101.6	1.03	Pass
93.6	89.7	1.04	Pass
93.6	91.5	1.02	Pass
96.5	93.8	1.03	Pass
100.6	91.8	1.10	Pass
91.3	93.7	1.03	Pass
93.8	93.5	1.00	Pass
89.1	92.1	1.03	Pass
96.6	95.2	1.01	Pass
92.8	91.0	1.02	Pass
94.4	92.6	1.02	Pass
91.4	91.9	1.01	Pass

Table 13. Rejected IC50 of two test results of SDS

IC50 (μg /mL) of SDS (1)	IC50 (μg/mL) of SDS (2)	High value/low value	Evaluation	The reason of rejection
37.0	17.1	2.16	Reject	The substance, 2,4-Difluoronitrobenzene affected the other wells by volatilization.

References

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Report on the selection of test substances for SIRC-CVS:TEA test validation study

2013/10/31
2013/11/20 revised
2014/12/10 revised
2015/2/13 revised
2015/3/18 revised
2015/4/6 revised

SIRC-CVS:TEA Validation Management Team (VMT)

This report describes the selection process for test substances used in the SIRC-CVS:TEA test validation study.

The objective of this study was to evaluate the within- and between-laboratory reproducibility and predictive capacity of the SIRC-CVS:TEA test on eye irritation (consistency with the two categories, Irritant and Non-irritant) as the initial step in a bottom-up approach.

In a complementary study, the validation management team (VMT) evaluated predictive capacity for the Category 1, Category 2, and Non-irritant classifications of the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) as well as four classifications used by the United States Environmental Protection Agency (EPA).

To this end, phase II-A, phase II-B and phase III studies were conducted by three laboratories using the test substances as shown in Table 1. These test substances were selected by the VMT without any participation by delegates from the three laboratories.

In addition, the list of these test substances included chemical categories or physical and chemical properties (molecular weight, solubility in the medium, etc.) to facilitate study of an optimal applicable domain.

Table 1: Breakdown of the SIRC-CSV:TEA test validation study

Phase	No. of test substances	No. of repetitions	Subject
II-A	5	3	Within- and between- laboratory reproducibility
II-B	15	3	
III	100 (Including common test substances)	1	Between- laboratory reproducibility and predictability

1. Phase II study

In the Phase II study, the twenty test substances shown in Table 1were selected by the VMT for use in assessing within- and between-laboratory reproducibility. Selections were made from the following lists with an eye toward maintaining a balance between UN GHS or EPA labeling and solid or liquid.

- Extant individual animal data for test substances were available for classifying the eye irritating hazard under UN GHS.
- Test substances had already been evaluated in other i*n vitro* eye irritation tests.

Twenty test substances comprising 10 irritants and 10 non-irritants are listed in Table 2. To assess within- and between-laboratory reproducibility, the VMT distributed 3 sets of each coded test substance to each laboratory. The three sets were tested separately, but the order in which they were tested was considered immaterial. The VMT distributed 15 coded test substances (5 different test substances) in the phase II-A study and 45 coded test substances (15 different test substances) in the phase II-B study to each laboratory.

Table 2-1 : List of the 20 substances selected for phase II in SIRC-CSV:TEA test validation study

Phase II-A study

No.	Test substance	CAS No.	Solid/ Liquid	Supplier	Storage	Lab. Code			GHS	EPA
						SA	SB	SC		
						Nihon Kolmar	Bozo	Biotoxtech		
1	piperonylbutoxide	51- 03- 6	Liquid	Sigma Aldrich	rt	SA008	SB010	SC011	No	Ⅲ
						SA013	SB001	SC004		
						SA002	SB009	SC006		
2	2,5-dimethylhexaediol	110-03-2	Solid	Sigma Aldrich	rt	SA001	SB005	SC010	I	I
						SA010	SB013	SC002		
						SA015	SB003	SC015		
3	1-(2-propoxy-1-methylet hoxy)-2-propanol	29911-27-1	Liquid	Sigma Aldrich	rt	SA005	SB015	SC008	2B	Ⅲ
						SA012	SB007	SC003		
						SA007	SB012	SC014		
4	ammonium nitrate	6484-52-2	Solid	Sigma Aldrich	rt	SA004	SB002	SC007	2B	Ⅲ
						SA011	SB006	SC013		
						SA009	SB014	SC005		
5	potassium tetrafluoroborate	14075-53-7	Solid	Sigma Aldrich	rt	SA014	SB011	SC009	No	Ⅳ
						SA003	SB004	SC012		
						SA006	SB008	SC001		

rt: room temp.

Set1

Set 2

Set 3

Table 2-2 : List of the 20 substances selected for phase II in SIRC-CSV:TEA test validation study

Phase II-B study

No.	Test substance	CAS No.	Solid/ Liquid	Supplier	Storage	Lab. Code			GHS	EPA
						SA	SB	SC		
						Nihon Kolmar	Bozo	Biotoxtech		
6	3,4,4'-trichlorocarbanilide	101-20-2	Solid	Sigma Aldrich	rt	SA049	SB017	SC031	No	Ⅳ
						SA029	SB054	SC021		
						SA057	SB060	SC043		
7	1-bromohexane	111-25-1	Liquid	Sigma Aldrich	rt	SA016	SB029	SC042	No	Ⅳ
						SA034	SB043	SC055		
						SA039	SB053	SC047		
8	4,4'-methylenebis (2,6-di-tert-butylphenol)	118-82-1	Solid	Sigma Aldrich	rt	SA022	SB057	SC016	No	Ⅳ
						SA048	SB037	SC030		
						SA028	SB044	SC053		
9	propylene glycol propyl ether	1569-01-3	Liquid	Sigma Aldrich	rt	SA038	SB028	SC033	2A	Ⅱ
						SA040	SB042	SC054		
						SA017	SB031	SC041		
10	ethyl thioglycolate	623-51-8	Liquid	Sigma Aldrich	rt	SA035	SB055	SC017	No	Ⅲ
						SA052	SB018	SC032		
						SA027	SB027	SC046		
11	sodium oxalate	62-76-0	Solid	Sigma Aldrich	rt	SA030	SB038	SC023	1	Ⅰ
						SA050	SB020	SC029		
						SA026	SB045	SC040		
12	2-phospho-L-ascorbic acid trisodium salt	66170-10-3	Solid	Sigma Aldrich	rt	SA018	SB034	SC022	No	Ⅲ
						SA045	SB052	SC034		
						SA031	SB030	SC044		
13	1-bromo-4-chlorobutane	6940-78-9	Liquid	Sigma Aldrich	rt	SA046	SB016	SC039	No	Ⅳ
						SA025	SB032	SC020		
						SA044	SB023	SC058		
14	sodium hydrogensulfite	7631-90-5	Solid	Sigma Aldrich	rt	SA019	SB041	SC024	No	Ⅲ
						SA037	SB046	SC045		
						SA032	SB019	SC048		
15	isobutyraldehyde	78-84-2	Liquid	Sigma Aldrich	**4ºC**	SA036	SB056	SC025	2B	Ⅲ
						SA033	SB022	SC035		
						SA021	SB050	SC038		
16	1-naphthaleneacetic acid	86-87-3	Solid	Wako Pure Chemicals	rt	SA041	SB024	SC056	1	Ⅰ
						SA053	SB021	SC026		
						SA024	SB047	SC049		
17	propyl 4-hydroxybenzoate	94-13-3	Solid	Sigma Aldrich	rt	SA054	SB039	SC057	No	Ⅲ
						SA020	SB026	SC060		
						SA059	SB051	SC018		
18	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	Solid	Sigma Aldrich	rt	SA051	SB035	SC036	2B	Ⅲ
						SA023	SB059	SC050		
						SA056	SB048	SC059		
19	camphene	79-92-5	Solid	Sigma Aldrich	rt	SA060	SB040	SC027	2B	Ⅲ
						SA042	SB033	SC052		
						SA058	SB049	SC051		
20	cyclopentanol	96-41-3	Liquid	Sigma Aldrich	rt	SA047	SB058	SC028	2B	Ⅱ
						SA055	SB025	SC037		
						SA043	SB036	SC019		

rt: room temp.

Set 1

Set 2

Set 3

2. Phase III study

According to the objective in the study plan, the 120 coded test substances (100 different test substances) were prepared to evaluate the predictability and to confirm between-laboratory reproducibility of SIRC-CVS:TEA validation studies. The 40 coded test substances (forty different test substances) were distributed to each laboratory for the Phase III validation study. Of the 40 test substances, the ten substances in Table 3 were used as common test substances. Therefore, a total of 100 test substances were tested to evaluate the predictive capacity in the Phase III study.

Of these 100 test substances, nearly 60 had been used in validation studies of a three-dimensional corneal model (such as EpiOcular) by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM)[1] and nearly 60 had been used in the Short Time Exposure (STE) test validation study by the Japanese Center for the Validation of Alternative Methods (JaCVAM) and Independent peer review by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[2,3,4].

In the Phase III study, all test substances were selected from the following lists with an eye toward maintaining a balance between UN GHS or EPA labeling and solid or liquid.

- Extant individual animal data for test substances were available for classifying the eye irritating hazard under UN GHS and EPA.
- A uniform balance between solids and liquids.
- Test substances had already been evaluated in other *in vitro* eye irritation tests.
- Test substances represented a variety of categories such as alcohol, ester, ketone, surfactant and so on.

Table 3：List of 100 test substances selected for phase III in SIRC-CVS:TEA test validation study

No.	Test substance	CAS No.	Solid/ Liquid	Supplier	Lab. Code	GHS	EPA	Source
1#	2-ethoxyethyl methacrylate	2370-63-0	Liquid	Sigma Aldrich	SB062	No	IV	ECETOC
2	iso-octylthioglycolate	25103-09-7	Liquid	Wako Pure	SC072	No	IV	ECETOX
3#	dipropyl disulfide	629-19-6	Liquid	Sigma Aldrich	SA082 SB079 SC061	No	IV	STE review
4	1-bromo-octane	111-83-1	Liquid	Sigma Aldrich	No	IV	STE review	Halogen compound
5#	2-(2-ethoxyethoxy)ethanol	111-90-0	Liquid	Sigma Aldrich	SA089, SB072 SC062	No	III	Cosing
6	dioctyl ether	629-82-3	Liquid	Sigma Aldrich	SC077	No	IV	Cognis
7	3-phenoxybenzyl alcohol	13826-35-2	Liquid	Sigma Aldrich	SC079	No	III	NICEATM
8	glycidyl methacrylate	106-91-2	Liquid	Sigma Aldrich	SB063	No	III	STE review
9	2-ethylhexylthioglycolate	7659-86-1	Liquid	Sigma Aldrich	SC080	No	IV	ECETOC
10#	n,n-dimethylguanidine sulfate	598-65-2	Solid	Sigma Aldrich	SA090, SB071, SC063	No	III	STE review
11	6-hydroxy-2,4,5-triaminopyrimidine sulfate	1603-02-7	Solid	Wako Pure	SC081	No	IV	Cosing
12#	polyethylene hydrogenated caster oil (40E.O.)	61788-85-0	Solid	Sigma Aldrich	SA084 SB077 SC064	No	IV	STE review
13	2,2'-methylenebis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	103597-45-1	Solid	Sigma Aldrich	SC082	No	IV	Ciba
14	cellulose, 2-(2-hydroxy-3-(trimethylammonio) propoxy) ethyl ether chloride	68610-92-4	Solid	Sigma Aldrich	SC083	No	III	J&J
15	3,4-dimethoxy benzaldehyde	120-14-9	Solid	Sigma Aldrich	SC084	No	III	NICEATM
16	3-chloropropionitrile	542-76-7	Liquid	Wako Pure	SC085	2B	III	ECETOC
17	2-methyl-1-pentanol	105-30-6	Liquid	Sigma Aldrich	SC087	2B	III	STE review
18	ethyl-2-methylacetoacetate	609-14-3	Liquid	Sigma Aldrich	SC088	2B	III	STE review
19#	diethyl toluamide	134-62-3	Liquid	Sigma Aldrich	SA088 SB073, SC065	2B	III	US-EPA
20#	4-nitrobenzoic acid	62-23-7	Solid	Sigma Aldrich	SA083 SB078, SC066	2B	III	NICEATM
21	sodium chloroacetate	3926-62-3	Solid	Sigma Aldrich	SC090	2B	III	STE review
22	2,4,11,13-tetraazatetra (chlorohexidine glucocinate)	18472-51-0	Liquid	Wako Pure	SA061	2A	II	NICEATM
23	-	-	-	-	-	-	-	-

24#	2-amino-3-hydroxy pyridine	16867-03-1	Solid	Sigma Aldrich	SA086, SB075, SC068	2A	III	Cosing
25	sodium benzoate	532-32-1	Solid	Sigma Aldrich	SC092	2A	II	Cosing
26	methylthioglycolate	2365-48-2	Liquid	Sigma Aldrich	SC093	1	II	ECETOC
27	3-(2-aminoethylamino)propyl]trimethoxysilane	1760-24-3	Liquid	Chemos	SA096	1	I	Evonik
28#	tetraethylene glycol	17831-71-9	Liquid	Sigma Aldrich	SA085 SB076 SC069	1	I	TSCA
29#	dodecanoic acid	143-07-7	Solid	Sigma Aldrich	SA087, SB074, SC070	1	I	ECETOC
30	1,2-benzisothiazol-3(2H)-one	2634-33-5	Solid	Wako Pure	SC097	1	I	Cosing
31	2-hydroxy-1,4-naphthoquinone	83-72-7	Solid	Sigma Aldrich	SC089	2B	III	Cosing
32	disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	27344-41-8	Solid	Wako Pure	SC098	1	II	Ciba
33#	gamma-butyrolactone	96-48-0	Liquid	Sigma Aldrich	SA081, SB080, SC067	2A	II	STE review
34	1-methylpropyl benzene	135-98-8	Liquid	Wako Pure	SC071	No	IV	STE review
35	4-(methylmercapto)benzaldehyde	3446-89-7	Liquid	Sigma Aldrich	SC073	No	IV	ECETOX
36	1,9-decaine	1647-16-1	Liquid	Sigma Aldrich	SC075	No	IV	STE review
37	2,4-dimethyl-3-pentanol	3970-62-5	Liquid	Sigma Aldrich	SC076	No	III	STE review
38	1-ethyl-3-methylimidazolium ethylsulfate	342573-75-5	Liquid	AlfaAesar	SC078	No	III	Evonik
39	1,2,4-triazole,sodium salt	41253-21-8	Solid	Sigma Aldrich	SC095	1	I	ECETOC
40	4,4'-(4,5,6,7-tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol]	4430-25-5	Solid	Sigma Aldrich	SC096	1	I	Coising
41	benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCl	3248-91-7	Solid	Sigma Aldrich	SC099	1	I	Cosing
42	1-(9H-carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	72956-09-3	Solid	LKT.Labs,Inc	SA062	No	IV	Glaxo
43	3-methyl-1,5-di(2,4-xylyl)-1,3,5-triazapenta-1,4-dien	33089-61-1	Solid	LKT.Labs,Inc	SB061	No	IV	US-EPA
44	isopropyl acetoacetate	542-08-5	Liquid	Wako Pure	SC086	2B	III	NICEATM
45	(3R,4R)-4-acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	76855-69-1	Solid	Sigma Aldrich	SA063	2A	II	Glaxo
46	1-octanol	111-87-5	Liquid	Wako Pure	SB064	2A	II	STE review
47	2-benzyloxyethanol	622-08-2	Liquid	Wako Pure	SB065	2A	II	STE review
48	butanol	71-36-3	Liquid	Wako Pure	SB066	1	I	STE review
49	isobutyl alcohol	78-83-1	Liquid	Sigma Aldrich	SB067	1	I	STE review
50	isopropyl alcohol	67-63-0	Liquid	Wako Pure	SB068	2A	III	STE review
51	myristyl alcohol	112-72-1	Solid	Wako Pure	SB069	2A	III	STE review
52	hexyl cinnamic aldehyde	101-86-0	Liquid	Wako Pure	SB070	2B	II	STE review

53	n-butanal	123-72-8	Liquid	Wako Pure	SB081	2B	III	STE review
54	monoethanolamine	141-43-5	Liquid	Sigma Aldrich	SB082	2B	III	NICEATM
55	m-phenylenediamine	108-45-2	Solid	TCI	SB083	1	I	STE review
56	ethyl acetate	141-78-6	Liquid	Sigma Aldrich	SB084	No	III	STE review
57	isopropyl myristate	110-27-0	Liquid	Wako Pure	SB085	No	IV	STE review
58	methoxyethyl acrylate	3121-61-7	Liquid	Wako Pure	SB086	1	>II	STE review
59	methyl acetate	79-20-9	Liquid	Sigma Aldrich	SB087	2A	II	STE review
60	methyl cyanoacetate	105-34-0	Liquid	Sigma Aldrich	SB088	2A	II	STE review
61	imidazole	288-32-4	Solid	Sigma Aldrich	SB089	1	I	STE review
62	pyridine	110-86-1	Liquid	Sigma Aldrich	SB090	1	I	STE review
63	isopropyl bromide	75-26-3	Liquid	Wako Pure	SB091	No	IV	STE review
64	cyclohexanone	108-94-1	Liquid	Sigma Aldrich	SB092	No	III	STE review
65	2-methylbuthyric acid	116-53-0	Liquid	Sigma Aldrich	SB093	1	I	STE review
66	calcium thioglycollate trihydrate	5793-98-6	Solid	TCI	SB094	1	I	Ohno(1999)
67	citric acid	77-92-9	Solid	Sigma Aldrich	SB095	2A?	II?	Kojima (2013)
68	potassium sorbate	24634-61-5	Solid	Sigma Aldrich	SB096	2A?	II?	Kojima (2013)
69	sodium salicylate	54-21-7	Solid	Wako Pure	SB097	1	I	STE review
70	distearyldimethylammonium chloride	107-64-2	Solid	TCI	SB098	1	I	STE review
71	n-lauroylsarcosine sodium salt	137-16-6	Solid	Wako Pure	SB099	2B	III	NICEATM
72	sodium lauryl sulfate	151-21-3	Solid	Wako Pure	SB100	2A?	III	STE review
73	triton X-100 (5%)	9002-93-1	Liquid	Sigma Aldrich	SA065	2B	III	NICEATM
74	2-ethylhexyl p-dimethyl-amino benzoate	21245-02-3	Liquid	Wako Pure	SA076	No	IV	STE review
75	promethazine hydrochloride	58-33-3	Solid	Sigma Aldrich	SA064	1	I	STE review
76	2-ethyl-1-hexanol	104-76-7	Liquid	Wako Pure	SA067	2A	II	STE review
77	3-methoxy-1.2-propanediol	623-39-2	Liquid	TCI	SA080	No	IV	STE review
78	cyclohexanol	108-93-0	Liquid	Sigma Aldrich	SA070	1	I	STE review
79	ethanol	64-17-5	Liquid	Wako Pure	SA091	2A	I	STE review
80	n-hexanol	111-27-3	Liquid	Sigma Aldrich	SA072	2A	II	STE review
81	3,3-dimethylpentane	562-49-2	Liquid	Sigma Aldrich	SA078	No	IV	STE review
82	methyl cyclopentane	96-37-7	Liquid	TCI	SA098	No	III	STE review
83	toluene	108-88-3	Liquid	Wako Pure	SA069	2B?	III	STE review
84	acetone	67-64-1	Liquid	Sigma Aldrich	SA092	2A	II	STE review
85	gluconolactone	90-80-2	Solid	Wako Pure	SA097	No	IV	NICEATM
86	methyl amyl ketone (2-heptanol)	110-43-0	Liquid	Wako Pure	SA071	No	III	STE review
87	methyl ethyl ketone (2-butanone)	78-93-3	Liquid	TCI	SA094	2A	III	STE review
88	methyl isobutyl ketone(4-methyl 2-pentanol)	108-10-1	Liquid	Sigma Aldrich	SA068	No	III	STE review
89	glycerol	56-81-5	Liquid	Wako Pure	SA079	No	IV	STE review
90	cetylpyridinium bromide	140-72-7	Solid	Sigma Aldrich	SA075	1	I	STE review

91	triton X-100	9002-93-1	Liquid	Sigma Aldrich	SC094	1	I	STE review
92	tween20	9005-64-5	Liquid	Sigma Aldrich	SC100	No	III	STE review
93	sodium hydroxide	1310-73-2	Solid	Wako Pure	SA074	1	I	STE review
94	glycolic acid	79-14-1	Solid	Sigma Aldrich	SA095	2B	III	NICEATM
95	3,3-dithiodipropionic acid	1119-62-6	Solid	Wako Pure	SC091 SA073	2B	II	NICEATM
96	sucrose fatty acid ester	Non	Solid	TCI	SA100	2A?	II?	STE review
97	methyl para-Hydroxybenzoate	99-76-3	Solid	Wako Pure	SA099	2？	II?	Ohno(1999)
98	silic acid, dehydrogate	7699-41-4	Solid	Wako Pure	SA093	No	IV	Ohno(1999)
99	benzyl alcohol	100-51-6	Liquid	Sigma Aldrich	SA066	1	I	STE review
100	lactic acid	50-21-5	Liquid	Wako Pure	SA077	1	I	STE review

#: Ten test substances (No.1-No.10) distributed to three participated laboratories as common test substances.

$: Tow test substances (No.35 and No.38) could not confirmed individual animal data

&: One test substance (No.60) duplicated.

3. Information on test substances selected for SIRC-CVS:TEA test validation study

The 116 test substances listed in Table 4 were used to analyze the predictive capacity of the SIRC-CVA:TEA test. These include the 20 test substances used in the Phase II study plus the 100 test substances used in the Phase III study. Of these 120 test substances, 3,3-dithiodipropionic acid was included twice by JaCVAM, so the duplication was exluded from the analysis. Citric acid and potassium sorbate did not have a clear source for individual animal data and were excluded from the analysis. A clear source for individual animal data was identified for the remaining 117 test substances in association of the NICEATM.

The UN GHS or EPA classifications of the 117 test substances selected for the validation study are shown in Table 5. The VMT considered this a reasonable balance of test substances. The ratio of solids to liquids for the 117 test substances selected for the validation study are shown in Table 6. This information may be useful in determining an optimal applicability domain for this assay.

Table 4: List of test substances used in SIRC-CVS:TEA test validation study

NO.	Code No.	Test substance	CAS	Solid: Liquid	Supplier	GHS	EPA	Source	Final chemical class
001	P2-016	1-naphthaleneacetic acid	86-87-3	Solid	Wako Pure	1	I	ECETOC	Carboxylic acid, Polycyclic compound
002	P3-030	1,2-benzisothiazol-3(2H)-one	2634-33-5	Solid	Wako Pure	1	I	Cosing	Heterocyclic compound, Thio compound, Amide
003	P3-039	1,2,4-triazole,sodium salt	41253-21-8	Solid	Sigma Aldrich	1	I	ECETOC	Heterocyclic compound
005	P3-065	2-methylbuthyric acid	116-53-0	Liquid	Sigma Aldrich	1	I	STE review	Carboxilic acid
004	P2-002	2,5-dimethylhexaediol	110-03-2	Solid	Sigma Aldrich	1	I	STE review	Alcohol
006	P3-027	3-(2-aminoethylamino)propyl]trimethoxysilane	1760-24-3	Liquid	Chemos	1	I	Evonik	Silicon compound
007	P3-040	4,4'-(4,5,6,7-tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol]	4430-25-5	Solid	Sigma Aldrich	1	I	Coising	Halogen compound, Phenol, Sulfonic acid
008	P3-041	benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCl	3248-91-7	Solid	Sigma Aldrich	1	I	Cosing	Organic salt
009	P3-099	benzyl alcohol	100-51-6	Liquid	Sigma Aldrich	1	I	STE review	Alcohol
010	P3-048	butanol	71-36-3	Liquid	Wako Pure	1	I	STE review	Alcohol
011	P3-066	calcium thioglycollate trihydrate	5793-98-6	Solid	TCI	1	I	STE review	Thio compound, Organic salt
012	P3-090	cetylpyridinium bromide	140-72-7	Solid	Sigma Aldrich	1	I	STE review	Surfactant (cationic)
013	P3-078	cyclohexanol	108-93-0	Liquid	Sigma Aldrich	1	I	STE review	Alcohol
014	P3-032	disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	27344-41-8	Solid	Wako Pure	1	II	Ciba	Sulfonic acid
015	P3-070	distearyldimethylammonium chloride	107-64-2	Solid	TCI	1	I	STE review	Quaternary ammonium compound, ~~Surfactant~~
016	P3-029	dodecanoic acid	143-07-7	Solid	Sigma Aldrich	1	I	ECETOC	Fatty acid
017	P3-061	imidazole	288-32-4	Solid	Sigma Aldrich	1	I	STE review	Heterocyclic compound, Amine
018	P3-049	isobutyl alcohol	78-83-1	Liquid	Sigma Aldrich	1	I	STE review	Alcohol
019	P3-100	lactic acid	50-21-5	Liquid	Wako Pure	1	I	STE review	Carboxylic acid
020	P3-058	methoxyethyl acrylate	3121-61-7	Liquid	Wako Pure	1	>II	STE review	Acrylate, Ether, Ester
021	P3-026	methylthioglycolate	2365-48-2	Liquid	Sigma Aldrich	1	II	ECETOC	Thio compound, Ester
022	P3-055	m-phenylenediamine	108-45-2	Solid	TCI	1	I	STE review	Amine
023	P3-075	promethazine hydrochloride	58-33-3	Solid	Sigma Aldrich	1	I	STE review	Heterocyclic compound, Organic salt
024	P3-062	pyridine	110-86-1	Liquid	Sigma Aldrich	1	I	STE review	Heterocyclic compound
025	P3-093	sodium hydroxide	1310-73-2	Solid	Wako Pure	1	I	STE review	Alkali
026	P2-011	sodium oxalate	62-76-0	Solid	Sigma Aldrich	1	I	ECETOC	Organic salt
027	P3-069	sodium salicylate	54-21-7	Solid	Wako Pure	1	I	STE review	Organic salt, Phenol
028	P3-028	tetraethylene glycol	17831-71-9	Liquid	Sigma Aldrich	1	I	TSCA	Acrylate, Ether, Ester
029	P3-091	triton X-100	9002-93-1	Liquid	Sigma Aldrich	1	I	STE review	Surfactant (nonionic)
030	P3-046	1-octanol	111-87-5	Liquid	Wako Pure	2A	II	STE review	Fatty alcohol

031	P3-024	2-amino-3-hydroxy pyridine	16867-03-1	Solid	Sigma Aldrich	2A	III	Cosing	Heterocyclic compound, Amine
032	P3-047	2-benzyloxyethanol	622-08-2	Liquid	Wako Pure	2A	II	STE review	Alcohol, Ether
033	P3-076	2-ethyl-1-hexanol	104-76-7	Liquid	Wako Pure	2A	II	STE review	Fatty alcohol
034	P3-022	2,4,11,13-tetraazatetra (chlorohexidine glucocinate)	18472-51-0	Liquid	Wako Pure	2A	II	NICEATM	Organic salt, Halogen Compound
035	P3-045	(3R,4R)-4-acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	76855-69-1	Solid	Sigma Aldrich	2A	II	Glaxo	Silicon compound
036	P3-084	acetone	67-64-1	Liquid	Sigma Aldrich	2A	II	STE review	Ketone
037	P3-079	ethanol	64-17-5	Liquid	Wako Pure	2A	I	STE review	Alcohol
038	P3-033	gamma-butyrolactone	96-48-0	Liquid	Sigma Aldrich	2A	II	STE review	Heterocyclic compound, Ketone
039	P3-050	isopropyl alcohol	67-63-0	Liquid	Wako Pure	2A	III	STE review	Alcohol
040	P3-059	methyl acetate	79-20-9	Liquid	Sigma Aldrich	2A	II	STE review	Ester
041	P3-060	methyl cyanoacetate	105-34-0	Liquid	Sigma Aldrich	2A	II	STE review	Ester, Nitrile compound
042	P3-087	methyl ethyl ketone (2-butanone)	78-93-3	Liquid	TCI	2A	III	STE review	Ketone
043	P3-097	methyl para-Hydroxybenzoate	99-76-3	Solid	Wako Pure	2?	II?	Ohno(1999)	Ester, Phenol
044	P3-051	myristyl alcohol	112-72-1	Solid	Wako Pure	2A	III	STE review	Fatty alcohol
045	P3-080	n-hexanol	111-27-3	Liquid	Sigma Aldrich	2A	II	STE review	Alcohol
046	P2-009	propylene glycol propyl ether	1569-01-3	Liquid	Sigma Aldrich	2A	II	NICEATM	Alcohol, Ether
047	P3-025	sodium benzoate	532-32-1	Solid	Sigma Aldrich	2A	II	Cosing	Organic salt
048	P3-072	sodium lauryl sulfate	151-21-3	Solid	Wako Pure	2A?	III	STE review	Surfactant (anionic)
049	P3-096	sucrose fatty acid ester	Non	Solid	TCI	2A?	II?	STE review	Polyol, Ester
050	P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	29911-27-1	Liquid	Sigma Aldrich	2B	III	STE review	Alcohol, Ether
051	P3-031	2-hydroxy-1,4-naphthoquinone	83-72-7	Solid	Sigma Aldrich	2B	III	Cosing	Phenol compound
052	P3-017	2-methyl-1-pentanol	105-30-6	Liquid	Sigma Aldrich	2B	III	STE review	Fatty alcohol
053	P3-016	3-chloropropionitrile	542-76-7	Liquid	Wako Pure	2B	III	ECETOC	Halogen compound, Nitrile compound
054	P3-023, P3-095	3,3-dithiodipropionic acid	1119-62-6	Solid	Wako Pure	2B	II	NICEATM	Carboxilic acid, Thio compound
055	P3-020	4-nitrobenzoic acid	62-23-7	Solid	Sigma Aldrich	2B	III	NICEATM	Carboxylic acid
056	P2-004	ammonium nitrate	6484-52-2	Solid	Sigma Aldrich	2B	III	NICEATM	Inorganic salt
057	P2-019	camphene	79-92-5	Solid	Sigma Aldrich	2B	III	STE review	Hydrocarbon
058	P2-020	cyclopentanol	96-41-3	Liquid	Sigma Aldrich	2B	II	ECETOC	Alcohol
059	P3-019	diethyl toluamide	134-62-3	Liquid	Sigma Aldrich	2B	III	US-EPA	Amide
060	P3-018	ethyl-2-methylacetoacetate	609-14-3	Liquid	Sigma Aldrich	2B	III	STE review	Ester, Ketone
061	P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	96568-04-6	Solid	Sigma Aldrich	2B	III	NICEATM	Halogen compound, Heterocyclic compound, Ester, Ketone
062	P3-094	glycolic acid	79-14-1	Solid	Sigma Aldrich	2B	III	NIEATM	Carboxylic acid
063	P3-052	hexyl cinnamic aldehyde	101-86-0	Liquid	Wako Pure	2B	II	STE review	Aldehyde

064	P2-015	isobutyraldehyde	78-84-2	Liquid	Sigma Aldrich	2B	III	STE review	Aldehyde
065	P3-044	isopropyl acetoacetate	542-08-5	Liquid	Wako Pure	2B	III	NICEATM	Ester, Ketone
066	P3-054	monoethanolamine	141-43-5	Liquid	Sigma Aldrich	2B	III	NICEATM	Alkanolamine
067	P3-053	n-butanal	123-72-8	Liquid	Wako Pure	2B	III	STE review	Aldehyde
068	P3-071	n-lauroylsarcosine sodium salt	137-16-6	Solid	Wako Pure	2B	III	NICEATM	Surfactant (anionic)
069	P3-021	sodium chloroacetate	3926-62-3	Solid	Sigma Aldrich	2B	III	STE review	Organic salt, Halogen compound
070	P3-073	triton X-100 (5%)	9002-93-1	Liquid	Sigma Aldrich	2B	III	NICEATM	Surfactant (nonionic)
071	P3-083	toluene	108-88-3	Liquid	Wako Pure	2B?	III	STE review	Hydrocarbon (aromatic)
072	P3-042	1-(9H-carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	72956-09-3	Solid	LKT.Labs, Inc	No	IV	Glaxo	Polycyclic compound, Alcohol
073	P2-013	1-bromo-4-chlorobutane	6940-78-9	Liquid	Sigma Aldrich	No	IV	STE review	Halogen compound
074	P2-007	1-bromohexane	111-25-1	Liquid	Sigma Aldrich	No	IV	STE review	Halogen compound
075	P3-004	1-bromo-octane	111-83-1	Liquid	Sigma Aldrich	No	IV	STE review	Halogen compound
076	P3-038	1-ethyl-3-methylimidazolium ethylsulfate	342573-75-5	Liquid	AlfaAesar	No	III	Evonik	Heterocyclic compound, Inorganic salt
077	P3-034	1-methylpropyl benzene	135-98-8	Liquid	Wako Pure	No	IV	STE review	Hydrocarbon(aromatic)
078	P3-036	1,9-decaine	1647-16-1	Liquid	Sigma Aldrich	No	IV	STE review	Alkene
079	P3-001	2-ethoxyethyl methacrylate	2370-63-0	Liquid	Sigma Aldrich	No	IV	ECETOC	Methacrylate, Ester, Ether
080	P3-074	2-ethylhexyl p-dimethyl-amino benzoate	21245-02-3	Liquid	Wako Pure	No	IV	STE review	PABA derivative
081	P3-009	2-ethylhexylthioglycolate	7659-86-1	Liquid	Sigma Aldrich	No	IV	ECETOC	Thiol compound, Ester
082	P2-012	2-phospho-L-ascorbic acid trisodium salt	66170-10-3	Solid	Sigma Aldrich	No	III	BASF	Heterocyclic compound, Organic salt, Phosphorus compound
083	P3-005	2-(2-ethoxyethoxy)ethanol	111-90-0	Liquid	Sigma Aldrich	No	III	Cosing	Alcohol, Ether
084	P3-013	2,2'-methylenebis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	103597-45-1	Solid	Sigma Aldrich	No	IV	Ciba	Phenol compound, Heterocyclic compound
085	P3-037	2,4-dimethyl-3-pentanol	3970-62-5	Liquid	Sigma Aldrich	No	III	STE review	Fatty alcohol
086	P3-077	3-methoxy-1.2-propanediol	623-39-2	Liquid	TCI	No	IV	STE review	Alcohol, Ether
087	P3-043	3-methyl-1,5-di(2,4-xylyl)-1,3,5-triazapenta-1,4-dien	33089-61-1	Solid	LKT.Labs, Inc	No	IV	US-EPA	Triazapentadien compound
088	P3-007	3-phenoxybenzyl alcohol	13826-35-2	Liquid	Sigma Aldrich	No	III	NICEATM	Alcohol
089	P3-081	3,3-dimethylpentane	562-49-2	Liquid	Sigma Aldrich	No	IV	STE review	Hydrocarbon
090	P3-015	3,4-dimethoxy benzaldehyde	120-14-9	Solid	Sigma Aldrich	No	III	NICEATM	Aldehyde
091	P2-006	3,4,4'-trichlorocarbanilide	101-20-2	Solid	Sigma Aldrich	No	IV	Cosing	Halogen compound, Amide
092	P3-035	4-(methylmercapto)benzaldehyde	3446-89-7	Liquid	Sigma Aldrich	No	IV	ECETOX	Thio compound, Aldehyde
093	P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	118-82-1	Solid	Sigma Aldrich	No	IV	ECETOC	Phenol compound
094	P3-011	6-hydroxy-2,4,5-triaminopyrimidine sulfate	1603-02-7	Solid	Wako Pure	No	IV	Cosing	Heterocyclic compound(salt)
095	P3-014	cellulose, 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride	68610-92-4	Solid	Sigma Aldrich	No	III	J&J	Quaternary ammonium compound, Synthetic polymer

096	P3-064	cyclohexanone	108-94-1	Liquid	Sigma Aldrich	No	III	STE review	Ketone, Hydrocarbon(cyclic)
097	P3-006	dioctyl ether	629-82-3	Liquid	Sigma Aldrich	No	IV	Cognis	Ether
098	P3-003	dipropyl disulfide	629-19-6	Liquid	Sigma Aldrich	No	IV	STE review	Disulfide compound
099	P3-056	ethyl acetate	141-78-6	Liquid	Sigma Aldrich	No	III	STE review	Ester
100	P2-010	ethyl thioglycolate	623-51-8	Liquid	Sigma Aldrich	No	III	NICEATM	Thiol compound, Ester
101	P3-085	gluconolactone	90-80-2	Solid	Wako Pure	No	IV	NICEATM	Polyol
102	P3-089	glycerol	56-81-5	Liquid	Wako Pure	No	IV	STE review	Polyol
103	P3-008	glycidyl methacrylate	106-91-2	Liquid	Sigma Aldrich	No	III	STE review	Methacrylate, Ester
104	P3-002	iso-octylthioglycolate	25103-09-7	Liquid	Wako Pure	No	IV	ECETOX	Thio compound, Ester
105	P3-063	isopropyl bromide	75-26-3	Liquid	Wako Pure	No	IV	STE review	Halogen compound
106	P3-057	isopropyl myristate	110-27-0	Liquid	Wako Pure	No	IV	STE review	Ester
107	P3-086	methyl amyl ketone (2-heptanol)	110-43-0	Liquid	Wako Pure	No	III	STE review	Ketone
108	P3-082	methyl cyclopentane	96-37-7	Liquid	TCI	No	III	STE review	Hydrocarbon
109	P3-088	methyl isobutyl ketone(4-methyl 2-pentanol)	108-10-1	Liquid	Sigma Aldrich	No	III	STE review	Ketone
110	P3-010	n,n-dimethylguanidine sulfate	598-65-2	Solid	Sigma Aldrich	No	III	STE review	Organic salt
111	P2-001	piperonylbutoxide	51- 03- 6	Liquid	Sigma Aldrich	No	III	US-EPA	Ether
112	P3-012	polyethylene hydrogenated caster oil (40E.O.)	61788-85-0	Solid	Sigma Aldrich	No	IV	STE review	Surfactant (nonionic)
113	P2-005	potassium tetrafluoroborate	14075-53-7	Solid	Sigma Aldrich	No	IV	ECETOC	Inorganic salt, Halogen compound
114	P2-017	propyl 4-hydroxybenzoate	94-13-3	Solid	Sigma Aldrich	No	III	LNS	Ester, Phenol
115	P3-098	silic acid, dehydrogenate	7699-41-4	Solid	Wako Pure	No	IV	Ohno(1999)	Silicon compound
116	P2-014	sodium hydrogensulfite	7631-90-5	Solid	Sigma Aldrich	No	III	NICEATM	Inorganic salt
117	P3-092	tween20	9005-64-5	Liquid	Sigma Aldrich	No	III	STE review	Surfactant (nonionic)

Table 5. Distribution of test substances (lank of *in vivo*) selected for SIRC-CVS:TEA test validation study

GHS				Total
Category 1	Category 2A	Category 2B	No	
29	20	22	46	117

EPA				Total
I	II	III	IV	
27	19	44	27	117

Table 6. Distribution of test substances (chemical properties) selected for SIRC-CVS:TEA test validation study

Solid	Liquid	Total
49	68	117

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Analysis for prediction

Table R-4.3.5. Eye irritation potential of test substances in the SIRC-CVS:TEA validation phase III study

Chemical code	Laboratory	Name of test substance	Run 1	Run 2	Final Evaluation
P3-001	B	2-ethoxyethyl methacrylate	P	P	P
P3-002	C	iso-octylthioglycolate	N	N	N
P3-003	A/B/C	dipropyl disulfide	P/P/N	P/P/N	P
P3-004	C	1-bromo-octane	P	P	P
P3-005	A/B/C	2-(2-ethoxyethoxy)ethanol	N/N/N	N/N/N	N
P3-006	C	dioctyl ether	P	P	P
P3-007	C	3-phenoxybenzyl alcohol	P	P	P
P3-008	B	glycidyl methacrylate	P	P	P
P3-009	C	2-ethylhexylthioglycolate	N	N	N
P3-010	A/B/C	n,n-dimethylguanidine sulfate	N/P/N	N/P/N	N
P3-011	C	6-hydroxy-2,4,5-triaminopyrimidine Sulfate	P	P	P
P3-012	A/B/C	polyethylene hydrogenated caster oil (40E.O.)	N/P/N	N/P/N	N
P3-013	C	2,2'-methylene-bis-(6-(2Hbenzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)	N	N	N
P3-014	C	cellulose 2-(2-hydroxy-3-(trimethylammonio)propoxy ethyl ether chloride	N	N	N
P3-015	C	3,4-dimethoxy benzaldehyde	P	P	P
P3-016	C	3-chloropropionitrile	P	P	P
P3-017	C	2-methyl-1-pentanol	N	N	N
P3-018	C	ethyl-2-methylacetoacetate	N	N	N
P3-019	A/B/C	diethyl toluamide	P/P/P	P/P/P	P
P3-020	A/B/C	4-nitrobenzoic acid	N/N/N	N/N/N	N
P3-021	C	sodium chloroacetate	P	P	P
P3-022	A	2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	P	P	P
P3-023	C	-	-	-	-
P3-024	A/B/C	2-amino-3-hydroxy pyridine	P/P/P	P/P/P	P
P3-025	C	sodium benzoate	N	N	N
P3-026	C	methylthioglycolate	P	P	P
P3-027	A	3-(2-aminoethylamino)propyl]trimethoxysilane	P	P	P
P3-028	A/B/C	tetraethylene glycol	P/P/P	P/P/P	P
P3-029	A/B/C	dodecanoic acid	P/P/P	P/P/P	P
P3-030	C	1,2-benzisothiazol-3(2H)-one	P	P	P
P3-031	C	2-hydroxy-1,4-naphthoquinone	P	P	P
P3-032	C	disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	P	P	P
P3-033	A/B/C	gamma-butyrolactone	N/N/N	N/N/N	N
P3-034	C	1-methylpropyl benzene	N	N	N
P3-035	C	4-(methylmercapto)benzaldehyde	P	P	P
P3-036	C	1,9-decaine	P	P	P
P3-037	C	2,4-dimethyl-3-pentanol	N	N	N
P3-038	C	1-ethyl-3-methylimidazolium ethylsulfate	N	N	N
P3-039	C	1,2,4-triazole,sodium salt	P	P	P
P3-040	C	4,4'-(4,5,6,7-tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl) bis [2,6-dibromophenol]	P	P	P
P3-041	C	benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCl	P	P	P
P3-042	A	1-(9H-carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	P	P	P
P3-043	B	3-methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	P	P	P
P3-044	C	isopropyl acetoacetate	N	N	N
P3-045	A	(3R,4R)-4-acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	P	P	P
P3-046	B	1-octanol	P	P	P
P3-047	B	2-benzyloxyethanol	N	N	N
P3-048	B	butanol	N	N	N
P3-049	B	isobutyl alcohol	P	P	P
P3-050	B	isopropyl alcohol	N	N	N

P3-051	B	myristyl alcohol	P	P	P
P3-052	B	hexyl cinnamic aldehyde	P	P	P
P3-053	B	n-butanal	P	P	P
P3-054	B	monoethanolamine	P	P	P
P3-055	B	m-phenylenediamine	P	P	P
P3-056	B	ethyl acetate	N	N	N
P3-057	B	isopropyl myristate	N	N	N
P3-058	B	methoxyethyl acrylate	P	P	P
P3-059	B	methyl acetate	N	N	N
P3-060	B	methyl cyanoacetate	N	N	N
P3-061	B	imidazole	P	P	P
P3-062	B	pyridine	N	N	N
P3-063	B	isopropyl bromide	N	N	N
P3-064	B	cyclohexanone	N	N	N
P3-065	B	2-methylbutyric acid	N	N	N
P3-066	B	calcium thioglycolate trihydrate	-	-	-
P3-067	B	citric acid	P	P	P
P3-068	B	potassium sorbate	N	N	N
P3-069	B	sodium salicylate	N	N	N
P3-070	B	distearyldimethylammonium chloride	P	P	P
P3-071	B	n-lauroylsarcosine sodium salt	P	P	P
P3-072	B	sodium lauryl sulfate	P	P	P
P3-073	A	triton X-100 (5%)	P	P	P
P3-074	A	2-ethylhexyl p-dimethyl-amino benzoate	P	P	P
P3-075	A	promethazine hydrochloride	P	P	P
P3-076	A	2-ethyl-1-hexanol	P	P	P
P3-077	A	3-methoxy-1.2-propanediol	N	N	N
P3-078	A	cyclohexanol	N	N	N
P3-079	A	ethanol	N	N	N
P3-080	A	n-hexanol	N	N	N
P3-081	A	3,3-dimethylpentane	P	P	P
P3-082	A	methyl cyclopentane	P	P	P
P3-083	A	toluene	N	N	N
P3-084	A	acetone	N	N	N
P3-085	A	gluconolactone	N	N	N
P3-086	A	methyl amyl ketone (2-heptanol)	N	N	N
P3-087	A	methyl ethyl ketone (2-butanone)	N	N	N
P3-088	A	methyl isobutyl ketone(4-methyl 2-pentanol)	N	N	N
P3-089	A	glycerol	N	N	N
P3-090	A	cetylpyridinium bromide	P	P	P
P3-091	C	triton X-100	P	P	P
P3-092	C	tween20	P	P	P
P3-093	A	sodium hydroxide	P	P	P
P3-094	A	glycolic acid	N	N	N
P3-095	A	3,3-dithiodipropionic acid	N	N	N
P3-096	A	sucrose fatty acid ester	P	P	P
P3-097	A	methyl para-Hydroxybenzoate	P	P	P
P3-098	A	silic acid	P	P	P
P3-099	A	benzyl alcohol	N	N	N
P3-100	A	lactic acid	N	N	N

*1: N; Negative, P; Positive

*2: Eye irritaion potential of common test substances were expressed as a representative of three laboratories.

*3: -; Inapplicable

Table R-4.1.1. Means and standard deviations of IC50s for test substances, relative controls and positive controls in the SIRC-CVS:TEA validation phase I study

Chemical No.	Name of test substance		Laboratory A IC50 ug/mL			Laboratory A (Retest) IC50 ug/mL			Laboratory B IC50 ug/mL			Laboratory C IC50 ug/mL		
			Test Sbstance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control
C01	acetoacetate	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	>5000	1677.70	172.07	3296.53	1234.47	83.17	3642.03	1551.63	87.20	>5000	1349.47	82.57
		SD	-	133.12	10.33	292.34	306.25	3.27	142.30	376.15	4.22	-	62.42	1.36
C02	Safflower oil	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	>5000	1613.37	170.33	>5000	1264.97	86.60	>5000	1579.80	84.67	>5000	1365.47	80.23
		SD	-	426.35	6.12	-	175.77	4.04	-	31.82	4.84	-	23.28	0.06
C03	3-Chloropropionitrile	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	60.60	2386.13	179.70	45.57	1370.83	84.40	38.93	1339.37	88.60	48.53	1390.33	86.70
		SD	10.12	965.97	5.99	6.25	176.47	8.34	6.92	285.34	1.30	1.07	51.83	7.35
C04	Sodium dehydroacetate	N	3	3	3	3	3	3	3	3	3	3	3	3
		Mean	2024.17	1915.33	161.63	854.27	1252.77	84.07	720.77	1646.50	87.50	1026.60	1425.80	78.50
		SD	485.58	314.52	38.54	100.83	188.79	3.50	235.31	75.72	2.78	46.42	33.36	0.44

* N; Number of runs

Table R-4.1.2. Means and standard deviations of IC50s for relative controls and positive controls

	Laboratory A		Laboratory A (Retest)		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	4	4	4	4	4	4	4	4
Mean	1898.13	170.93	1280.76	84.56	1529.33	86.99	1382.77	82.00
SD	350.30	7.42	61.34	1.46	132.74	1.66	33.25	3.55

* N; Number of relative controls and positive controls

* IC50 was expressed as ug/mL.

Table R-4.1.3. Eye irritation potential of test substances in the SIRC-CVS:TEA validation phase I study

Chemical No.	Name of test substances	Laboratory A			Laboratory A (Retest)			Laboratory B			Laboratory C			Lead laboratory
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
C01	acetoacetate	N	N	N	N	N	N	N	N	N	N	N	N	N
C02	Safflower oil	N	N	N	N	N	N	N	N	N	N	N	N	N
C03	3-Chloropropionitrile	P	P	P	P	P	P	P	P	P	P	P	P	P
C04	Sodium dehydroacetate	N	N	N	P	P	P	P	P	P	P	P	P	P

* N; Negative, P; Positive,

Table R-4.2.1. The IC50s for test substances, relative controls and positive controls in the SIRC-CVS:TEA validation phase II

Chemical code	Set	Laboratory A			Laboratory B			Laboratory C		
		IC50 ug/mL			IC50 ug/mL			IC50 ug/mL		
		Test Substance	Relative Control	Positive Control	Test Substance	Relative Control	Positive Control	Test Sbstance	Relative Control	Positive Control
Phase II-A										
P2-001	1	141.00	1478.77	85.83	288.07	1295.93	88.77	298.30	1512.17	84.57
	2	71.73	1235.60	88.27	260.17	1327.03	93.77	266.57	1453.57	88.40
	3	101.80	1589.07	87.90	452.27	1177.97	91.83	282.87	1304.20	84.83
	Mean	104.84	1434.48	87.33	333.50	1266.98	91.46	282.58	1423.31	85.93
P2-002	1	>5000	1602.33	84.53	>5000	1402.37	89.93	>5000	1755.00	93.67
	2	>5000	1281.53	89.30	>3989.1	1330.83	96.23	>5000	1198.70	92.47
	3	>5000	1384.27	88.20	>5000	1274.03	92.30	>5000	1525.30	89.47
	Mean	>5000	1422.71	87.34	>3989.1	1335.74	92.82	>5000	1493.00	91.87
P2-003	1	4130.03	1517.03	86.20	3188.87	1320.57	90.90	>4673.4	1808.87	90.63
	2	3899.03	1452.13	88.73	3654.77	1357.83	93.17	>5000	1348.53	89.73
	3	3931.70	1513.40	88.67	3025.03	1290.27	92.37	>5000	1338.87	86.60
	Mean	3986.92	1494.19	87.87	3289.56	1322.89	92.14	>4673.4	1498.76	88.99
P2-004	1	1342.27	1518.23	89.37	1147.50	1413.97	93.20	1409.60	1525.10	80.33
	2	925.50	1352.43	90.73	778.47	1184.47	92.53	1216.40	1531.50	85.33
	3	1151.90	1440.13	86.43	1061.47	1295.83	90.80	1099.60	1508.13	86.57
	Mean	1139.89	1436.93	88.84	995.81	1298.09	92.18	1241.87	1521.58	84.08
P2-005	1	1791.60	1362.47	84.63	1949.60	1273.83	88.43	>5000	1837.10	95.87
	2	1783.17	1288.30	88.20	3630.77	1379.60	88.43	>5000	1532.13	90.93
	3	1868.60	1341.87	85.50	>3506.9	1256.43	90.27	>4952.0	1264.60	91.33
	Mean	1814.46	1330.88	86.11	>1949.6	1303.29	89.04	>4952.0	1544.61	92.71
Phase II-B										
P2-006	1	<39.1	1223.40	85.67	<39.1	1323.80	86.00	<39.1	1768.27	91.50
	2	<39.1	1334.50	83.60	<39.1	1122.43	92.80	<39.1	1692.43	98.77
	3	<39.1	1221.33	82.13	<39.1	1256.37	94.83	<39.1	1710.83	87.37
	Mean	<39.1	1259.74	83.80	<39.1	1234.20	91.21	<39.1	1723.84	92.54
P2-007	1	266.20	1452.87	85.27	99.30	1227.27	82.93	519.13	1613.90	90.67
	2	506.50	1312.67	86.23	110.30	1214.47	88.37	421.67	1718.20	92.53
	3	906.33	1373.43	78.30	408.30	1242.57	93.40	421.50	1432.00	85.20
	Mean	559.68	1379.66	83.27	205.97	1228.10	88.23	454.10	1588.03	89.47
P2-008	1	>5000	1417.17	88.13	>2345.6	1221.30	86.53	>5000	1672.70	88.97
	2	3365.35	1356.67	80.43	>5000	1224.97	89.97	>5000	1715.73	93.53
	3	3670.67	1239.17	81.17	>5000	1104.43	90.80	>5000	1510.83	85.27
	Mean	>3365.35	1337.67	83.24	>2345.6	1183.57	89.10	>5000	1633.09	89.26
P2-009	1	>4865.3	1345.63	86.83	3561.93	1227.47	89.87	>5000	1524.27	93.67
	2	>3048.1	1215.83	83.63	3528.17	1248.63	89.30	>5000	1681.47	95.30
	3	>4537.5	1457.47	83.90	3661.80	1166.23	92.73	>5000	1689.60	93.07
	Mean	>3048.1	1339.64	84.79	3583.97	1214.11	90.63	>5000	1631.78	94.01
P2-010	1	<39.1	1618.87	89.40	<51.4	1220.37	89.23	<39.1	1421.63	96.70
	2	<39.1	1333.93	85.30	<39.1	1237.93	90.80	<39.1	1619.03	92.90
	3	<39.1	1407.60	85.90	138.30	1103.27	92.80	<39.1	1691.83	91.17
	Mean	<39.1	1453.47	86.87	<138.3	1187.19	90.94	<39.1	1577.50	93.59

P2-011	1	239.43	1427.67	85.70	109.83	1160.10	89.67	227.00	1755.53	93.23
	2	123.70	1298.27	83.53	121.50	1094.97	91.40	243.67	1543.40	97.40
	3	130.17	1322.27	86.03	115.00	1222.50	93.93	176.37	1449.23	86.73
	Mean	164.43	1349.40	85.09	115.44	1159.19	91.67	215.68	1582.72	92.46
P2-012	1	3575.53	1372.63	84.27	3615.73	1188.53	87.27	4386.23	1652.83	107.63
	2	3630.43	1268.97	82.40	3721.63	1256.27	91.40	4246.53	1738.87	95.70
	3	2965.90	1298.63	82.43	4259.13	1049.27	94.17	4589.23	1455.00	87.20
	Mean	3390.62	1313.41	83.03	3865.50	1164.69	90.94	4407.33	1615.57	96.84
P2-013	1	434.83	1470.87	87.40	398.80	1197.70	88.63	352.80	1670.07	95.80
	2	1055.60	1329.70	85.57	544.13	1339.73	92.37	298.50	1600.60	95.03
	3	703.80	1127.00	82.33	336.27	1090.73	91.73	177.87	1326.67	89.80
	Mean	731.41	1309.19	85.10	426.40	1209.39	90.91	276.39	1532.44	93.54
P2-014	1	91.93	1434.87	84.70	<45.1	1135.30	90.93	55.20	1639.87	91.10
	2	82.47	1247.37	84.90	64.77	1248.77	91.00	70.30	1683.13	90.50
	3	115.20	1471.33	80.77	<44.4	1190.53	91.20	100.67	1803.03	91.07
	Mean	96.53	1384.52	83.46	<64.77	1191.53	91.04	75.39	1708.68	90.89
P2-015	1	664.00	1473.57	81.47	452.27	1142.77	82.47	1288.70	1553.47	89.97
	2	1152.17	1172.77	83.87	395.00	1203.53	93.83	1054.47	1495.20	94.37
	3	809.23	1232.50	82.27	283.93	1180.93	94.23	1279.93	1754.03	87.53
	Mean	875.13	1292.94	82.53	377.07	1175.74	90.18	1207.70	1600.90	90.62
P2-016	1	796.67	1300.10	82.53	618.03	1203.53	88.97	1419.93	1669.03	95.47
	2	715.13	1364.63	87.13	632.63	1168.57	87.80	1191.07	1850.53	96.93
	3	605.57	1385.97	82.33	629.70	1149.63	92.83	1311.20	1853.70	92.63
	Mean	705.79	1350.23	84.00	626.79	1173.91	89.87	1307.40	1791.09	95.01
P2-017	1	57.67	1298.20	86.83	68.53	1282.07	90.20	49.43	1699.50	97.00
	2	92.73	1332.43	83.87	44.03	1177.10	92.10	90.17	1487.10	94.13
	3	66.53	1260.83	83.23	43.67	1202.43	91.50	<39.1	1589.73	90.93
	Mean	72.31	1297.16	84.64	52.08	1220.53	91.27	<90.17	1592.11	94.02
P2-018	1	<46.4	1226.20	86.13	<39.1	1305.83	93.77	<39.1	1606.07	94.53
	2	69.93	1372.13	82.80	<39.1	1145.77	88.13	<39.1	1644.93	92.83
	3	<65.0	1416.70	84.97	<39.1	1150.93	93.90	<39.1	1477.17	100.90
	Mean	<69.93	1338.34	84.63	<39.1	1200.84	91.93	<39.1	1576.06	96.09
P2-019	1	359.37	1272.43	84.77	385.97	1387.10	89.87	1471.77	1679.23	98.20
	2	567.03	1301.33	85.80	94.77	1311.97	88.73	1268.13	1676.53	89.30
	3	397.97	1254.83	79.63	418.47	1198.10	93.30	1208.87	1720.37	90.50
	Mean	441.46	1276.20	83.40	299.73	1299.06	90.63	1316.26	1692.04	92.67
P2-020	1	3074.00	1232.70	87.07	1729.57	1392.87	88.00	4013.20	1721.57	94.60
	2	2633.47	1331.47	86.57	2187.27	1228.00	90.53	3593.00	1851.43	95.83
	3	3002.77	1364.20	80.87	2109.37	1196.90	92.03	3504.93	1749.60	94.63
	Mean	2903.41	1309.46	84.83	2008.73	1272.59	90.19	3703.71	1774.20	95.02

*; Each IC50 for test substances, relative controls and positive controls was expressed as an average every set.

Table R-4.2.2. Means and standard deviations of IC50s for relative controls and

	Laboratory A		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	60	60	60	60	60	60
Mean	1355.51	85.01	1232.08	90.82	1605.07	91.98
SD	106.68	2.69	84.18	2.68	154.61	4.57

* N; Numbers of each test substances, relative controls and positive controls
* IC50 was expressed as ug/mL.

Table R-4.2.3. Eye irritation potential of test substances in the SIRC-CVS:TEA validation phase II study

Chemical code	Name of test substance	Set	Laboratory A			Laboratory B			Laboratory C			Final Evaluation
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
P2-001	piperonylbutoxide	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-002	2,5-dimethylhexaediol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-004	ammonium nitrate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-005	potassium tetrafluoroborate	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-006	3,4,4'-trichlorocarbanilide	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-007	1-bromohexane	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-009	propylene glycol propyl ether	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-010	ethyl thioglycolate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-011	sodium oxalate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-012	2-phospho-L-ascorbic acid trisodium salt	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	
P2-013	1-bromo-4-chlorobutane	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-014	sodium hydrogensulfite	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-015	isobutyraldehyde	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-016	1-naphthaleneacetic acid	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-017	propyl 4-hydroxybenzoate	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-019	camphene	1	P	P	P	P	P	P	P	P	P	P
		2	P	P	P	P	P	P	P	P	P	
		3	P	P	P	P	P	P	P	P	P	
P2-020	cyclopentanol	1	N	N	N	N	N	N	N	N	N	N
		2	N	N	N	N	N	N	N	N	N	
		3	N	N	N	N	N	N	N	N	N	

*N; Negative, P; Positive

Table R-4.3.1. The IC50s for test substances, relative controls and positive controls at laboratory A in the SIRC-CVS:TEA validation phase III study

Chemical Code	Chemical Codei in Laboratory A	Test Sbstance (IC50 ug/mL)			Relative Control (IC50 ug/mL)			Positive Control (IC50 ug/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
P3-003*2	**SA82**	212.80	259.20	236.00	1069.30	1081.90	1075.60	93.70	90.20	91.95
P3-005*2	**SA89**	>5000	>5000	>5000	1057.70	1275.50	1166.60	86.70	95.50	91.10
P3-010*2	**SA90**	1323.30	1653.30	1488.30	1040.30	1053.70	1047.00	88.30	91.40	89.85
P3-012*2	**SA84**	1460.90	1541.20	1501.05	1040.10	1088.50	1064.30	87.30	93.80	90.55
P3-019*2	**SA88**	155.80	202.50	179.15	1096.70	1219.70	1158.20	86.30	90.60	88.45
P3-020*2	**SA83**	1347.40	1588.50	1467.95	1076.00	1044.60	1060.30	85.60	94.40	90.00
P3-022	**SA61**	<39.1	42.40	<42.4	1095.40	1159.10	1127.25	86.90	90.80	88.85
P3-024*2	**SA86**	151.80	182.90	167.35	1039.00	1095.20	1067.10	89.20	91.40	90.30
P3-027	**SA96**	484.90	869.10	677.00	1040.50	1417.70	1229.10	86.70	91.20	88.95
P3-028*2	**SA85**	<39.1	<39.1	<39.1	1037.20	1101.00	1069.10	89.90	90.50	90.20
P3-029*2	**SA87**	42.20	46.00	44.10	1073.70	1082.10	1077.90	89.80	91.50	90.65
P3-033*2	**SA81**	>5000	>5000	>5000	1010.50	1257.20	1133.85	94.00	85.90	89.95
P3-042	**SA62**	<39.1	<39.1	<39.1	1206.60	1133.10	1169.85	83.70	92.20	87.95
P3-045	**SA63**	117.70	128.70	123.20	1031.80	1121.70	1076.75	78.10	91.90	85.00
P3-073	**SA65**	444.10	470.60	457.35	1085.60	1084.00	1084.80	80.30	90.70	85.50
P3-074	**SA76**	52.10	47.50	49.80	1056.30	1063.60	1059.95	88.20	85.20	86.70
P3-075	**SA64**	<39.1	<39.1	<39.1	1203.10	1010.60	1106.85	85.20	87.00	89.10
P3-076	**SA67**	946.30	761.90	854.10	1038.10	1054.50	1046.30	94.20	80.60	87.40
P3-077	**SA80**	>5000	>5000	>5000	1194.40	1253.60	1224.00	91.50	92.00	91.75
P3-078	**SA70**	1941.10	2253.70	2097.40	1068.90	1138.00	1103.45	96.80	91.60	94.20
P3-079	**SA91**	>5000	>5000	>5000	1033.50	1412.30	1222.90	84.20	92.70	88.45
P3-080	**SA72**	1082.20	1666.50	1374.35	1010.20	1030.00	1020.10	90.90	85.80	88.35
P3-081	**SA78**	84.60	352.00	218.30	1114.00	1130.40	1122.20	90.80	91.20	91.00
P3-082	**SA98**	777.30	857.30	817.30	1152.50	1335.80	1244.15	85.70	91.70	88.70
P3-083	**SA69**	>5000	>5000	>5000	1090.90	1168.30	1129.60	92.10	93.30	92.70
P3-084	**SA92**	4903.10	>5000	>4903.1	1073.70	1446.40	1260.05	87.30	89.70	88.50
P3-085	**SA97**	3331.80	3672.40	3502.10	1036.10	1149.10	1092.60	84.40	92.80	88.60
P3-086	**SA71**	2243.50	3624.50	2934.00	1119.60	1151.00	1135.30	92.80	92.30	92.55
P3-087	**SA94**	>5000	3648.00	>3648	1032.80	1408.90	1220.85	87.60	88.00	87.80
P3-088	**SA68**	>5000	>5000	>5000	1085.90	1201.10	1143.50	86.60	90.20	88.40
P3-089	**SA79**	>5000	>5000	>5000	1059.50	1076.60	1068.05	90.70	93.20	91.95
P3-090	**SA75**	<39.1	<39.1	<39.1	1172.00	1186.00	1179.00	89.10	90.80	89.95
P3-093	**SA74**	682.60	866.20	774.40	1053.80	1186.70	1120.25	93.00	93.10	93.05
P3-094	**SA95**	1429.50	1504.20	1466.85	1043.00	1277.70	1160.35	87.20	95.80	91.50

P3-095	**SA73**	1864.40	1696.90	1780.65	1149.40	1065.10	1107.25	91.40	92.40	91.90
P3-096	**SA100**	94.30	67.00	80.65	1058.70	1040.70	1049.70	88.10	89.50	88.80
P3-097	**SA99**	132.40	274.50	203.45	1085.70	1103.20	1094.45	88.70	84.60	86.65
P3-098	**SA93**	190.00	168.80	179.40	1146.30	1024.90	1085.60	87.10	89.40	88.25
P3-099	**SA66**	1133.60	1574.30	1353.95	1016.00	1209.40	1112.70	86.80	92.30	89.55
P3-100	**SA77**	2043.90	2606.80	2325.35	1031.60	1100.90	1066.25	91.00	91.00	91.00

*1; Each IC50 for test substances, relative controls and positive controls was expressed as an average every set.

*2; Ten test substances were shared in Laboratory A, B and C.

Table R-4.3.2. The IC50s for test substances, relative controls and positive controls at laboratory B in the SIRC-CVS:TEA validation phase III study

Chemical Code	Chemical Code in Laboratory B	Test Sbstance (IC50 ug/mL)			Relative Control (IC50 ug/mL)			Positive Control (IC50 ug/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
P3-001	**SB62**	119.60	122.60	121.10	1673.80	1571.90	1622.85	89.80	90.40	90.10
P3-003*2	**SB79**	695.20	672.80	684.00	1352.70	1038.20	1195.45	93.90	91.40	92.65
P3-005*2	**SB72**	>5000	>5000	>5000	1077.80	1260.80	1169.30	87.30	86.80	87.05
P3-008	**SB63**	17.70	22.80	20.25	1186.90	1573.00	1379.95	91.60	95.40	93.50
P3-010*2	**SB71**	626.80	535.20	581.00	1394.20	1488.50	1441.35	91.80	91.40	91.60
P3-012*2	**SB77**	814.20	768.80	791.50	1089.70	1433.60	1261.65	89.40	86.90	88.15
P3-019*2	**SB73**	265.50	187.40	226.45	1193.40	1296.80	1245.10	92.30	87.10	89.70
P3-020*2	**SB78**	2923.40	2017.90	2470.65	1026.60	1305.70	1166.15	79.60	85.80	82.70
P3-024*2	**SB75**	71.70	63.10	67.40	1155.30	1095.60	1125.45	92.40	89.70	91.05
P3-028*2	**SB76**	6.90	11.70	9.30	1455.30	1580.90	1518.10	86.80	93.50	90.15
P3-029*2	**SB74**	<39.1	<39.1	<39.1	1141.60	1274.10	1207.85	80.80	88.60	84.70
P3-033*2	**SB80**	4864.90	4126.60	4495.75	1120.40	1081.20	1100.80	92.10	85.30	88.70
P3-043	**SB61**	163.30	191.90	177.60	1572.90	1387.20	1480.05	78.10	91.50	84.80
P3-046	**SB64**	783.50	346.30	564.90	1281.80	1239.30	1260.55	92.80	91.30	92.05
P3-047	**SB65**	1599.20	1570.60	1584.90	1282.40	1430.40	1356.40	91.90	89.30	90.60
P3-048	**SB66**	2203.10	2105.00	2154.05	1298.60	1277.30	1287.95	91.90	92.60	92.25
P3-049	**SB67**	772.60	414.80	593.70	1668.10	1571.90	1620.00	78.40	89.70	84.05
P3-050	**SB68**	>5000	>5000	>5000	1275.10	1154.20	1214.65	92.10	86.70	89.40
P3-051	**SB69**	128.70	312.50	220.60	1334.10	1571.00	1452.55	94.90	93.10	94.00
P3-052	**SB70**	92.10	98.30	95.20	1302.20	1534.70	1418.45	94.40	89.00	91.70
P3-053	**SB81**	720.40	213.40	466.90	1068.60	1704.30	1386.45	81.60	92.80	87.20
P3-054	**SB82**	195.50	169.90	182.70	1319.00	1133.40	1226.20	89.00	91.10	90.05
P3-055	**SB83**	17.30	20.60	18.95	1071.60	1527.10	1299.35	89.90	89.80	89.85
P3-056	**SB84**	>5000	>5000	>5000	1359.10	1262.40	1310.75	87.00	84.80	85.90
P3-057	**SB85**	>5000	>5000	>5000	1173.10	1365.70	1269.40	92.30	92.50	92.40
P3-058	**SB86**	11.30	13.90	12.60	1188.30	1569.80	1379.05	87.30	88.70	88.00
P3-059	**SB87**	>5000	>5000	>5000	1101.00	1408.10	1254.55	88.90	89.50	89.20
P3-060	**SB88**	1343.60	1473.80	1408.70	1103.50	1431.30	1267.40	78.40	87.00	82.70
P3-061	**SB89**	620.50	604.40	612.45	1084.00	1028.60	1056.30	89.50	82.70	86.10
P3-062	**SB90**	1729.40	1824.40	1776.90	1291.70	1472.40	1382.05	92.50	89.70	91.10
P3-063	**SB91**	>2500	>2500	>2500	1251.80	1457.50	1354.65	88.90	90.20	89.55
P3-064	**SB92**	1619.00	1403.10	1511.05	1262.80	1329.40	1296.10	89.90	90.00	89.95
P3-065	**SB93**	1604.10	1429.40	1516.75	1396.40	1067.30	1231.85	88.50	88.70	88.60
P3-066	**SB94**	-	-	-	-	-	-	-	-	-

P3-067	**SB95**	875.30	807.70	841.50	1257.50	1405.50	1331.50	78.10	92.00	85.05
P3-068	**SB96**	1584.60	1468.40	1526.50	1176.90	1395.80	1286.35	93.30	87.90	90.60
P3-069	**SB97**	1276.00	1587.50	1431.75	1112.00	1368.80	1240.40	93.80	90.60	92.20
P3-070	**SB98**	3.60	14.00	8.80	1553.30	1683.60	1618.45	80.30	91.10	85.70
P3-071	**SB99**	97.50	70.70	84.10	1445.10	1194.80	1319.95	95.50	90.00	92.75
P3-072	**SB100**	57.20	60.10	58.65	1076.20	1605.60	1340.90	93.40	91.40	92.40

*1; Each IC50 of test substances, relative controls and positive controls was expressed as an average every set.

*2; Ten test substances were shared in Laboratory A, B and C.

*3: -; Inapplicable

Table R-4.3.3. The IC50s for test substances, relative controls and positive controls at laboratory C in the SIRC-CVS:TEA validation phase III study

Chemical Code	Chemical Code in Laboratory C	Test Sbstance (IC50 ug/mL)			Relative Control (IC50 ug/mL)			Positive Control (IC50 ug/mL)		
		Run 1	Run 2	Mean	Run 1	Run 2	Mean	Run 1	Run 2	Mean
P3-002	**SC72**	>2500	>2500	>2500	1628.00	1753.10	1690.55	126.10	123.50	124.80
P3-003*2	**SC61**	>2500	>2500	>2500	1177.80	1413.70	1295.75	87.50	102.00	94.75
P3-004	**SC74**	105.80	244.30	175.05	1085.20	1618.10	1351.65	123.80	126.50	125.15
P3-005*2	**SC62**	>5000	>5000	>5000	1256.90	1375.10	1316.00	109.00	119.60	114.30
P3-006	**SC77**	845.80	1302.60	1074.20	1248.60	1555.90	1402.25	129.50	126.00	127.75
P3-007	**SC79**	77.40	35.40	56.40	1181.10	1747.40	1464.25	136.50	129.90	133.20
P3-009	**SC80**	>2500	>2500	>2500	1256.90	1665.80	1461.35	109.00	111.90	110.45
P3-010*2	**SC63**	3464.60	2748.70	3106.65	1831.10	1108.60	1469.85	120.60	87.50	104.05
P3-011	**SC81**	<39.1	<39.1	<39.1	1285.60	1418.20	1351.90	180.80	137.30	159.05
P3-012*2	**SC64**	3210.00	2765.90	2987.95	1851.80	1415.30	1633.55	117.10	119.50	118.30
P3-013	**SC82**	>5000	>5000	>5000	1186.40	1123.90	1155.15	125.70	140.60	133.15
P3-014	**SC83**	>5000	>5000	>5000	1400.10	1064.40	1232.25	114.80	133.40	124.10
P3-015	**SC84**	328.00	218.10	273.05	1071.90	1250.00	1160.95	141.60	133.20	137.40
P3-016	**SC85**	<39.1	40.40	<40.4	1017.50	1013.80	1015.65	140.10	130.60	135.35
P3-017	**SC87**	>2500	>2500	>2500	1353.90	1365.50	1359.70	123.70	138.30	131.00
P3-018	**SC88**	>5000	>5000	>5000	1154.10	1269.40	1211.75	116.70	121.10	118.90
P3-019*2	**SC65**	285.10	246.00	265.55	1159.40	1913.30	1536.35	121.20	118.80	120.00
P3-020*2	**SC66**	1946.00	2991.20	2468.60	1864.20	1573.00	1718.60	129.60	113.20	121.40
P3-021	**SC90**	<39.1	39.80	<39.8	1115.00	1166.50	1140.75	120.20	143.20	131.70
P3-023	**SC91**	-	-	-	-	-	-	-	-	-
P3-024*2	**SC68**	172.90	55.30	114.10	1182.30	1678.20	1430.25	136.10	90.90	113.50
P3-025	**SC92**	>5000	>5000	>5000	1017.10	1112.30	1064.70	137.20	124.90	131.05
P3-026	**SC93**	<39.1	<39.1	<39.1	1674.10	1106.50	1390.30	120.20	129.00	124.60
P3-028*2	**SC69**	<39.1	<39.1	<39.1	1822.50	1787.80	1805.15	116.70	82.60	99.65
P3-029*2	**SC70**	55.70	33.20	44.45	1786.40	1433.90	1610.15	128.00	113.90	120.95
P3-030	**SC97**	<19.5	<19.5	<19.5	1061.00	1169.40	1115.20	124.90	136.40	130.65
P3-031	**SC89**	85.90	86.50	86.20	1259.60	1112.60	1186.10	111.50	123.10	117.30
P3-032	**SC98**	41.70	55.90	48.80	1279.50	1369.20	1324.35	123.90	129.10	126.50
P3-033*2	**SC67**	>5000	>5000	>5000	1133.00	1794.70	1463.85	114.70	83.90	99.30
P3-034	**SC71**	>2500	>2500	>2500	1244.80	1743.90	1494.35	141.30	98.90	120.10
P3-035	**SC73**	103.30	184.50	143.90	1269.40	1754.20	1511.80	105.90	109.20	107.55
P3-036	**SC75**	931.40	940.20	935.80	1418.20	1676.30	1547.25	148.00	119.40	133.70
P3-037	**SC76**	>2500	>2500	>2500	1389.20	1181.20	1285.20	114.00	122.70	118.35
P3-038	**SC78**	1786.60	2253.10	2019.85	1070.70	1288.20	1179.45	121.60	119.00	120.30

P3-039	**SC95**	919.10	922.50	920.80	1286.30	1143.10	1214.70	126.80	131.70	129.25
P3-040	**SC96**	62.50	56.20	59.35	1173.40	1116.60	1145.00	134.00	123.10	128.55
P3-041	**SC99**	<39.1	<39.1	<39.1	1456.50	1159.60	1308.05	138.80	146.30	142.55
P3-044	**SC86**	3114.80	2076.00	2595.40	1801.20	1154.50	1477.85	118.40	127.20	122.80
P3-091	**SC94**	<39.1	<39.1	<39.1	1356.10	1241.50	1298.80	129.10	135.60	132.35
P3-092	**SC100**	149.60	443.10	296.35	1193.80	1143.70	1168.75	119.00	121.40	120.20

*1; Each IC50 of test substances, relative controls and positive controls was expressed as an average every set.

*2; Ten test substances were shared in Laboratory A, B and C.

*3: -; Inapplicable

Table R-4.3.4. Mean and standard deviation of IC50s for relative controls and positive controls of Phase III in the SIRC-CVS:TEA validation

	Laboratory A		Laboratory B		Laboratory C	
	Relative Control	Positive Control	Relative Control	Positive Control	Relative Control	Positive Control
N	40	40	39	39	39	39
Mean	1119.58	89.65	1317.34	89.19	1358.71	123.18
SD	61.58	2.05	134.27	3.04	189.60	12.34

* N; Numbers of each test substances, relative controls and positive controls
* IC50 was expressed as ug/mL.

Table R-4.4. Transferability of the SIRC-CVS:TEA method using Phase I study

Chemical No.	Name of test substances	Laboratory A	Laboratory B	Laboratory C (Retest)	Transferability
C01	Ethyl-2-methyl acetoacetate	N	N	N	Good
C02	Safflower oil	N	N	N	Good
C03	3-Chloropropionitrile	P	P	P	Good
C04	Sodium dehydroacetate	P	P	P	Good

* N; Negative, P; Positive,

Table R-4.5.1.1. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using Phase II study in laboratory A

Chemical code	Name of test substance	Laboratory A			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	piperonylbutoxide	P	P	P	1
P2-002	2,5-dimethylhexaediol	N	N	N	1
P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	ammonium nitrate	P	P	P	1
P2-005	potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-trichlorocarbanilide	P	P	P	1
P2-007	1-bromohexane	P	P	P	1
P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	propylene glycol propyl ether	N	N	N	1
P2-010	ethyl thioglycolate	P	P	P	1
P2-011	sodium oxalate	P	P	P	1
P2-012	2-phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-bromo-4-chlorobutane	P	P	P	1
P2-014	sodium hydrogensulfite	P	P	P	1
P2-015	isobutyraldehyde	P	P	P	1
P2-016	1-naphthaleneacetic acid	P	P	P	1
P2-017	propyl 4-hydroxybenzoate	P	P	P	1
P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	P	P	P	1
P2-019	camphene	P	P	P	1
P2-020	cyclopentanol	N	N	N	1

*1: N; Negative, P; Positive

*2: 1; All sets' judge agreed.

Table R-4.5.1.2. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using Phase II study in laboratory B

Chemical code	Name of test substance	LaboratoryB			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	piperonylbutoxide	P	P	P	1
P2-002	2,5-dimethylhexaediol	N	N	N	1
P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	ammonium nitrate	P	P	P	1
P2-005	potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-trichlorocarbanilide	P	P	P	1
P2-007	1-bromohexane	P	P	P	1
P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	propylene glycol propyl ether	N	N	N	1
P2-010	ethyl thioglycolate	P	P	P	1
P2-011	sodium oxalate	P	P	P	1
P2-012	2-phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-bromo-4-chlorobutane	P	P	P	1
P2-014	sodium hydrogensulfite	P	P	P	1
P2-015	isobutyraldehyde	P	P	P	1
P2-016	1-naphthaleneacetic acid	P	P	P	1
P2-017	propyl 4-hydroxybenzoate	P	P	P	1
P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	P	P	P	1
P2-019	camphene	P	P	P	1
P2-020	cyclopentanol	N	N	N	1

*1: N; Negative, P; Positive

*2: 1; All sets' judge agreed.

Table R-4.5.1.3. Intra-laboratory reproducibility of the SIRC-CVS:TEA method using Phase II study in laboratory C

Chemical code	Name of test substance	Laboratory C			
		Set 1	Set 2	Set 3	Intra-laboratory reproducibility
P2-001	piperonylbutoxide	P	P	P	1
P2-002	2,5-dimethylhexaediol	N	N	N	1
P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	ammonium nitrate	P	P	P	1
P2-005	potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-trichlorocarbanilide	P	P	P	1
P2-007	1-bromohexane	P	P	P	1
P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	propylene glycol propyl ether	N	N	N	1
P2-010	ethyl thioglycolate	P	P	P	1
P2-011	sodium oxalate	P	P	P	1
P2-012	2-phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-bromo-4-chlorobutane	P	P	P	1
P2-014	sodium hydrogensulfite	P	P	P	1
P2-015	isobutyraldehyde	P	P	P	1
P2-016	1-naphthaleneacetic acid	P	P	P	1
P2-017	propyl 4-hydroxybenzoate	P	P	P	1
P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	P	P	P	1
P2-019	camphene	P	P	P	1
P2-020	cyclopentanol	N	N	N	1

*1: N; Negative, P; Positive

*2: 1; All sets' judge agreed.

Table R-4.5.2.1. Inter-laboratory reproducibility of the SIRC-CVS:TEA method in Phase II study

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P2-001	piperonylbutoxide	P	P	P	1
P2-002	2,5-dimethylhexaediol	N	N	N	1
P2-003	1-(2-propoxy-1-methylethoxy)-2-propanol	N	N	N	1
P2-004	ammonium nitrate	P	P	P	1
P2-005	potassium tetrafluoroborate	N	N	N	1
P2-006	3,4,4'-trichlorocarbanilide	P	P	P	1
P2-007	1-bromohexane	P	P	P	1
P2-008	4,4'-methylenebis(2,6-di-tert-butylphenol)	N	N	N	1
P2-009	propylene glycol propyl ether	N	N	N	1
P2-010	ethyl thioglycolate	P	P	P	1
P2-011	sodium oxalate	P	P	P	1
P2-012	2-phospho-L-ascorbic acid trisodium salt	N	N	N	1
P2-013	1-bromo-4-chlorobutane	P	P	P	1
P2-014	sodium hydrogensulfite	P	P	P	1
P2-015	isobutyraldehyde	P	P	P	1
P2-016	1-naphthaleneacetic acid	P	P	P	1
P2-017	propyl 4-hydroxybenzoate	P	P	P	1
P2-018	ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepro	P	P	P	1
P2-019	camphene	P	P	P	1
P2-020	cyclopentanol	N	N	N	1

*1: N; Negative, P; Positive

*2: 1; All laboratories' judge agreed.

Table R-4.5.2.2. Inter-laboratory reproducibility of the SIRC-CVS:TEA method in Phase III study

Chemical code	Name of test substance	Laboratory A	Laboratory B	Laboratory C	Inter-laboratory reproducibility
P3-003	dipropyl disulfide	P	P	N	0
P3-005	2-(2-ethoxyethoxy)ethanol	N	N	N	1
P3-010	n,n-dimethylguanidine sulfate	N	P	N	0
P3-012	polyethylene hydrogenated caster oil (40E.O.)	N	P	N	0
P3-019	diethyl toluamide	P	P	P	1
P3-020	4-nitrobenzoic acid	N	N	N	1
P3-024	2-amino-3-hydroxy pyridine	P	P	P	1
P3-028	tetraethylene glycol	P	P	P	1
P3-029	dodecanoic acid	P	P	P	1
P3-033	gamma-butyrolactone	N	N	N	1

*1: N; Negative, P; Positive

*2: 1; All laboratories' judge agreed, 0; Only two laboratories' judge agreed.

Definition of chemical classes

The chemical classes were basically defined by existence of functional group in this validation. Only surfactants were classified on the basis of function in accordance with the actual condition. The information of function was obtained from International Cosmetic Ingredient Dictionary. For example, Triton X-100 was classified as surfactant, and was not classified as alcohol or alkoxylated alcohol from Chemical class.
Even if those surfactants are re-classified by chemical class such as alcohol, ether…., the predictive capacity is not changed in the study considering applicability domain. Because those molecular weights are over 180, those surfactants are not excluded in the study of predictive capacity in this validation.

Table 1 Functional group and so on for classification of chemical class

Chemical class	Functional group and so on for classification
Acrylate	Derivatives of acrylic acid (CH_2=CH-COOH).
Alcohol	R-OH
Aldehyde	R-CHO
Alkali	Basic ionic salt of an alkali metal or alkali earth metal chemical element
Alkanolamine	Organic compound containing both hydroxyl group (–OH) and amine (–NH2 –NHR or –NR2)
Alkene	Unsaturated hydrocarbon that contains at least one carbon-carbon double bond
Amide	-CO-NH_2 (or -CO-NHR, -CO-NR_2)
Amine	-NH_2 (or -NHR, -NR_2,-NR_3)
Carboxylic acid (salt)	-COOH, -COO^-
Disulfide compound	-S-S-
Ester	R-O-CO-R
Ether	R-O-R
Fatty acid	-C-COOH
Halogen compound	Organic compound containing halogen
Heterocyclic compound	Cyclic compound that has atoms of at least two different elements as members of its ring(s).

Hydrocarbon	Organic compound consisting entirely of hydrogen and carbon
Inorganic salt	Salt consisting of Inorganic compound
Ketone	R-CO-R
Metacrylate	Derivative of methacylic acid (CH_2=CCH_3-COOH).
Nitrile compound	R-C≡N
Organic salt	Salt containing an organic ion
PABA derivative	Derivative of PABA
Phenol compound	C_6H_5-OH
Phosphorus compound	Phosphorus-containing compound
Polycyclic compound	Organic compound featuring several closed rings of atoms, primarily carbon
Polyol	Alcohol containing multiple hydroxyl groups
Quaternaryammonium compound	-NR_4^+
Silicon compound	Compound containing silicon
Surfactant	Compound described as surfactant at “Function” of “International Cosmetic Ingredient Dictionary”. The substance that is not contained in INCI dictionary is classified by the same manner. For example, cetylpyridinium bromide was classified as surfactant on the basis of the information of cetylpyridinium chloride.
Surfonic acid	Organosulfur compounds with the general formula RS(=O)2–OH
Synthetic polymer	Synthetic polymer
Thiol compound	Compound containing thiol (-SH)
Triazapentadien compound	-N=C-NR-C=N-

Analysis of overlapped data of this validation and Shiseido

The comparison of data between this validation and Shiseido was performed by overlapped 21 substances, as shown in table 1and 2. The difference of results was found in four substances, that were 2,4- dimethyl-3-pentanol, iso-octylthioglycolate, 3,3-dithiodipropionic acid and n,n-dimethylguanidine sulfate. They contained two volatile substances, 2,4- dimethyl-3-pentanol and iso-octylthioglycolate. Also, the IC50s of 3,3-Dithiodipropionic acid and n,n-Dimethylguanidine sulfate were considerably near IC50 of triethanolamine.

Table 1 Overlapped data of this validation and Shiseido

Substance	CAS	MW	In vitro evaluation in this validation	In vitro evaluation in Shiseido	in vivo
Ammonium nitrate	6484-52-2	80.0	P	P	P
Cyclopentanol	96-41-3	86.1	N	N	P
3-Chloropropionitrile	542-76-7	89.5	P	P	P
Toluene	108-88-3	92.1	N	N	N
2-Methyl-1-pentanol	105-30-6	102.2	N	N	P
3-Methoxy-1,2-propanediol	623-39-2	106.1	N	N	N
2,4-Dimethyl-3-pentanol	3970-62-5	116.2	N	P	N
Propylene glycol propyl ether	1569-01-3	118.2	N	N	P
iso-Propyl bromide	75-26-3	123.0	N	N	N
Potassium tetrafluoroborate	14075-53-7	125.9	N	N	N
Ethyl-2-methyl acetoacetate	609-14-3	144.2	N	N	P
1-(2-Propoxy-1-methylethoxy) -2-propanol	29911-27-1	176.3	N	N	P
iso-Octylthioglycolate	25103-09-7	204.3	N	P	N
3,3-Dithiodipropionic acid	1119-62-6	210.3	N	P	P
Hexyl cinnamic aldehyde	101-86-0	216.3	P	P	P
Triton X-100	9002-93-1	250.4	P	P	P
Isopropyl Myristate	110-27-0	270.5	N	N	N
n,n-Dimethylguanidine sulfate	598-65-2	272.3	N	P	N
Ethyl 2,6-Dichloro-5-fluoro-beta -oxo-3-pyridinepropanoate	96568-04-6	280.1	P	P	P
N-Lauroylsarcosine sodium salt	137-16-6	293.4	P	P	P
2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	18472-51-0	897.8	P	P	P

Table 2 IC50 and evaluated results of 21 substances in this validation and Shiseido

Substance	In vitro evaluation in this validation					In vitro evaluation in Shiseido					In vivo
	IC50 of the first measurement (μg/mL)		IC50 of the second measurement (μg/mL)		In vitro evaluation	IC50 of the first measurement (μg/mL)		IC50 of the second measurement (μg/mL)		In vitro evaluation	
	Substance	Triethanolamine	Substance	Triethanolamine		Substance	Triethanolamine	Substance	Triethanolamine		
Ammonium nitrate (Lab. A) (Lab. B) (Lab. C)	1342.3 1147.5 1409.6	1518.2 1414.0 1525.1	925.5 778.5 1216.4	1352.4 1184.5 1531.5	P P P	1999.0	2000.5	1439.6	1808.3	P	P
Cyclopentanol (Lab. A) (Lab. B) (Lab. C)	3074.0 1729.6 4013.2	1232.7 1392.9 1721.6	2633.5 2187.3 3593.0	1331.5 1228.0 1851.4	N N N	2684.1	1656.6	2366.4	1687.6	N	P
3-Chloropropionitrile	<39.1	1017.5	40.4	1013.8	P	47.2	1757.2	50.1	1604.0	P	P
Toluene	>5000	1090.9	>5000	1168.3	N	>5000	1349.7	>5000	1782.0	N	N
2-Methyl-1-pentanol	>2500	1353.9	>2500	1365.5	N	1665.9	1558.9	1688.2	1386.8	N	P
3-Methoxy-1,2-propane diol	>5000	1194.4	>5000	1253.6	N	>5000	1820.5	>5000	1451.3	N	N
2,4-Dimethyl-3 -pentanol	>2500	1389.2	>2500	1181.2	N	1399.8	1500.2	976.2	1429.1	P	N
Propylene glycol propyl ether (Lab. A) (Lab. B) (Lab. C)	>4865.3 3561.9 >5000	1345.6 1227.5 1524.3	>3048.1 3528.2 >5000	1215.8 1248.6 1681.5	N N N	3889.9	1868.2	3816.8	1663.4	N	P
iso-Propyl bromide	>2500	1251.8	>2500	1457.5	N	>5000	1763.3	>5000	1206.8	N	N
Potassium tetrafluoroborate (Lab. A) (Lab. B) (Lab. C)	1791.6 1949.6 >5000	1362.5 1273.8 1837.1	1783.2 3630.8 >5000	1288.3 1379.6 1532.1	N N N	4595.1	1525.0	>5000	1683.3	N	N
Ethyl-2-methyl acetoacetate	.>5000	1154.1	>5000	1269.4	N	2978.4	2164.2	3410.9	1620.4	N	P
1-(2-Propoxy-1-methyl ethoxy)-2-propanol (Lab. A) (Lab. B) (Lab. C)	4130.0 3188.9 >4673.4	1517.0 1320.6 1808.9	3899.0 3654.8 >5000	1452.1 1357.8 1348.5	N N N	2729.9	1675.7	3646.0	1401.5	N	P
iso-Octylthioglycolate	>2500	1628.0	>2500	1753.1	N	399.6	1614.6	219.1	1452.7	P	N
3,3-Dithiodipropionic acid (Lab. A) (Lab. C)	1864.4 1938.6	1149.4 1340.7	1696.9 1664.5	1065.1 1025.1	N N	1436.8	1940.1	1313.8	1674.5	P	P
Hexyl cinnamic aldehyde	92.1	1302.2	98.3	1534.7	P	49.1	1709.2	125.5	1704.8	P	P
Triton X-100	<39.1	1356.1	<39.1	1241.5	P	<39.1	1945.1	<39.1	1599.5	P	P
Isopropyl Myristate	>5000	1173.1	>5000	1365.7	N	>5000	1531.6	3606.0	1452.4	N	N
n,n-Dimethylguanidine sulfate (Lab. A) (Lab. B) (Lab. C)	1323.3 626.8 3464.6	1040.3 1394.2 1831.1	1653.3 535.2 2748.7	1053.7 1488.5 1108.6	N P N	1380.8	1526.8	1018.5	1690.2	P	N
Ethyl 2,6-dichloro -5-fluoro-beta-oxo -3-pyridinepropanoate (Lab. A) (Lab. B) (Lab. C)	<46.4 86.1 93.8	1226.2 1305.8 1606.1	69.9 82.8 88.1	1372.1 1145.8 1644.9	P P P	<39.1	1932.9	84.2	1461.8	P	P
N-Lauroylsarcosine sodium salt	97.5	1445.1	70.7	1194.8	P	53.3	2228.9	55.1	1694.8	P	P
2,4,11,13-tetraazatetra (Chlorohexidine glucocinate)	<39.1	1095.4	42.4	1159.1	P	<39.1	1408.8	<39.1	1437.1	P	P

Examination of difference by lot of triethanolamine and serum

Triethanolamine from different manufacturing lots provided consistent results. Also, differences in manufacturers or production lots of serum and SDS did not have any significant effect on test results.

Result of triethanolamine (1) Phase I & IIA

IC50 (μg/mL)	Lab.	Year	Manufacturer of triethanolamine	Lot of triethanolamine	Manufacturer of serum	Lot of serum
1416.5	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1338.9	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1293.0	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1378.3	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1379.5	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1338.6	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1335.9	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1396.0	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1439.1	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1455.5	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1389.7	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1432.2	A	2012	Sigma-Aldrich	BCBC2078	GIBCO	909463
1777.0	B	2012	Wako	DCK2809	Nichirei	9E0887
1117.4	B	2012	Wako	DCK2809	Nichirei	9E0887
1760.5	B	2012	Wako	DCK2809	Nichirei	9E0887
1543.5	B	2012	Wako	DCK2809	Nichirei	9E0887
1602.9	B	2012	Wako	DCK2809	Nichirei	9E0887
1593.0	B	2012	Wako	DCK2809	Nichirei	9E0887
1138.6	B	2012	Wako	DCK2809	Nichirei	9E0887
1213.5	B	2012	Wako	DCK2809	Nichirei	9E0887
1666.0	B	2012	Wako	DCK2809	Nichirei	9E0887
1559.9	B	2012	Wako	DCK2809	Nichirei	9E0887
1700.2	B	2012	Wako	DCK2809	Nichirei	9E0887
1679.4	B	2012	Wako	DCK2809	Nichirei	9E0887
1587.2	C #	2012	Wako	DCK2809	Nichirei	9E0887
1036.4	C #	2012	Wako	DCK2809	Nichirei	9E0887
1079.8	C #	2012	Wako	DCK2809	Nichirei	9E0887
1392.7	C #	2012	Wako	DCK2809	Nichirei	9E0887
1064.5	C #	2012	Wako	DCK2809	Nichirei	9E0887
1337.7	C #	2012	Wako	DCK2809	Nichirei	9E0887
1193.0	C #	2012	Wako	DCK2809	Nichirei	9E0887
1373.6	C #	2012	Wako	DCK2809	Nichirei	9E0887
1545.9	C #	2012	Wako	DCK2809	Nichirei	9E0887
1117.8	C #	2012	Wako	DCK2809	Nichirei	9E0887
1468.5	C #	2012	Wako	DCK2809	Nichirei	9E0887
1172.0	C #	2012	Wako	DCK2809	Nichirei	9E0887

IC50 (μg/mL)	Lab.	Year	Manufacturer of triethanolamine	Lot of triethanolamine	Manufacturer of serum	Lot of serum
1676.6	A	2012	Wako	DCK3718	GIBCO	1073767
1461.0	A	2012	Wako	DCK3718	GIBCO	1073767
1298.7	A	2012	Wako	DCK3718	GIBCO	1073767
1078.8	A	2012	Wako	DCK3718	GIBCO	1073767
1006.3	A	2012	Wako	DCK3718	GIBCO	1073767
1621.7	A	2012	Wako	DCK3718	GIBCO	1073767
1802.0	A	2012	Wako	DCK3718	GIBCO	1073767
1524.8	A	2012	Wako	DCK3718	GIBCO	1073767
1440.4	A	2012	Wako	DCK3718	GIBCO	1073767
1762.6	A	2012	Wako	DCK3718	GIBCO	1073767
1590.1	A	2012	Wako	DCK3718	GIBCO	1073767
1454.3	A	2012	Wako	DCK3718	GIBCO	1073767
1541.3	A	2012	Wako	DCK3718	GIBCO	1073767
1067.9	A	2012	Wako	DCK3718	GIBCO	1073767
1235.4	A	2012	Wako	DCK3718	GIBCO	1073767
1702.5	A	2012	Wako	DCK3718	GIBCO	1073767
1380.5	A	2012	Wako	DCK3718	GIBCO	1073767
1069.8	A	2012	Wako	DCK3718	GIBCO	1073767
1484.8	A	2012	Wako	DCK3718	GIBCO	1073767
1355.0	A	2012	Wako	DCK3718	GIBCO	1073767
1711.3	A	2012	Wako	DCK3718	GIBCO	1073767
1704.7	A	2012	Wako	DCK3718	GIBCO	1073767
1040.1	A	2012	Wako	DCK3718	GIBCO	1073767
1611.6	A	2012	Wako	DCK3718	GIBCO	1073767
1715.7	A	2012	Wako	DCK3718	GIBCO	1073767
1511.3	A	2012	Wako	DCK3718	GIBCO	1073767
1313.2	A	2012	Wako	DCK3718	GIBCO	1073767
1708.9	A	2012	Wako	DCK3718	GIBCO	1073767
1741.3	A	2012	Wako	DCK3718	GIBCO	1073767
1104.5	A	2012	Wako	DCK3718	GIBCO	1073767
1616.2	A	2012	Wako	DCK3718	GIBCO	1073767
1054.6	A	2012	Wako	DCK3718	GIBCO	1073767
1386.5	A	2012	Wako	DCK3718	GIBCO	1073767
1750.1	A	2012	Wako	DCK3718	GIBCO	1073767
1468.9	A	2012	Wako	DCK3718	GIBCO	1073767
1101.4	A	2012	Wako	DCK3718	GIBCO	1073767
1394.3	A	2012	Wako	DCK3718	GIBCO	1073767
1503.8	A	2012	Wako	DCK3718	GIBCO	1073767
1189.3	A	2012	Wako	DCK3718	GIBCO	1073767
1635.4	A	2012	Wako	DCK3718	GIBCO	1073767
1029.0	A	2012	Wako	DCK3718	GIBCO	1073767
1200.5	A	2012	Wako	DCK3718	GIBCO	1073767
1607.7	A	2012	Wako	DCK3718	GIBCO	1073767
1337.4	A	2012	Wako	DCK3718	GIBCO	1073767
1080.5	A	2012	Wako	DCK3718	GIBCO	1073767

Result of triethanolamine (2) Phase IIA

IC50 (μg/mL)	Lab.	Year	Manufacturer of triethanolamine	Lot of triethanolamine	Manufacturer of serum	Lot of serum
1153.5	B	2012	Wako	DCK3718	GIBCO	1073767
1575.0	B	2012	Wako	DCK3718	GIBCO	1073767
1159.3	B	2012	Wako	DCK3718	GIBCO	1073767
1282.0	B	2012	Wako	DCK3718	GIBCO	1073767
1388.1	B	2012	Wako	DCK3718	GIBCO	1073767
1311.0	B	2012	Wako	DCK3718	GIBCO	1073767
1228.9	B	2012	Wako	DCK3718	GIBCO	1073767
1267.0	B	2012	Wako	DCK3718	GIBCO	1073767
1038.0	B	2012	Wako	DCK3718	GIBCO	1073767
1401.0	B	2012	Wako	DCK3718	GIBCO	1073767
1558.4	B	2012	Wako	DCK3718	GIBCO	1073767
1247.7	B	2012	Wako	DCK3718	GIBCO	1073767
1497.4	B	2012	Wako	DCK3718	GIBCO	1073767
1130.8	B	2012	Wako	DCK3718	GIBCO	1073767
1364.3	B	2012	Wako	DCK3718	GIBCO	1073767
1231.5	B	2012	Wako	DCK3718	GIBCO	1073767
1314.4	B	2012	Wako	DCK3718	GIBCO	1073767
1276.2	B	2012	Wako	DCK3718	GIBCO	1073767
1279.2	B	2012	Wako	DCK3718	GIBCO	1073767
1596.1	B	2012	Wako	DCK3718	GIBCO	1073767
1086.4	B	2012	Wako	DCK3718	GIBCO	1073767
1450.8	B	2012	Wako	DCK3718	GIBCO	1073767
1046.0	B	2012	Wako	DCK3718	GIBCO	1073767
1576.7	B	2012	Wako	DCK3718	GIBCO	1073767
1218.0	B	2012	Wako	DCK3718	GIBCO	1073767
1345.4	B	2012	Wako	DCK3718	GIBCO	1073767
1307.4	B	2012	Wako	DCK3718	GIBCO	1073767
1259.8	B	2012	Wako	DCK3718	GIBCO	1073767
1701.7	B	2012	Wako	DCK3718	GIBCO	1073767
1280.4	B	2012	Wako	DCK3718	GIBCO	1073767
1048.7	B	2012	Wako	DCK3718	GIBCO	1073767
1119.0	B	2012	Wako	DCK3718	GIBCO	1073767
1385.7	B	2012	Wako	DCK3718	GIBCO	1073767
1286.4	B	2012	Wako	DCK3718	GIBCO	1073767
1431.1	B	2012	Wako	DCK3718	GIBCO	1073767
1170.0	B	2012	Wako	DCK3718	GIBCO	1073767
1261.3	B	2012	Wako	DCK3718	GIBCO	1073767
1556.7	B	2012	Wako	DCK3718	GIBCO	1073767
1003.5	B	2012	Wako	DCK3718	GIBCO	1073767
1449.2	B	2012	Wako	DCK3718	GIBCO	1073767
1344.8	B	2012	Wako	DCK3718	GIBCO	1073767
1344.8	B	2012	Wako	DCK3718	GIBCO	1073767
1260.4	B	2012	Wako	DCK3718	GIBCO	1073767
1232.7	B	2012	Wako	DCK3718	GIBCO	1073767
1276.2	B	2012	Wako	DCK3718	GIBCO	1073767

IC50 (μg/mL)	Lab.	Year	Manufacturer of triethanolamine	Lot of triethanolamine	Manufacturer of serum	Lot of serum
1774.8	C	2012	Wako	DCK3718	GIBCO	1073767
1411.5	C	2012	Wako	DCK3718	GIBCO	1073767
1350.2	C	2012	Wako	DCK3718	GIBCO	1073767
1430.9	C	2012	Wako	DCK3718	GIBCO	1073767
1570.4	C	2012	Wako	DCK3718	GIBCO	1073767
1359.4	C	2012	Wako	DCK3718	GIBCO	1073767
1373.3	C	2012	Wako	DCK3718	GIBCO	1073767
1051.3	C	2012	Wako	DCK3718	GIBCO	1073767
1488.0	C	2012	Wako	DCK3718	GIBCO	1073767
1725.7	C	2012	Wako	DCK3718	GIBCO	1073767
1721.0	C	2012	Wako	DCK3718	GIBCO	1073767
1818.3	C	2012	Wako	DCK3718	GIBCO	1073767
1054.3	C	2012	Wako	DCK3718	GIBCO	1073767
1263.3	C	2012	Wako	DCK3718	GIBCO	1073767
1278.5	C	2012	Wako	DCK3718	GIBCO	1073767
1439.3	C	2012	Wako	DCK3718	GIBCO	1073767
1594.3	C	2012	Wako	DCK3718	GIBCO	1073767
1542.3	C	2012	Wako	DCK3718	GIBCO	1073767
1780.3	C	2012	Wako	DCK3718	GIBCO	1073767
1696.0	C	2012	Wako	DCK3718	GIBCO	1073767
1950.3	C	2012	Wako	DCK3718	GIBCO	1073767
1405.1	C	2012	Wako	DCK3718	GIBCO	1073767
1303.2	C	2012	Wako	DCK3718	GIBCO	1073767
1337.3	C	2012	Wako	DCK3718	GIBCO	1073767
1182.2	C	2012	Wako	DCK3718	GIBCO	1073767
1481.0	C	2012	Wako	DCK3718	GIBCO	1073767
1353.4	C	2012	Wako	DCK3718	GIBCO	1073767
1556.4	C	2012	Wako	DCK3718	GIBCO	1073767
1433.1	C	2012	Wako	DCK3718	GIBCO	1073767
1585.8	C	2012	Wako	DCK3718	GIBCO	1073767
1564.9	C	2012	Wako	DCK3718	GIBCO	1073767
1512.8	C	2012	Wako	DCK3718	GIBCO	1073767
1516.8	C	2012	Wako	DCK3718	GIBCO	1073767
1595.8	C	2012	Wako	DCK3718	GIBCO	1073767
1522.1	C	2012	Wako	DCK3718	GIBCO	1073767
1406.5	C	2012	Wako	DCK3718	GIBCO	1073767
1638.7	C	2012	Wako	DCK3718	GIBCO	1073767
1895.5	C	2012	Wako	DCK3718	GIBCO	1073767
1977.1	C	2012	Wako	DCK3718	GIBCO	1073767
1566.5	C	2012	Wako	DCK3718	GIBCO	1073767
1590.9	C	2012	Wako	DCK3718	GIBCO	1073767
1439.0	C	2012	Wako	DCK3718	GIBCO	1073767
1071.9	C	2012	Wako	DCK3718	GIBCO	1073767
1317.7	C	2012	Wako	DCK3718	GIBCO	1073767
1404.2	C	2012	Wako	DCK3718	GIBCO	1073767

Result of triethanolamine (3) Others

IC50 (μg/mL)	Lab.	Year	Manufacturer of triethanolamine	Lot of triethanolamine	Manufacturer of serum	Lot of serum
1540.0	MHW-B	1995	Kanto	611E1858	GIBCO$	30P1033
1320.0	MHW-B	1995	Kanto	611E1858	GIBCO$	30P1033
1850.0	MHW-C	1995	Kanto	611E1858	GIBCO$	30P1033
1650.0	MHW-C	1995	Kanto	611E1858	GIBCO$	30P1033
1910.0	MHW-D	1995	Kanto	611E1858	GIBCO$	30P1033
2075.0	MHW-D	1995	Kanto	611E1858	GIBCO$	30P1033
3200.0	MHW-E	1995	Kanto	611E1858	GIBCO$	30P1033
4500.0	MHW-E	1995	Kanto	611E1858	GIBCO$	30P1033
1580.0	MHW-A(Shiseido)	1995	Kanto	611E1858	GIBCO$	30P1033
1300.0	MHW-A(Shiseido)	1995	Kanto	611E1858	GIBCO$	30P1033
2164.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
2000.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1675.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1757.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1656.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1940.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1709.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
2228.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1558.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1868.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1669.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1932.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1945.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1424.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1666.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1526.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1501.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1763.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1773.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1614.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1435.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1500.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1525.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1820.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1349.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1786.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1664.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1338.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
2145.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1861.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1770.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1611.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1550.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1408.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1260.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1267.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1695.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1495.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1339.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1218.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1484.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1468.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1531.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1222.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1737.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1662.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1706.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1436.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1446.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1471.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1545.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1584.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1413.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1439.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1622.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1621.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1464.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1857.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1403.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1713.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1513.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1631.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1825.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1685.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1769.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1642.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1620.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1808.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1401.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1604.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1687.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1674.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1704.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1694.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1386.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1663.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1576.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1461.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1599.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1251.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1347.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1690.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1448.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1206.8	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1808.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1452.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1295.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1429.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1683.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1451.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1782.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1757.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1118.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1452.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1669.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1330.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1488.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1534.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
2290.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1437.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1441.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1374.6	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1354.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1486.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1303.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1662.7	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1485.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1696.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1452.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1557.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1555.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1647.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1283.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1700.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1508.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
2276.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1565.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1552.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1498.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1601.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1009.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1499.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1381.5	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1628.4	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1424.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1781.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1550.3	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1341.0	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1586.1	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1576.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1446.2	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1549.9	Shiseido	2009	Kanto	810W1077	JRH	12603C-500ML
1012.3	Shiseido	2010	Kanto	810W1077	JRH	12603C-500ML
1595.4	Shiseido	2010	Kanto	810W1077	JRH	12603C-500ML

JRH:JRH Bioscience
Kanto:Kanto Chemical CO., INC.
Nichirei:Nichirei Biosciences Inc.
Wako: Wako Pure Chemical Industries, Ltd.
#:Retest
$:Calf serum was obtained from GIBCO Lavoratories (NY, USA)

Difference by manufacturer and lot of TEA

Manufacturer of TEA	Lot of TEA	n	IC50 (μg/mL)		Other difference of test condition
			Average	SD	
Wako	DCK2809	24	1405.0	247.2	-
Wako	DCK3718	135	1408.5	224.4	Lab., Lot of serum
Sigma-Aldrich	BCBC2078	12	1382.8	49.016	Lab., Manufacturer of serum
Kanto	810W1077	134	1578.5	222.7	Lab., Manufacturer of serum
Kanto	611E1858	10	2092.5	1005.7	Lab.., Type of serum (Calf serum)

Difference by manufacturer and lot of serum

Manufacturer of serum	Lot of serum	n	IC50 (μg/mL)		Other difference of test condition
			Average	SD	
GIBCO	909463	12	1382.8	49.0	-
GIBCO	1073767	135	1408.5	224.4	Lab., Lot of TEA
Nichirei	9E0887	24	1405.0	247.19	Lab., Lot of TEA
JRH	810W1077	134	1578.5	222.7	Lab., Manufacturer of TEA
GIBCO[3] (Calf serum)	30P1033	10	2092.5	1005.7	Lab.(5labs), Manufacturer of TEA

Result of positive control (SDS) (1) Phase I & IIA

IC50 (μg/mL)	Lab.	Year	Manufacturer of SDS	Lot of SDS	Manufacturer of Serum	Lot of serum
83.4	A	2012	Wako	SDF8154	GIBCO	909463
81.0	A	2012	Wako	SDF8154	GIBCO	909463
83.3	A	2012	Wako	SDF8154	GIBCO	909463
80.2	A	2012	Wako	SDF8154	GIBCO	909463
80.2	A	2012	Wako	SDF8154	GIBCO	909463
80.3	A	2012	Wako	SDF8154	GIBCO	909463
93.9	A	2012	Wako	SDF8154	GIBCO	909463
87.0	A	2012	Wako	SDF8154	GIBCO	909463
79.2	A	2012	Wako	SDF8154	GIBCO	909463
78.2	A	2012	Wako	SDF8154	GIBCO	909463
78.3	A	2012	Wako	SDF8154	GIBCO	909463
79.0	A	2012	Wako	SDF8154	GIBCO	909463
83.5	B	2012	Wako	SDF8154	Nichirei	9E0887
86.3	B	2012	Wako	SDF8154	Nichirei	9E0887
91.8	B	2012	Wako	SDF8154	Nichirei	9E0887
88.7	B	2012	Wako	SDF8154	Nichirei	9E0887
79.3	B	2012	Wako	SDF8154	Nichirei	9E0887
86.0	B	2012	Wako	SDF8154	Nichirei	9E0887
89.9	B	2012	Wako	SDF8154	Nichirei	9E0887
87.3	B	2012	Wako	SDF8154	Nichirei	9E0887
88.6	B	2012	Wako	SDF8154	Nichirei	9E0887
85.0	B	2012	Wako	SDF8154	Nichirei	9E0887
87.0	B	2012	Wako	SDF8154	Nichirei	9E0887
90.5	B	2012	Wako	SDF8154	Nichirei	9E0887
81.8	C #	2012	Wako	SDF8154	Nichirei	9E0887
80.8	C #	2012	Wako	SDF8154	Nichirei	9E0887
86.9	C #	2012	Wako	SDF8154	Nichirei	9E0887
83.3	C #	2012	Wako	SDF8154	Nichirei	9E0887
85.4	C #	2012	Wako	SDF8154	Nichirei	9E0887
91.1	C #	2012	Wako	SDF8154	Nichirei	9E0887
81.5	C #	2012	Wako	SDF8154	Nichirei	9E0887
93.8	C #	2012	Wako	SDF8154	Nichirei	9E0887
77.9	C #	2012	Wako	SDF8154	Nichirei	9E0887
80.5	C #	2012	Wako	SDF8154	Nichirei	9E0887
84.2	C #	2012	Wako	SDF8154	Nichirei	9E0887
87.5	C #	2012	Wako	SDF8154	Nichirei	9E0887

IC50 (μg/mL)	Lab.	Year	Manufacturer of SDS	Lot of SDS	Manufacturer of Serum	Lot of serum
82.1	A	2012	Wako	SDF8154	GIBCO	1073767
88.7	A	2012	Wako	SDF8154	GIBCO	1073767
86.7	A	2012	Wako	SDF8154	GIBCO	1073767
88.8	A	2012	Wako	SDF8154	GIBCO	1073767
85.7	A	2012	Wako	SDF8154	GIBCO	1073767
90.3	A	2012	Wako	SDF8154	GIBCO	1073767
89.4	A	2012	Wako	SDF8154	GIBCO	1073767
86.3	A	2012	Wako	SDF8154	GIBCO	1073767
88.0	A	2012	Wako	SDF8154	GIBCO	1073767
80.9	A	2012	Wako	SDF8154	GIBCO	1073767
88.4	A	2012	Wako	SDF8154	GIBCO	1073767
84.3	A	2012	Wako	SDF8154	GIBCO	1073767
94.3	A	2012	Wako	SDF8154	GIBCO	1073767
86.2	A	2012	Wako	SDF8154	GIBCO	1073767
87.4	A	2012	Wako	SDF8154	GIBCO	1073767
86.7	A	2012	Wako	SDF8154	GIBCO	1073767
86.4	A	2012	Wako	SDF8154	GIBCO	1073767
91.5	A	2012	Wako	SDF8154	GIBCO	1073767
87.7	A	2012	Wako	SDF8154	GIBCO	1073767
86.4	A	2012	Wako	SDF8154	GIBCO	1073767
84.5	A	2012	Wako	SDF8154	GIBCO	1073767
89.1	A	2012	Wako	SDF8154	GIBCO	1073767
85.7	A	2012	Wako	SDF8154	GIBCO	1073767
91.4	A	2012	Wako	SDF8154	GIBCO	1073767
87.9	A	2012	Wako	SDF8154	GIBCO	1073767
90.8	A	2012	Wako	SDF8154	GIBCO	1073767
87.3	A	2012	Wako	SDF8154	GIBCO	1073767
88.0	A	2012	Wako	SDF8154	GIBCO	1073767
87.5	A	2012	Wako	SDF8154	GIBCO	1073767
92.6	A	2012	Wako	SDF8154	GIBCO	1073767
90.4	A	2012	Wako	SDF8154	GIBCO	1073767
90.1	A	2012	Wako	SDF8154	GIBCO	1073767
91.7	A	2012	Wako	SDF8154	GIBCO	1073767
88.3	A	2012	Wako	SDF8154	GIBCO	1073767
87.0	A	2012	Wako	SDF8154	GIBCO	1073767
84.0	A	2012	Wako	SDF8154	GIBCO	1073767
81.9	A	2012	Wako	SDF8154	GIBCO	1073767
86.8	A	2012	Wako	SDF8154	GIBCO	1073767
85.2	A	2012	Wako	SDF8154	GIBCO	1073767
87.7	A	2012	Wako	SDF8154	GIBCO	1073767
87.5	A	2012	Wako	SDF8154	GIBCO	1073767
89.4	A	2012	Wako	SDF8154	GIBCO	1073767
89.4	A	2012	Wako	SDF8154	GIBCO	1073767
79.6	A	2012	Wako	SDF8154	GIBCO	1073767
87.5	A	2012	Wako	SDF8154	GIBCO	1073767

Result of positive control (SDS) (2) Phase IIA

IC50 (μg/mL)	Lab.	Year	Manufacturer of SDS	Lot of SDS	Manufacturer of Serum	Lot of serum
89.9	B	2012	Wako	SDF8154	GIBCO	1073767
91.8	B	2012	Wako	SDF8154	GIBCO	1073767
84.6	B	2012	Wako	SDF8154	GIBCO	1073767
95.8	B	2012	Wako	SDF8154	GIBCO	1073767
92.1	B	2012	Wako	SDF8154	GIBCO	1073767
93.4	B	2012	Wako	SDF8154	GIBCO	1073767
91.9	B	2012	Wako	SDF8154	GIBCO	1073767
91.5	B	2012	Wako	SDF8154	GIBCO	1073767
92.1	B	2012	Wako	SDF8154	GIBCO	1073767
92.3	B	2012	Wako	SDF8154	GIBCO	1073767
92.9	B	2012	Wako	SDF8154	GIBCO	1073767
84.6	B	2012	Wako	SDF8154	GIBCO	1073767
95.7	B	2012	Wako	SDF8154	GIBCO	1073767
100.0	B	2012	Wako	SDF8154	GIBCO	1073767
93.0	B	2012	Wako	SDF8154	GIBCO	1073767
91.7	B	2012	Wako	SDF8154	GIBCO	1073767
93.0	B	2012	Wako	SDF8154	GIBCO	1073767
92.2	B	2012	Wako	SDF8154	GIBCO	1073767
91.8	B	2012	Wako	SDF8154	GIBCO	1073767
94.0	B	2012	Wako	SDF8154	GIBCO	1073767
86.9	B	2012	Wako	SDF8154	GIBCO	1073767
93.5	B	2012	Wako	SDF8154	GIBCO	1073767
95.0	B	2012	Wako	SDF8154	GIBCO	1073767
91.0	B	2012	Wako	SDF8154	GIBCO	1073767
92.9	B	2012	Wako	SDF8154	GIBCO	1073767
94.6	B	2012	Wako	SDF8154	GIBCO	1073767
89.6	B	2012	Wako	SDF8154	GIBCO	1073767
95.3	B	2012	Wako	SDF8154	GIBCO	1073767
94.9	B	2012	Wako	SDF8154	GIBCO	1073767
89.4	B	2012	Wako	SDF8154	GIBCO	1073767
89.2	B	2012	Wako	SDF8154	GIBCO	1073767
97.3	B	2012	Wako	SDF8154	GIBCO	1073767
91.1	B	2012	Wako	SDF8154	GIBCO	1073767
91.2	B	2012	Wako	SDF8154	GIBCO	1073767
91.8	B	2012	Wako	SDF8154	GIBCO	1073767
89.4	B	2012	Wako	SDF8154	GIBCO	1073767
90.9	B	2012	Wako	SDF8154	GIBCO	1073767
94.2	B	2012	Wako	SDF8154	GIBCO	1073767
80.2	B	2012	Wako	SDF8154	GIBCO	1073767
92.3	B	2012	Wako	SDF8154	GIBCO	1073767
86.5	B	2012	Wako	SDF8154	GIBCO	1073767
86.5	B	2012	Wako	SDF8154	GIBCO	1073767
86.1	B	2012	Wako	SDF8154	GIBCO	1073767
92.5	B	2012	Wako	SDF8154	GIBCO	1073767
92.2	B	2012	Wako	SDF8154	GIBCO	1073767

IC50 (μg/mL)	Lab.	Year	Manufacturer	Lot of SDS	Manufacturer of Serum	Lot of serum
92.8	C	2012	Wako	SDF8154	GIBCO	1073767
83.2	C	2012	Wako	SDF8154	GIBCO	1073767
77.7	C	2012	Wako	SDF8154	GIBCO	1073767
89.5	C	2012	Wako	SDF8154	GIBCO	1073767
89.1	C	2012	Wako	SDF8154	GIBCO	1073767
86.6	C	2012	Wako	SDF8154	GIBCO	1073767
86.8	C	2012	Wako	SDF8154	GIBCO	1073767
80.7	C	2012	Wako	SDF8154	GIBCO	1073767
87.0	C	2012	Wako	SDF8154	GIBCO	1073767
96.1	C	2012	Wako	SDF8154	GIBCO	1073767
93.4	C	2012	Wako	SDF8154	GIBCO	1073767
91.5	C	2012	Wako	SDF8154	GIBCO	1073767
95.3	C	2012	Wako	SDF8154	GIBCO	1073767
93.2	C	2012	Wako	SDF8154	GIBCO	1073767
88.9	C	2012	Wako	SDF8154	GIBCO	1073767
92.6	C	2012	Wako	SDF8154	GIBCO	1073767
84.1	C	2012	Wako	SDF8154	GIBCO	1073767
91.7	C	2012	Wako	SDF8154	GIBCO	1073767
90.7	C	2012	Wako	SDF8154	GIBCO	1073767
88.9	C	2012	Wako	SDF8154	GIBCO	1073767
92.3	C	2012	Wako	SDF8154	GIBCO	1073767
94.5	C	2012	Wako	SDF8154	GIBCO	1073767
90.4	C	2012	Wako	SDF8154	GIBCO	1073767
84.3	C	2012	Wako	SDF8154	GIBCO	1073767
85.9	C	2012	Wako	SDF8154	GIBCO	1073767
88.0	C	2012	Wako	SDF8154	GIBCO	1073767
85.9	C	2012	Wako	SDF8154	GIBCO	1073767
81.9	C	2012	Wako	SDF8154	GIBCO	1073767
79.6	C	2012	Wako	SDF8154	GIBCO	1073767
79.5	C	2012	Wako	SDF8154	GIBCO	1073767
78.0	C	2012	Wako	SDF8154	GIBCO	1073767
85.9	C	2012	Wako	SDF8154	GIBCO	1073767
92.1	C	2012	Wako	SDF8154	GIBCO	1073767
88.3	C	2012	Wako	SDF8154	GIBCO	1073767
84.0	C	2012	Wako	SDF8154	GIBCO	1073767
87.4	C	2012	Wako	SDF8154	GIBCO	1073767
98.9	C	2012	Wako	SDF8154	GIBCO	1073767
95.8	C	2012	Wako	SDF8154	GIBCO	1073767
92.9	C	2012	Wako	SDF8154	GIBCO	1073767
90.9	C	2012	Wako	SDF8154	GIBCO	1073767
94.3	C	2012	Wako	SDF8154	GIBCO	1073767
87.6	C	2012	Wako	SDF8154	GIBCO	1073767
93.3	C	2012	Wako	SDF8154	GIBCO	1073767
83.0	C	2012	Wako	SDF8154	GIBCO	1073767
97.7	C	2012	Wako	SDF8154	GIBCO	1073767

Result of positive control (SDS) (3) Others

IC50 (μg/mL)	Lab.	Year	Manufacturer	Lot.	Manufacturer of serum	Lot of serum
168	MHW-B	1994	Nikko	2802	GIBCO#	45K4613
176	MHW-B	1994	Nikko	2802	GIBCO#	45K4613
172	MHW-B	1994	Nikko	2802	GIBCO#	45K4613
117	MHW-C	1994	Nikko	2802	GIBCO#	45K4613
117	MHW-C	1994	Nikko	2802	GIBCO#	45K4613
117	MHW-C	1994	Nikko	2802	GIBCO#	45K4613
190	MHW-D	1994	Nikko	2802	GIBCO#	45K4613
190	MHW-D	1994	Nikko	2802	GIBCO#	45K4613
187	MHW-D	1994	Nikko	2802	GIBCO#	45K4613
201	MHW-E	1994	Nikko	2802	GIBCO#	45K4613
194	MHW-E	1994	Nikko	2802	GIBCO#	45K4613
198	MHW-E	1994	Nikko	2802	GIBCO#	45K4613
140	MHW-F	1994	Nikko	2802	GIBCO#	45K4613
157	MHW-F	1994	Nikko	2802	GIBCO#	45K4613
123	MHW-F	1994	Nikko	2802	GIBCO#	45K4613
174	MHW-A(Shiseido)	1994	Nikko	2802	GIBCO#	45K4613
189	MHW-A(Shiseido)	1994	Nikko	2802	GIBCO#	45K4613
176	MHW-A(Shiseido)	1994	Nikko	2802	GIBCO#	45K4613
102.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
87.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
103.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
101.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
98.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
101.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
108	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
104.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
100.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
97.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
103.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
113.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
107.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
85.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
86.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
98.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
100.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML

IC50 (μg/mL)	Lab.	Year	Manufacturer	Lot.	Manufacturer of serum	Lot of serum
90.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
86	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
88.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
86	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
96.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
87.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
113.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.4	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
88.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
94.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
109.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.3	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
87.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
101.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
89.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.8	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.7	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
93.5	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.1	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
95.2	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
92.6	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
91.9	Shiseido	2009	Wako	TCG8149	JRH	12603C-500ML
90.7	Shiseido	2010	Wako	TCG8149	JRH	12603C-500ML
95.1	Shiseido	2010	Wako	TCG8149	JRH	12603C-500ML

JRH:JRH Bioscience
Nichirei:Nichirei Biosciences Inc.
Wako: Wako Pure Chemical Industries, Ltd.
#:Retest
$:Calf serum was obtained from GIBCO Lavoratories (NY, USA)

Difference by manufacturer and lot of SDS

Manufacturer of SDS	Lot of SDS	n	IC50 (μg/mL)		Other difference of test condition
			Average	SD	
Wako	SDF8155	171	88.3	4.8	-
Wako	TCG8149	134	93.5	4.9	Lab., Manufacturer of serum
Nikko Chemicals	2802	18	165.9	30.0	Lab., Type of serum (Calf serum)

Difference by manufacturer and lot of serum

Manufacturer of serum	Lot of serum	n	IC50 (μg/mL)		Other difference of test condition
			Average	SD	
GIBCO	909463	12	82.0	4.5	-
GIBCO	1073767	135	89.2	4.4	Lab.
Nichirei	9E0887	24	85.8	4.2	Lab.
JRH	2603C-500ML	134	93.5	4.9	Lab., Lot of SDS
GIBCO[5] (Calf serum)	45K4613	18	165.9	30.0	Lab., Manufacturer of SDS

Effect of solvents in the validation study

The average ±standard deviation of the O.D. showing viable cells after application of each solvent was analyzed from the viewpoint of effect of solvents. The negative control was 0.64 ±0.08 in the Medium (n = 52) and 0.66 ±0.08 in medium containing DMSO (n = 28), as calculated from Phase III data obtained at Lab A, and 0.97 ±0.09 in the Medium (n = 76) and 0.93 ±0.10 in medium containing ethanol (n = 4), as calculated from Phase III data obtained at Lab B. Neither Lab A nor Lab C used ethanol as a solvent, nor did Lab B use DMSO as solvent. No effect of used solvents was confirmed from this validation data.

(1)Effect of DMSO

No.	IC50 (μg/mL)	IC50 (μg/mL)	Solvent (M：Medium, D:Medium containing DMSO)	OD	OD
P3-003	212.8	259.2	M	0.636	0.649
P3-005	>5000	>5000	M	0.708	0.669
P3-010	1323.3	1653.3	M	0.691	0.583
P3-012	1460.9	1541.2	M	0.648	0.711
P3-019	155.8	202.5	D	0.681	0.652
P3-020	1347.4	1588.5	D	0.602	0.543
P3-022	<39.1	42.4	M	0.879	0.69
P3-024	151.8	182.9	M	0.732	0.534
P3-027	484.9	869.1	M	0.724	0.651
P3-028	<39.1	<39.1	M	0.657	0.608
P3-029	42.2	46	D	0.698	0.591
P3-033	>5000	>5000	M	0.489	0.58
P3-042	<39.1	<39.1	D	0.834	0.594
P3-045	117.7	128.7	D	0.814	0.563
P3-073	444.1	470.6	M	0.78	0.654
P3-074	52.1	47.5	M	0.696	0.556
P3-075	<39.1	<39.1	D	0.711	0.631
P3-076	946.3	761.9	M	0.644	0.631
P3-077	>5000	>5000	M	0.589	0.62
P3-078	>5000	>5000	M	0.736	0.576

P3-079	>5000	>5000	M	0.688	0.524
P3-080	1082.2	1666.5	D	0.606	0.536
P3-081	84.6	352	D	0.559	0.551
P3-082	777.3	857.3	D	0.705	0.653
P3-083	>5000	>5000	M	0.628	0.537
P3-084	4903.1	>5000	M	0.688	0.596
P3-085	2243.5	3624.5	M	0.789	0.599
P3-086	2243.5	3624.5	M	0.606	0.539
P3-087	>5000	3648	M	0.684	0.688
P3-088	1941.1	2253.7	M	0.699	0.534
P3-089	>5000	>5000	M	0.541	0.561
P3-090	<39.1	<39.1	D	0.704	0.628
P3-093	682.6	866.2	M	0.664	0.539
P3-094	1429.5	1504.2	M	0.784	0.646
P3-095	1864.4	1696.9	D	0.648	0.615
P3-096	94.3	67	D	0.837	0.703
P3-097	132.4	274.5	D	0.748	0.657
P3-098	190	168.8	D	0.674	0.724
P3-099	1133.6	1574.3	M	0.818	0.633
P3-100	2043.9	2606.8	M	0.64	0.545

	N	Average	SD
Medium	52	0.64	0.08
DMSO	28	0.66	0.08

(2)Effect of Ethanol

No.	IC50 (μg/mL)	IC50 (μg/mL)	Solvent (M:Medium, E:Medium containing Ethanol)	OD	OD
P3-001	119.6	122.6	M	0.981	1.036
P3-003	695.2	672.8	E	0.862	0.97
P3-005	>5000	>5000	M	1.02	1.149
P3-008	17.7	22.8	M	1.062	0.91
P3-010	626.8	535.2	M	0.976	0.99

P3-012	814.2	768.8	M	1.029	1.094
P3-019	265.5	187.4	M	1.072	0.977
P3-020	2923.4	2017.9	M	0.981	0.959
P3-024	71.7	63.1	M	0.853	0.966
P3-028	6.9	11.7	M	0.821	0.842
P3-029	<39.1	<39.1	M	0.992	0.853
P3-033	4864.9	4126.6	M	1.095	1.05
P3-043	163.3	191.9	M	1.006	0.919
P3-046	783.5	346.3	M	0.865	0.913
P3-047	1599.2	1570.6	M	0.913	0.848
P3-048	2203.1	2105	M	1.021	1.037
P3-049	772.6	414.8	M	0.847	0.957
P3-050	>5000	>5000	M	0.961	1.151
P3-051	128.7	312.5	M	1.011	0.93
P3-052	92.1	98.3	M	0.954	0.9
P3-053	720.4	213.4	M	0.858	0.751
P3-054	195.5	169.9	M	0.961	0.951
P3-055	17.3	20.6	M	1.065	0.946
P3-056	>5000	>5000	M	1.102	1.074
P3-057	>5000	>5000	M	0.989	0.888
P3-058	11.3	13.9	M	1.098	0.972
P3-059	>5000	>5000	M	1.037	0.967
P3-060	1343.6	1473.8	M	0.968	0.973
P3-061	620.5	604.4	M	1.027	1.136
P3-062	1729.4	1824.4	M	0.805	1.036
P3-063	>2500	>2500	M	1.028	0.857
P3-064	1619	1403.1	M	0.87	1.081
P3-065	1604.1	1429.4	M	0.805	0.891
P3-066	>315	>315	M	0.899	0.969
P3-067	875.3	807.7	M	0.97	1.038
P3-068	1584.6	1468.4	M	0.883	1.024
P3-069	1276	1587.5	M	0.935	0.812
P3-070	3.6	14	E	1.049	0.838
P3-071	97.5	70.7	M	0.923	0.971
P3-072	57.2	60.1	M	1.067	0.824

	N	Average	SD
Medium	76	0.97	0.09
Ethanol	4	0.93	0.10

Analysis of predictive capacity by the data from this validation study and the additional data from Shiseido

The predictive capacity of SIRC-CVS:TEA test was analyzed by the data from this validation study and the additional data from Shiseido. Shiseido's data were taken from the report used in the peer review by JaCVAM eye irritation test evaluating committee in 2009-2011, and Their data sheets was checked during the peer review. Table 1 shows the data of 33 substances (Purity≥80%) for the analysis, except for the overlapped 21 substances of this validation and Shiseido. The predictive capacity by 33 substances was an accuracy of 63.6% (21/33), a sensitivity of 76.5% (13/17), and a specificity of 50.0% (8/16), as shown in table 2. Also, when excluding chemicals such as alcohols, esters, ethers, ketones, heterocyclic compounds, and carboxylic acid compounds with a molecular weight of less than 180, the predictive capacity was an accuracy of 63.6% (14/22), a sensitivity of 100% (11/11), and a specificity of 27.3% (3/11), as shown in table 3.

The predictive capacity by the 57 data from this validation study and the additional 22 data from Shiseido was an accuracy of 64.6% (51/79), a sensitivity of 94.6% (35/37), and a specificity of 38.1% (16/42). Also, false negative rate was 5.4% (2/37) and false positive rate was 61.9% (26/42), as shown in table 4 and 5.

Table 1 Additional data for analysis of predictive capacity of SIRC-CVS:TEA test

	Substance	CAS	in vitro	in vivo[1)]	MW	Purity[2)]
1	Butylene glycol	107-88-0	N	N	90.1	min.98.0%
2	Propylene carbonate	108-32-7	N	N	102.1	min.97.0%
3	2,4-Pentanediol	625-69-4	N	N	104.2	98%
4	Resorcinol	108-46-3	P	P	110.1	99.0+%
5	Butoxyethanol	111-76-2	N	P	118.2	min.99.0%
6	Hexylene glycol	107-41-5	N	P	118.2	99.0+%
7	Phenethyl alcohol	60-12-8	P	P	122.2	98.0+%
8	Methoxyisopropyl acetate	108-65-6	N	P	132.2	99%
9	6-Methyl purine	2004-03-7	P	P	134.1	≧99%
10	Phenoxyethanol	122-99-6	N	P	138.2	min.99.0%
11	Di-iso-butyl ketone	108-83-8	N	N	142.2	≧99%

12	Triethylene glycol	112-27-6	N	N	150.2	95.0+%
13	Chloroxylenol	88-04-0	P	P	156.6	98.0+%
14	2,4-Difluoronitrobenzene	446-35-5	P	N	159.1	99%
15	iso-Octyl acrylate	29590-42-9	P	N	184.3	>90%
16	Sodium dehydroacetate	4418-26-2	P	N	190.1	95.0+%
17	Triisopropanolamine	122-20-3	P	P	191.3	95.0+%
18	2-Bromo-2-Nitropropane-1,3-Diol	52-51-7	P	P	200.0	98+%
19	Benzophenone-1	131-56-6	P	P	214.2	98.0+%
20	Triacetin	102-76-1	P	N	218.2	98.0+%
21	Chlorophene	120-32-1	P	P	218.7	≧97.0%
22	Sodium naphthalenesulfonate	532-02-5	P	P	230.2	min.90.0%
23	Diisopropyl adipate	6938-94-9	P	N	230.3	98.0+%
24	tetra-Aminopyrimidine sulfate	5392-28-9	P	N	238.2	97%
25	Cetyl alcohol	36653-82-4	P	N	242.4	95.0+%
26	Benzophenone-2	131-55-5	P	P	246.2	95.0+%
27	Oleyl alcohol	143-28-2	P	N	268.5	99%
28	Isopropyl Palmitate	142-91-6	N	N	298.5	95.0+%
29	Cetrimonium chloride	112-02-7	P	P	320.0	95.0+%
30	Diethylhexyl adipate	103-23-1	N	N	370.6	99.0+%
31	Squalane	111-01-3	N	N	422.8	98.0+%
32	Stearalkonium chloride	122-19-0	P	P	424.2	85.0+%
33	Dioctyl sodium sulfosuccinate	577-11-7	P	P	488.5	96%

1) In vivo data is taken from the previous paper.

2) Purity is that of the substances used at in vitro test.

Table 2 Predictive capacity of 33 substances

N=33		In vitro	
		Positive	Negative
In vivo	Positive	13	4
	Negative	8	8

Table 3 Predictive capacity of 22 substances except for alcohols, esters, ethers, ketones, heterocyclic compounds, and carboxylic acid compounds with a molecular weight of less than 180

N=22		In vitro	
		Positive	Negative
In vivo	Positive	11	0
	Negative	8	3

Table 4 The predictive capacity by the data from this validation study and the additional data from Shiseido

N=79		In vitro	
		Positive	Negative
In vivo	Positive[#]	35	2
	Negative	26	16

Table 5 The predictive capacity of test substances (Purity>=80%) except for alcohol, ester, ether, ketone, heterocyclic compound and carboxylic acid of molecular weight <180

Regulatory System	Analysis in applicability domain
Accuracy	64.6% (51/79)
Sensitivity	94.6% (35/37)
Specificity	38.1% (16/42)
False Negative Rate	5.4% (2/37)
False Positive Rate	61.9% (26/42)

Data by biostatistician

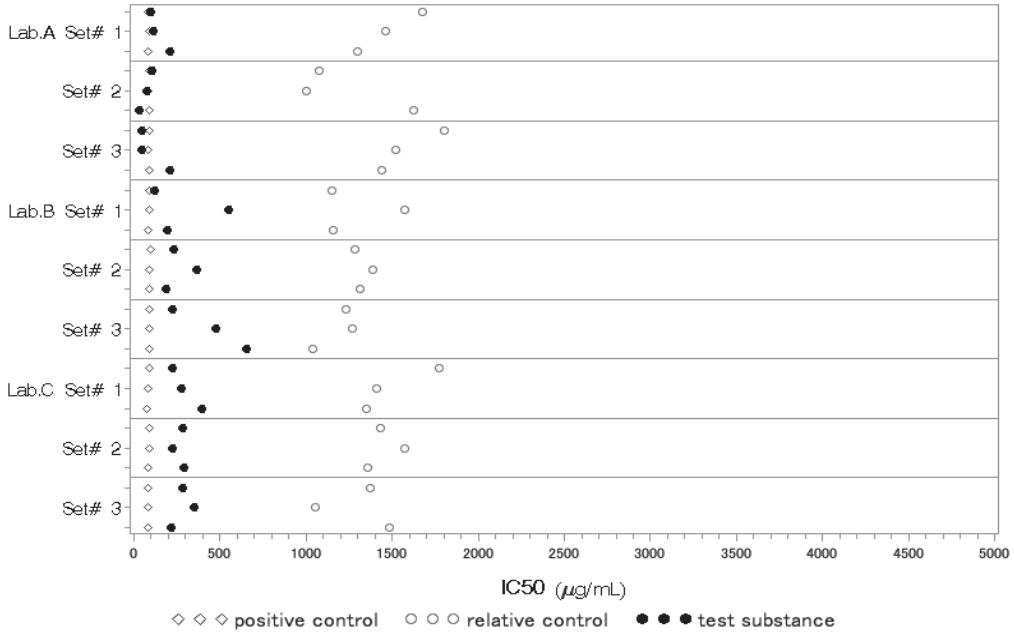


Fig.1. IC50s of the test substance (P2-001), relative controls and positive controls within each laboratory.

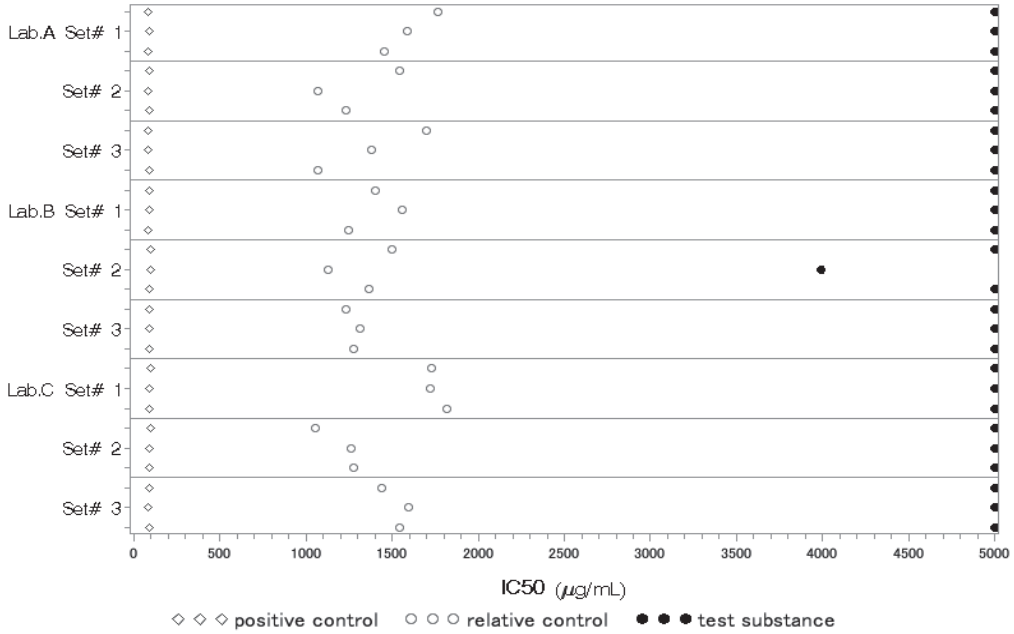


Fig.2. IC50s of the test substance (P2-002), relative controls and positive controls within each laboratory.

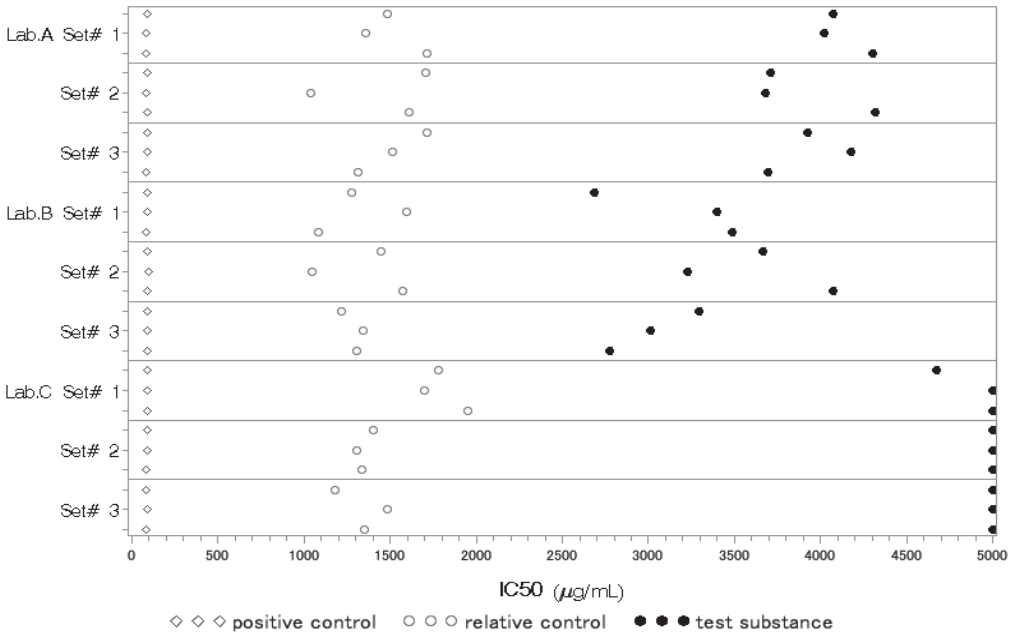


Fig.3. IC50s of the test substance (P2-003), relative controls and positive controls within each laboratory.

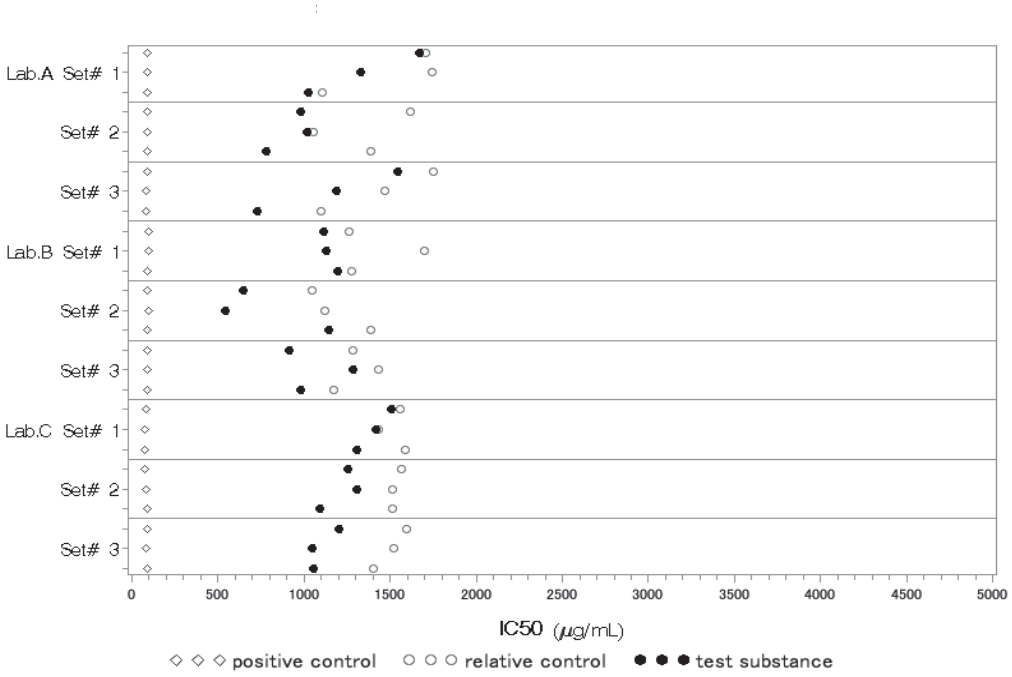


Fig.4. IC50s of the test substance (P2-004), relative controls and positive controls within each laboratory.

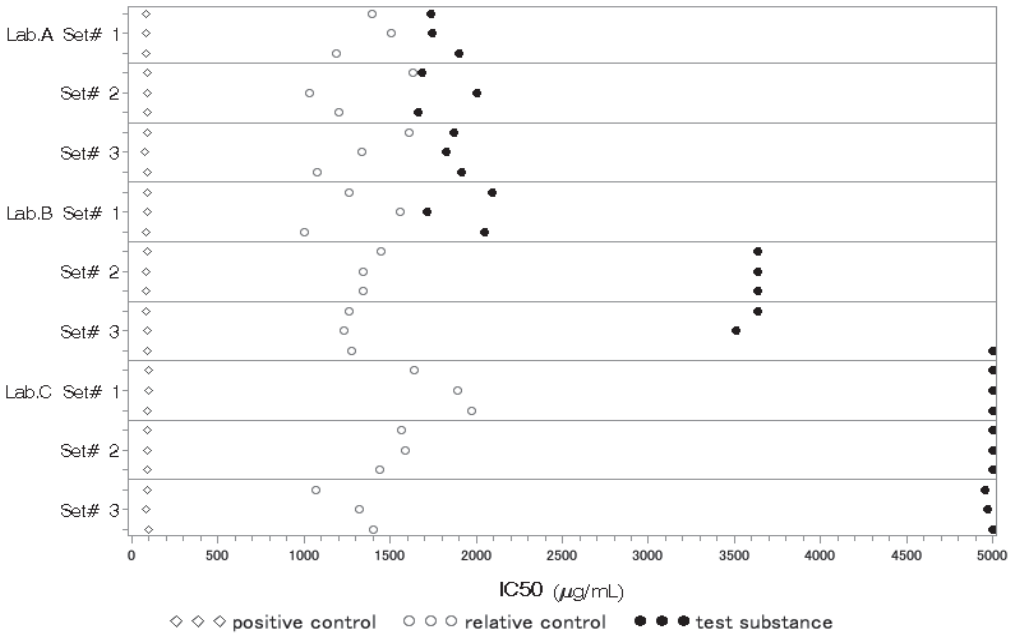


Fig.5. IC50s of the test substance (P2-005), relative controls and positive controls within each laboratory.

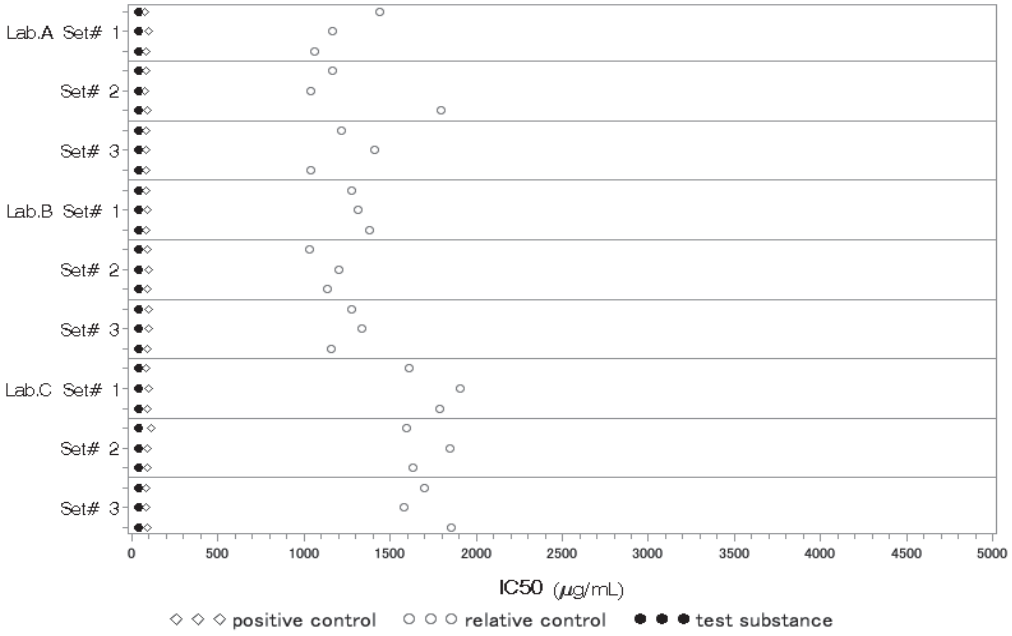


Fig.6. IC50s of the test substance (P2-006), relative controls and positive controls within each laboratory.

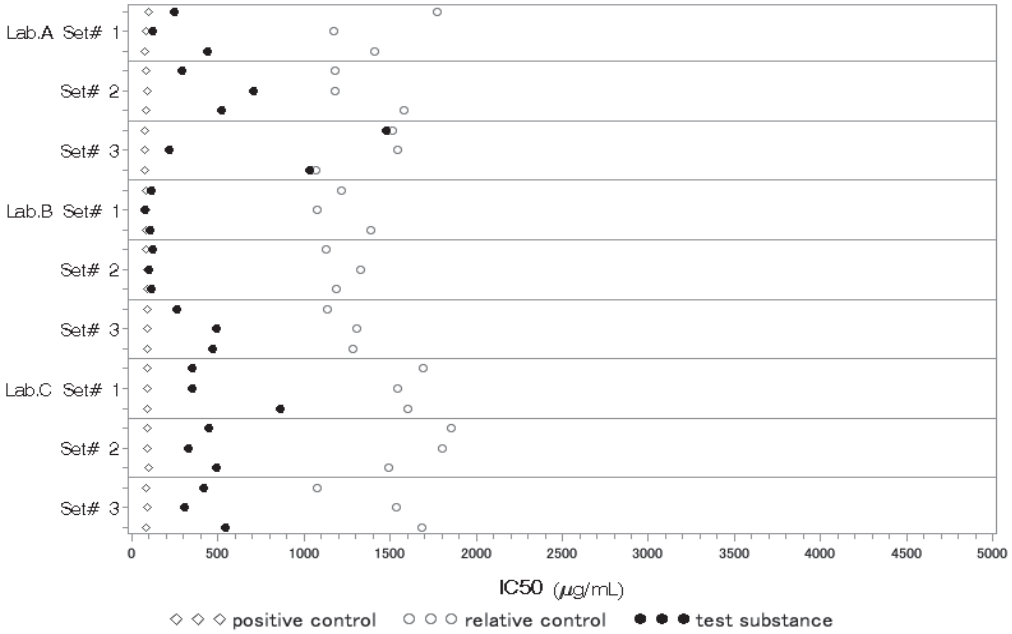


Fig.7. IC50s of the test substance (P2-007), relative controls and positive controls within each laboratory.

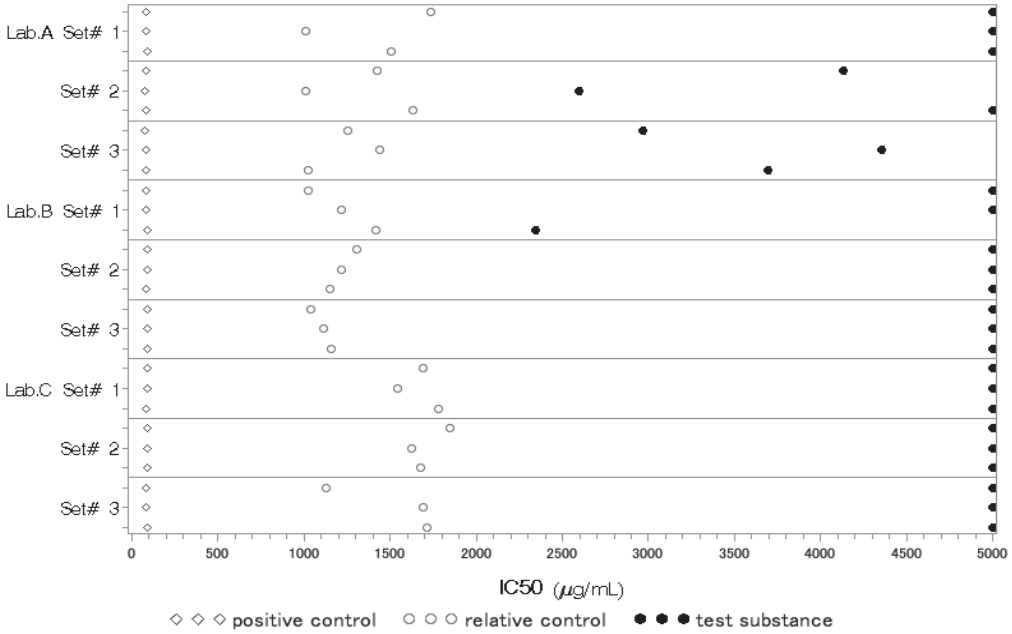


Fig.8. IC50s of the test substance (P2-008), relative controls and positive controls within each laboratory.

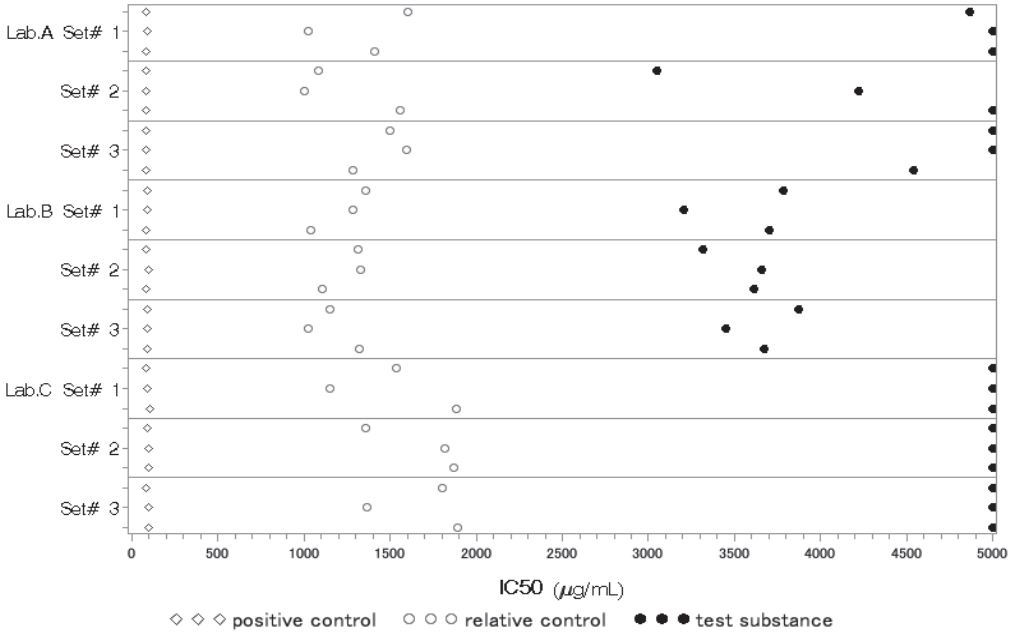


Fig.9. IC50s of the test substance (P2-009), relative controls and positive controls within each laboratory.

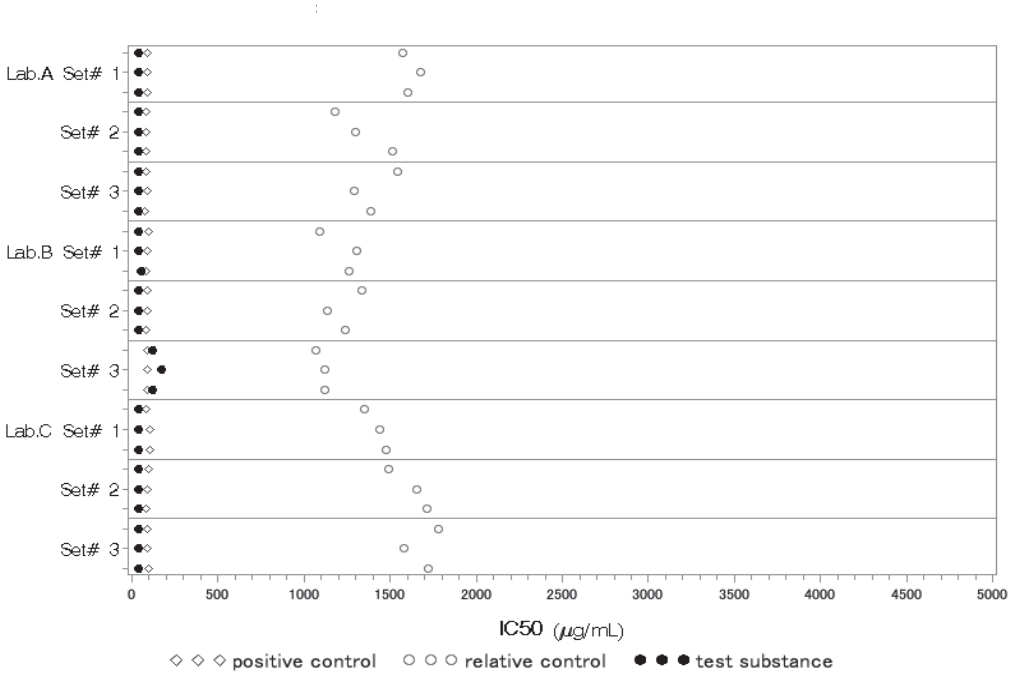


Fig.10. IC50s of the test substance (P2-010), relative controls and positive controls within each laboratory.

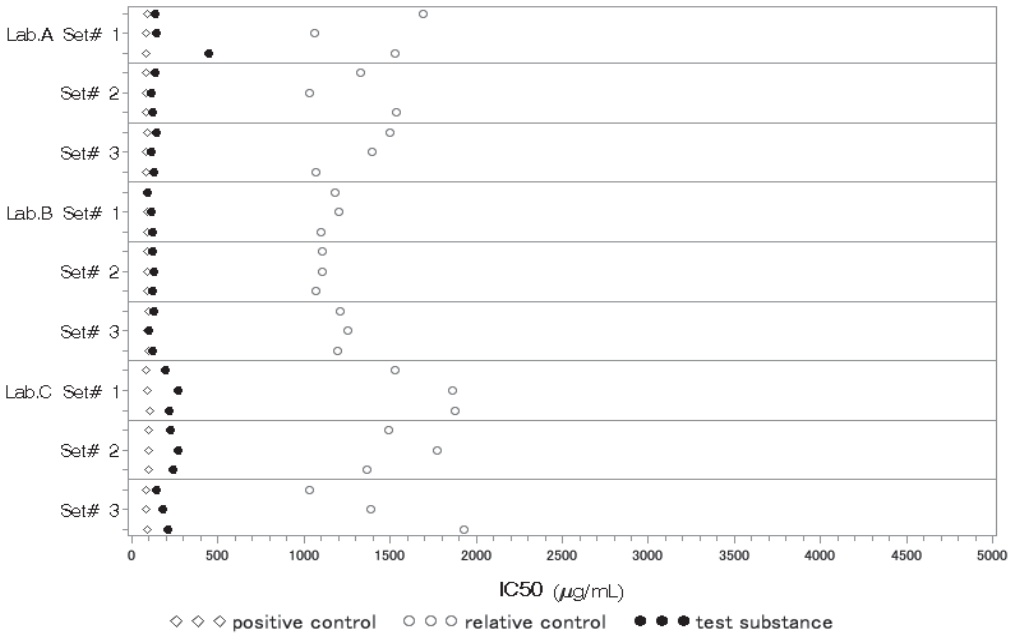


Fig.11. IC50s of the test substance (P2-011), relative controls and positive controls within each laboratory.

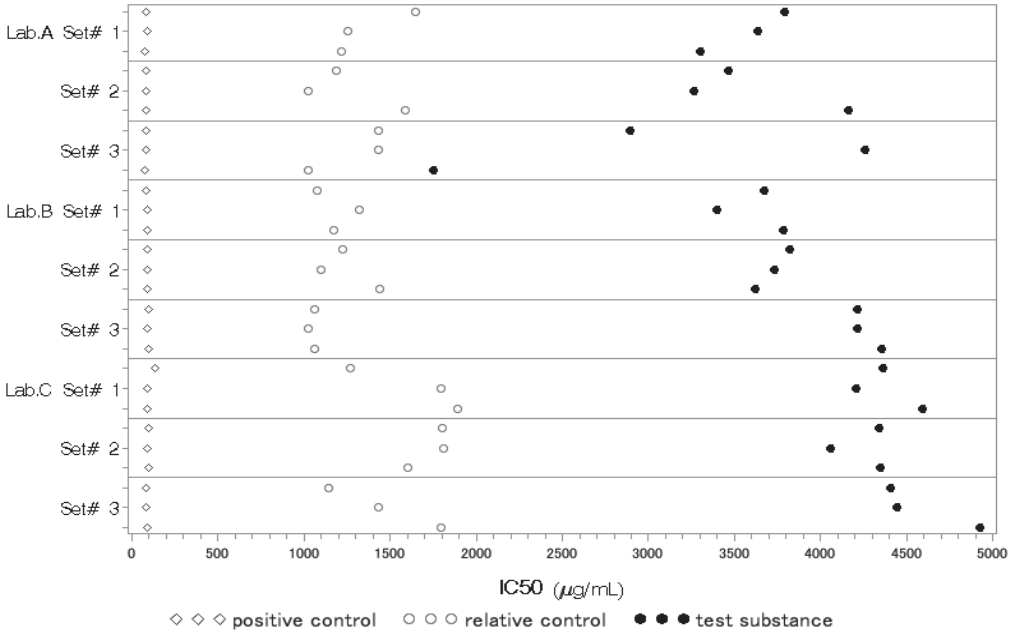


Fig.12. IC50s of the test substance (P2-012), relative controls and positive controls within each laboratory.

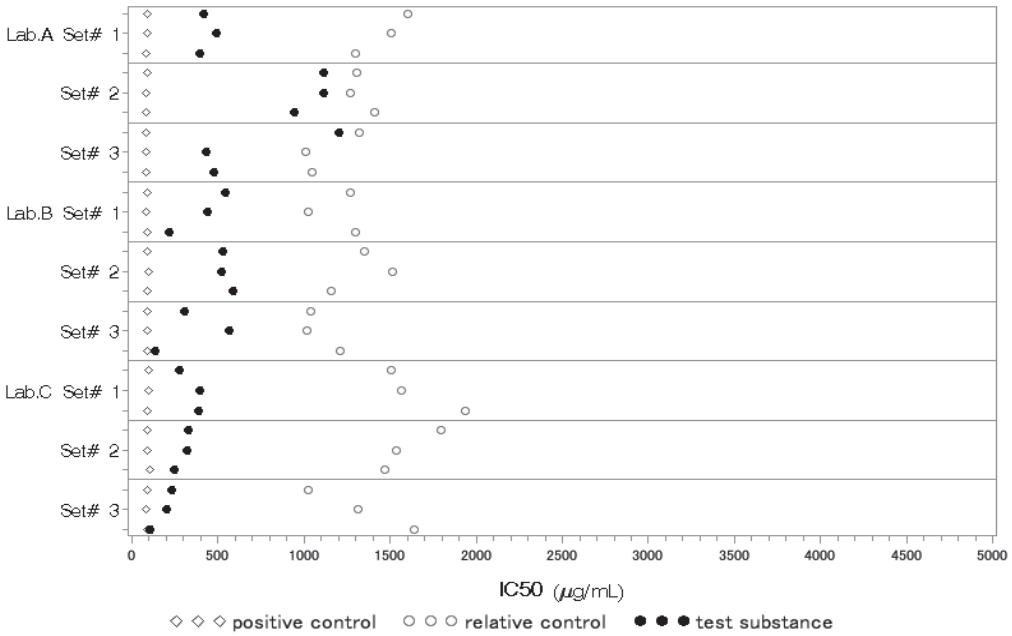


Fig.13. IC50s of the test substance (P2-013), relative controls and positive controls within each laboratory.

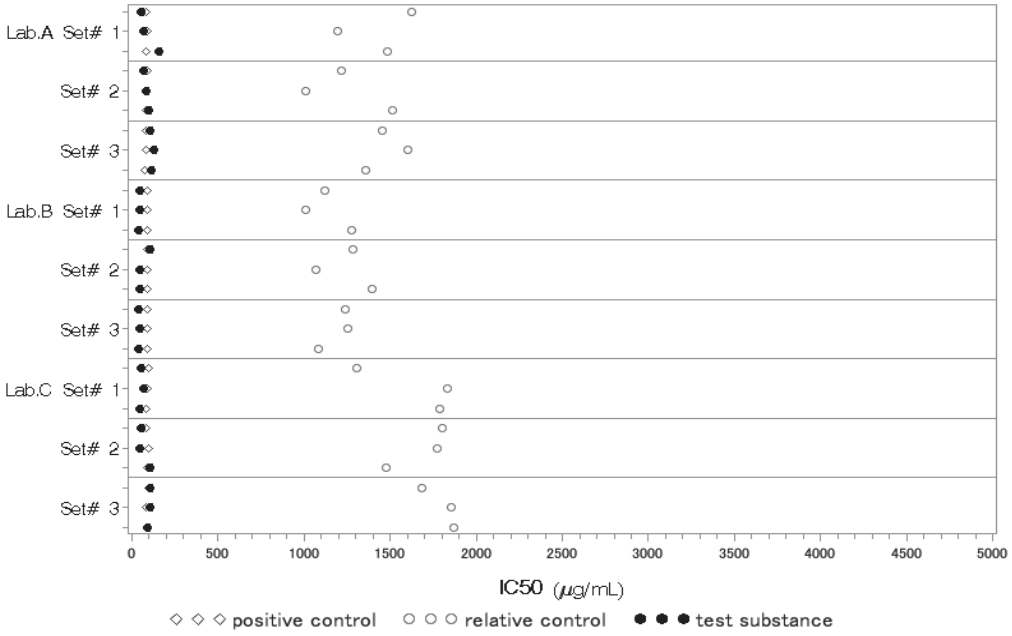


Fig.14. IC50s of the test substance (P2-014), relative controls and positive controls within each laboratory.

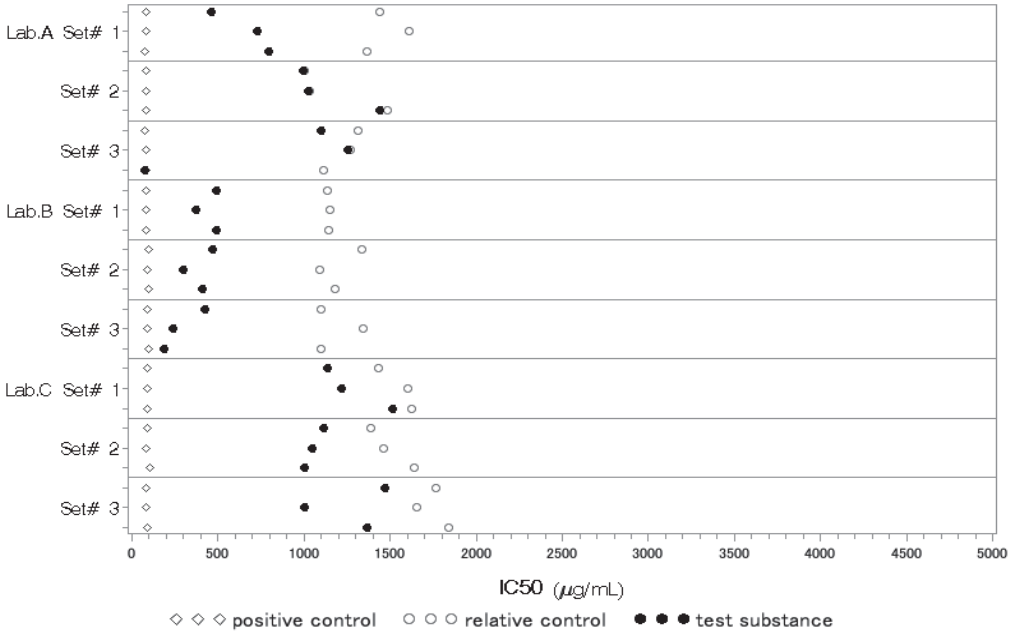


Fig.15. IC50s of the test substance (P2-015), relative controls and positive controls within each laboratory.

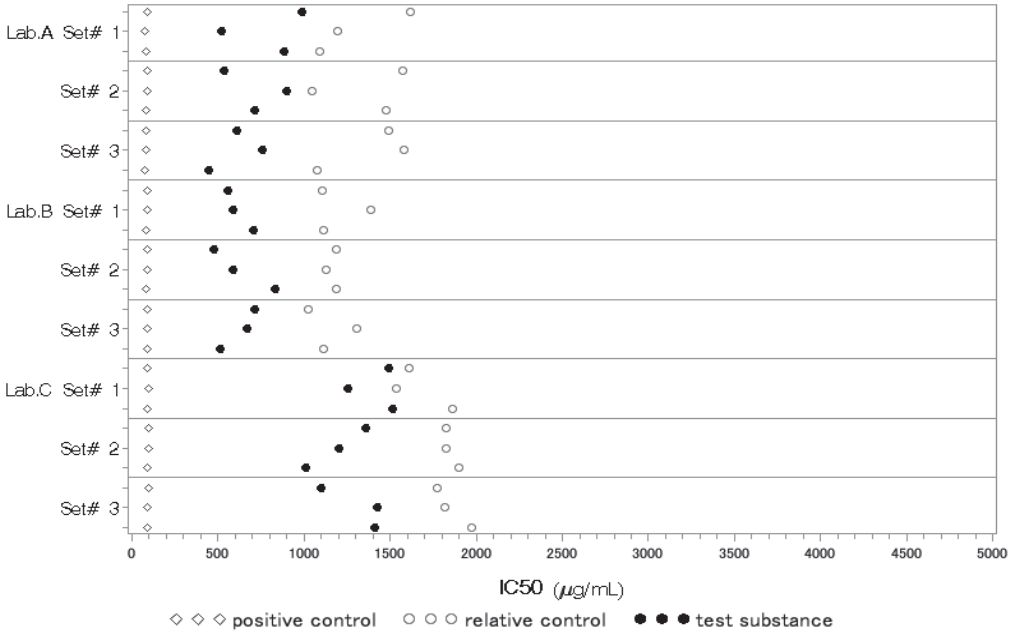


Fig.16. IC50s of the test substance (P2-016), relative controls and positive controls within each laboratory.

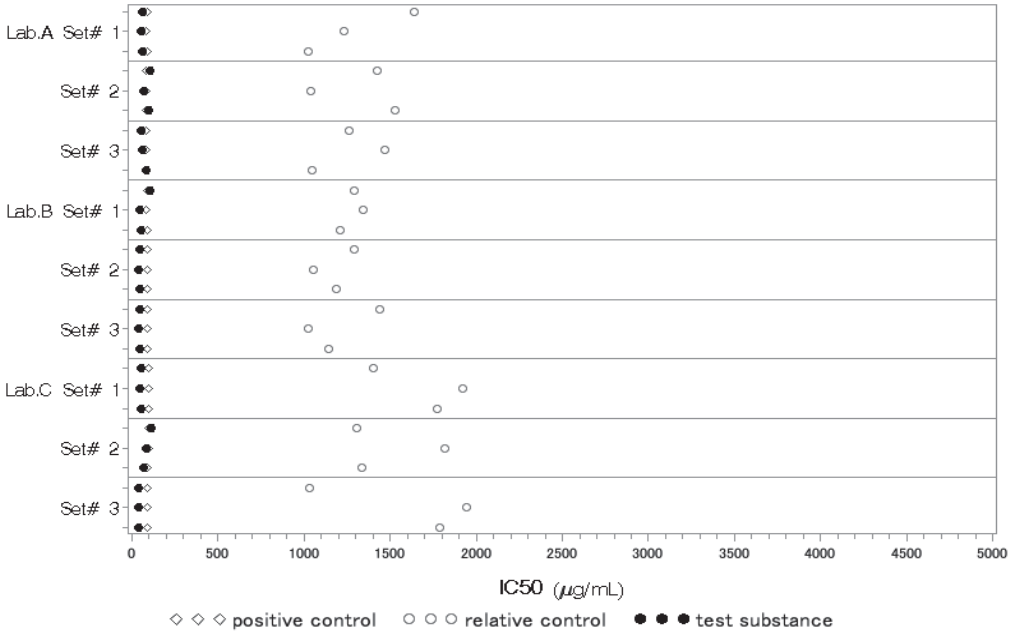


Fig.17. IC50s of the test substance (P2-017), relative controls and positive controls within each laboratory.

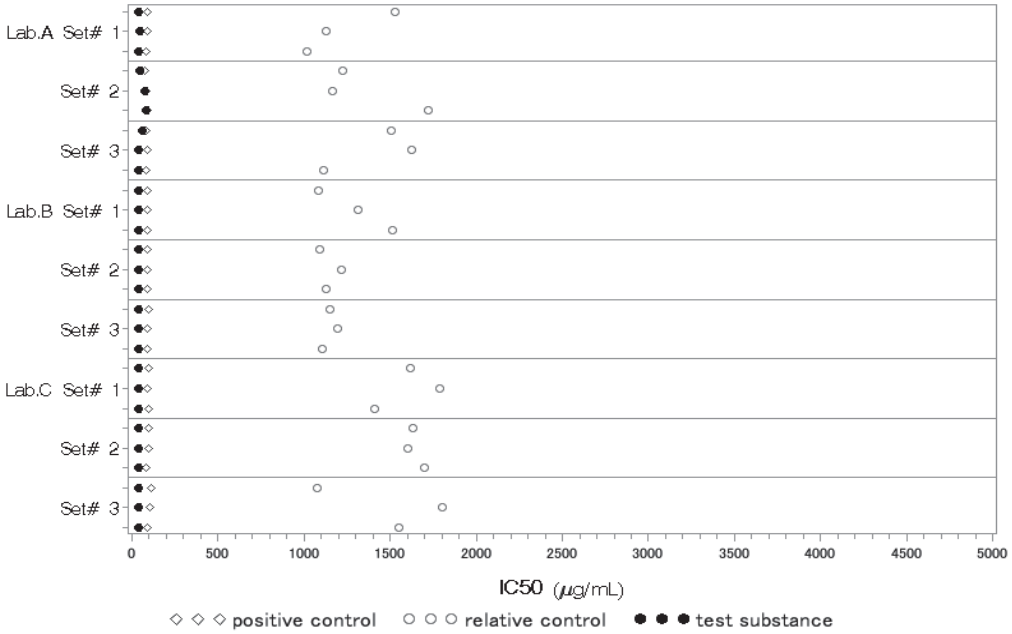


Fig.18. IC50s of the test substance (P2-018), relative controls and positive controls within each laboratory.

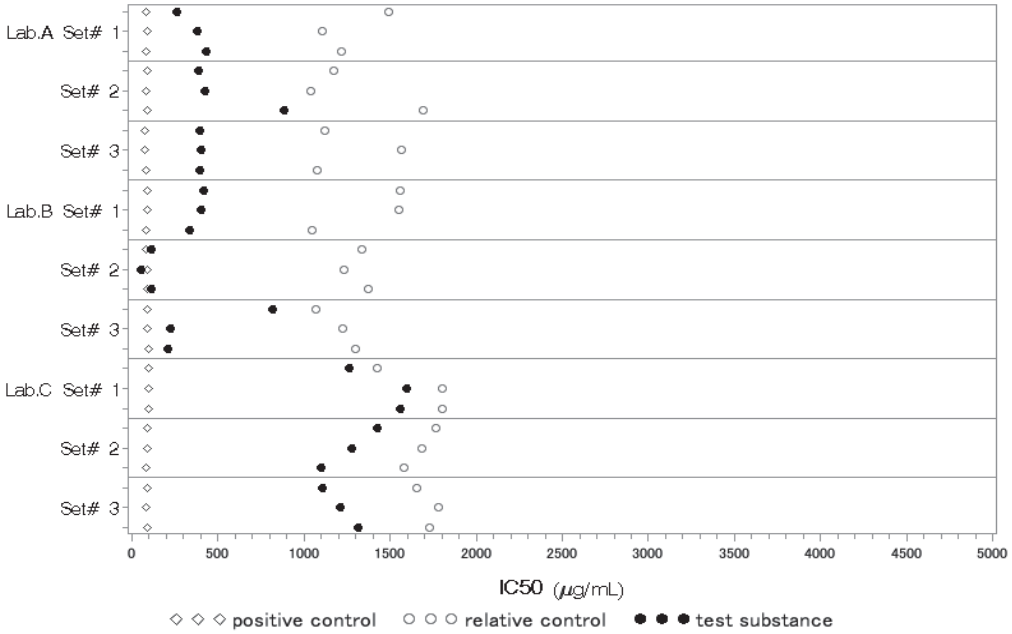


Fig.19. IC50s of the test substance (P2-019), relative controls and positive controls within each laboratory.

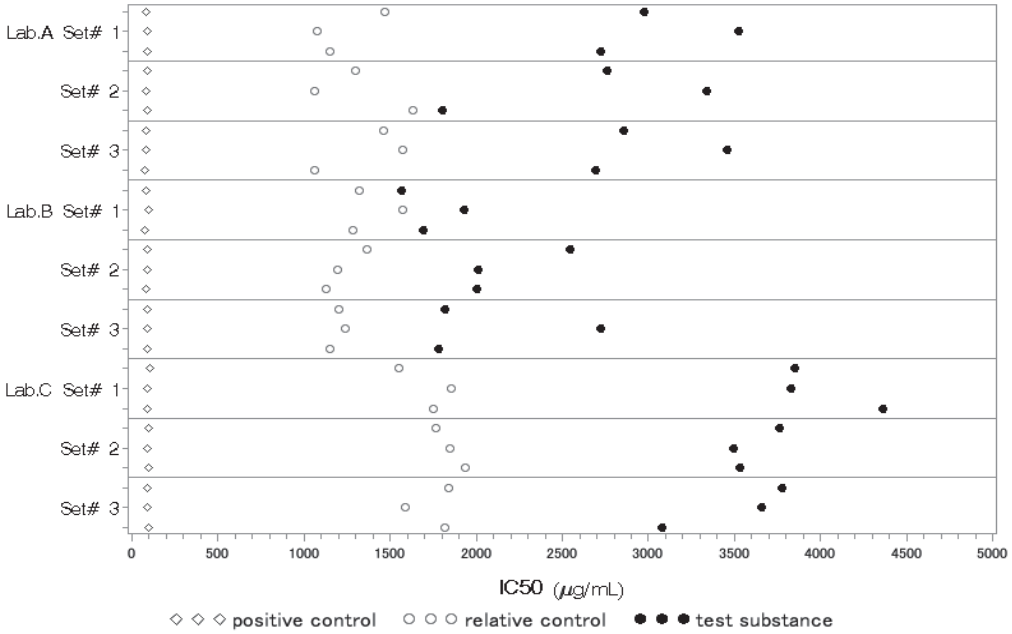


Fig.20. IC50s of the test substance (P2-020), relative controls and positive controls within each laboratory.

350

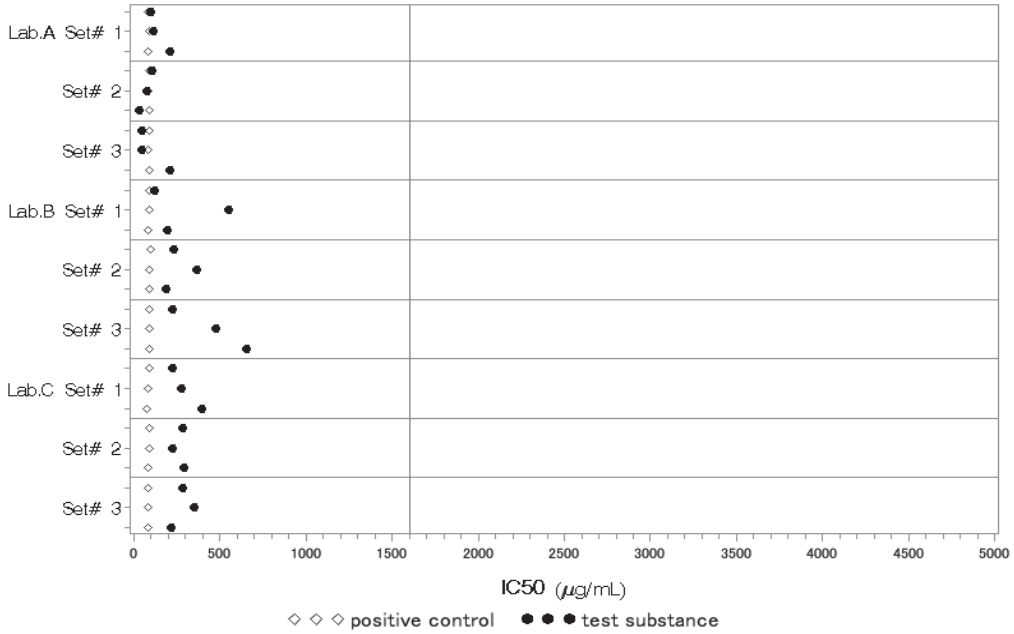


Fig.21. IC_{50}s of the test substance (P2-001) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

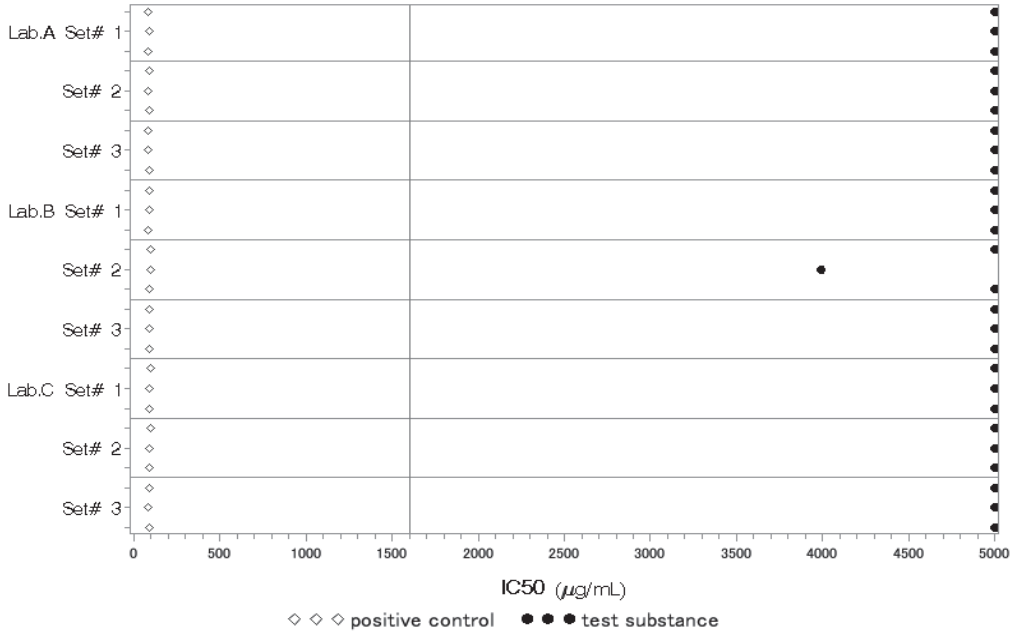


Fig.22. IC_{50}s of the test substance (P2-002) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

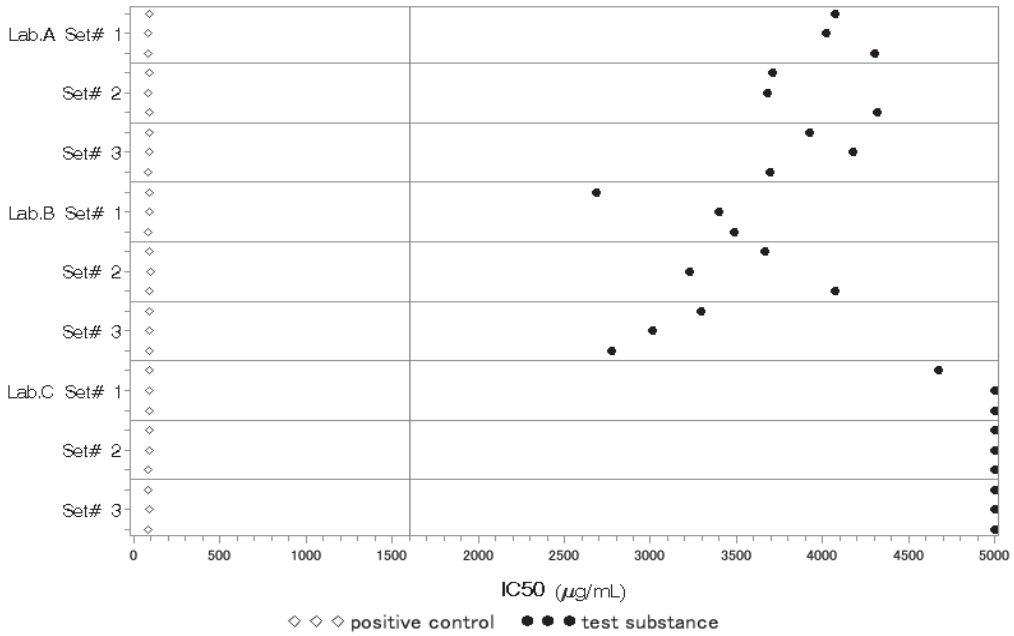


Fig.23. IC_{50}s of the test substance (P2-003) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

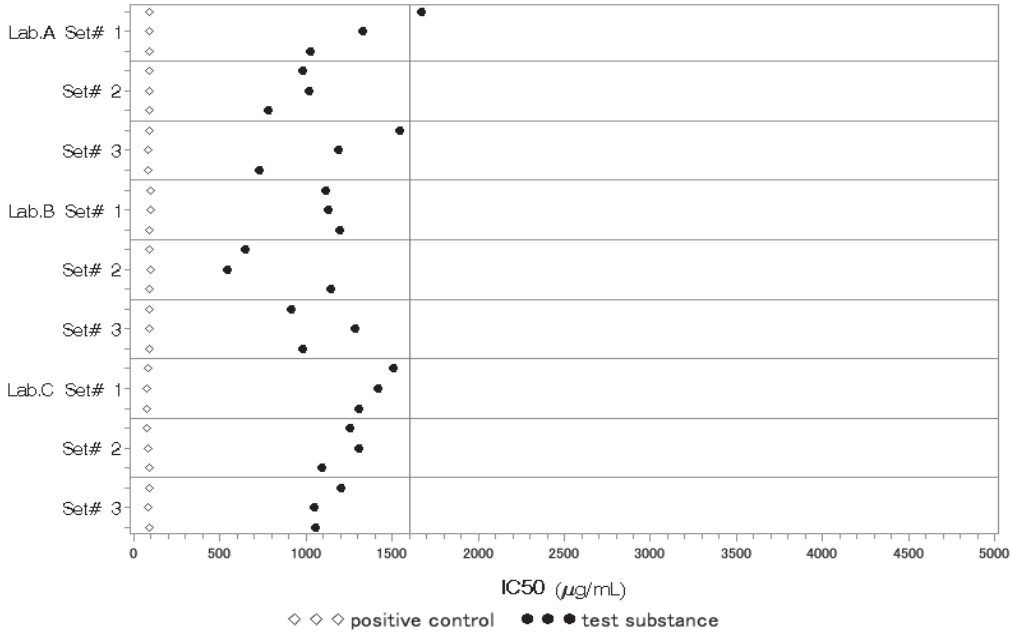


Fig.24. IC_{50}s of the test substance (P2-004) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

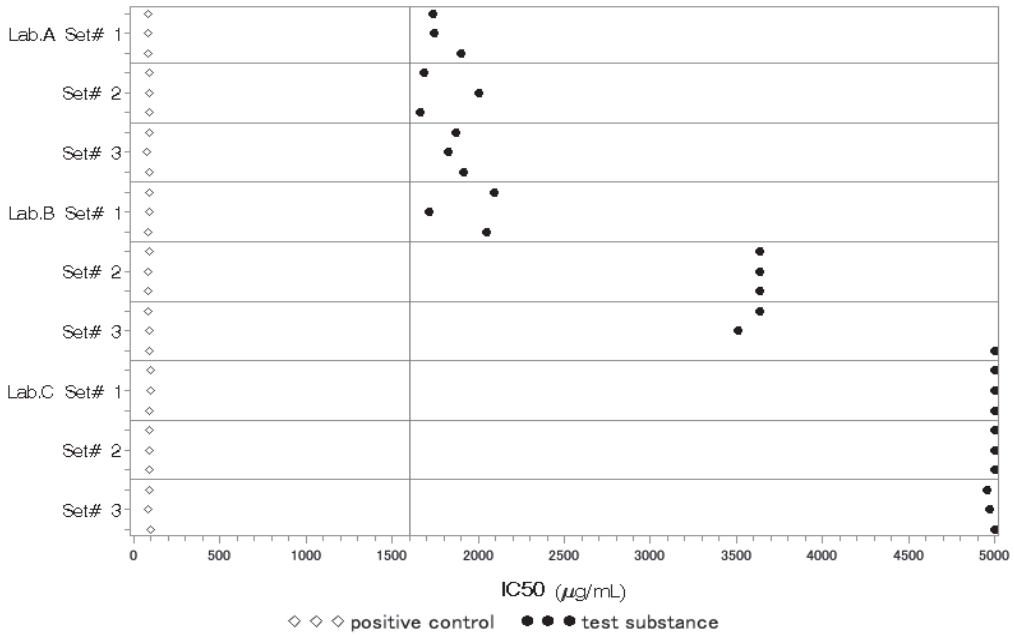


Fig.25. IC_{50}s of the test substance (P2-005) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

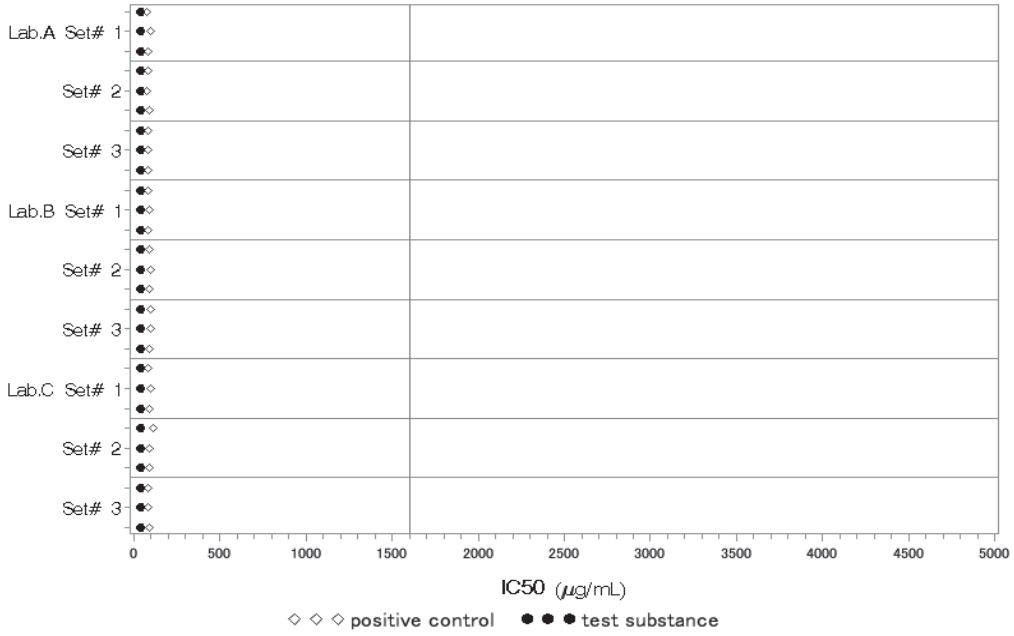


Fig.26. IC_{50}s of the test substance (P2-006) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

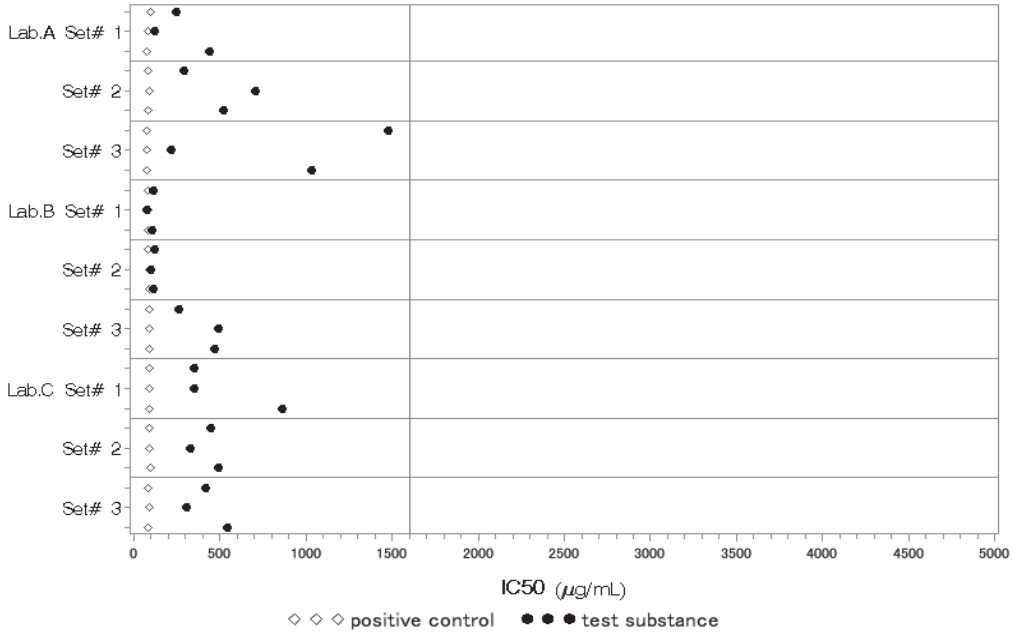


Fig.27. IC_{50}s of the test substance (P2-007) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

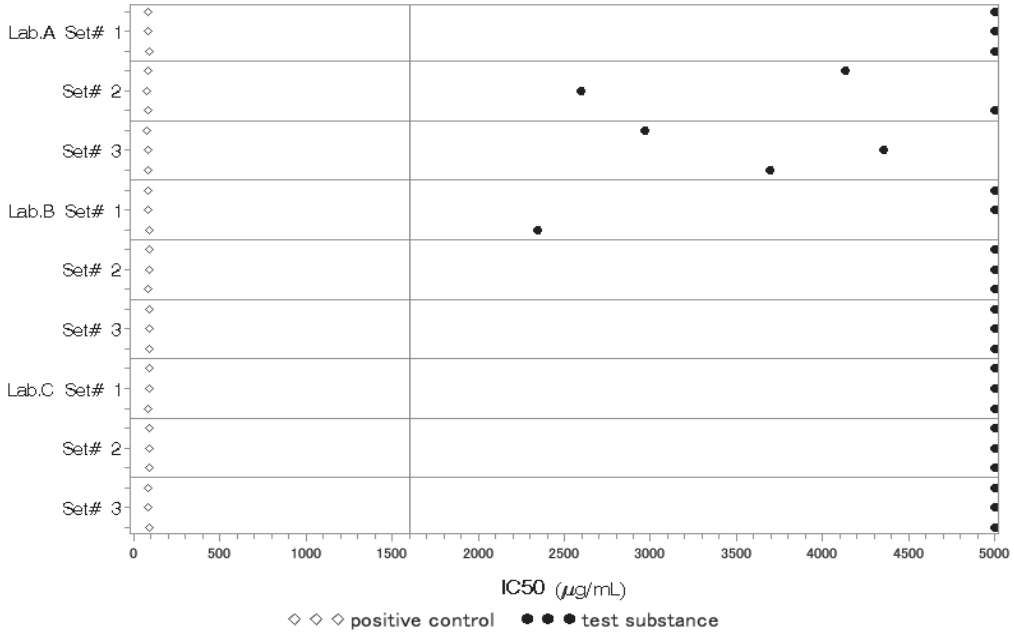


Fig.28. IC_{50}s of the test substance (P2-008) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

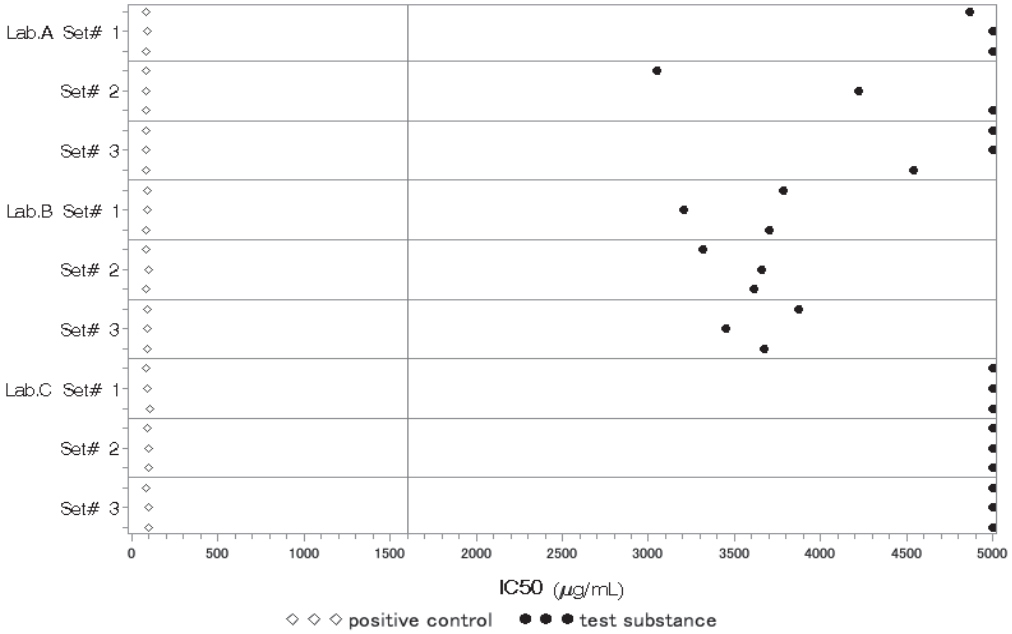


Fig.29. IC_{50}s of the test substance (P2-009) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

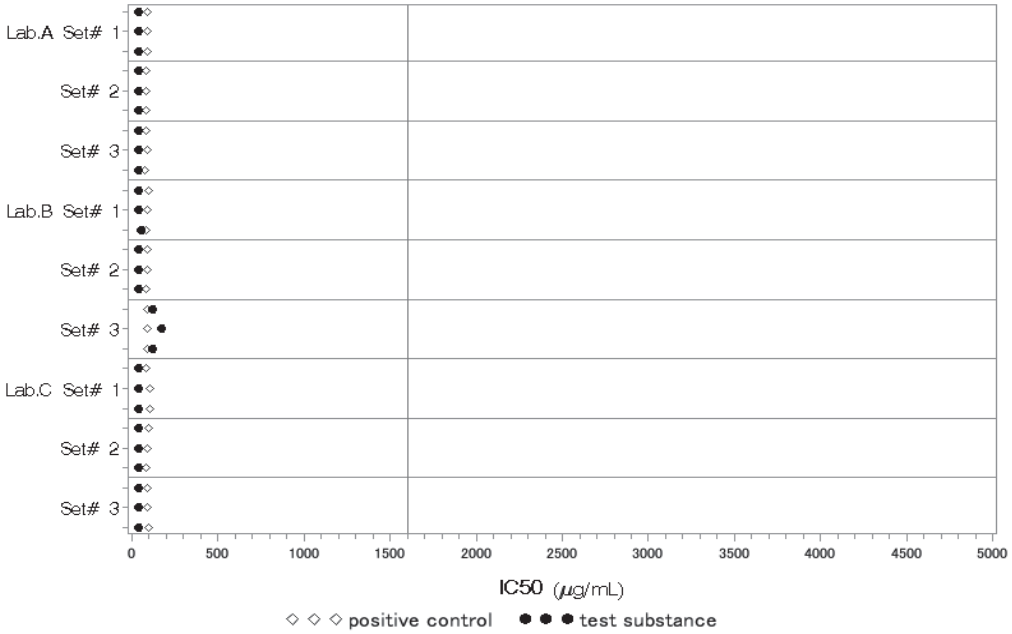


Fig.30. IC_{50}s of the test substance (P2-010) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

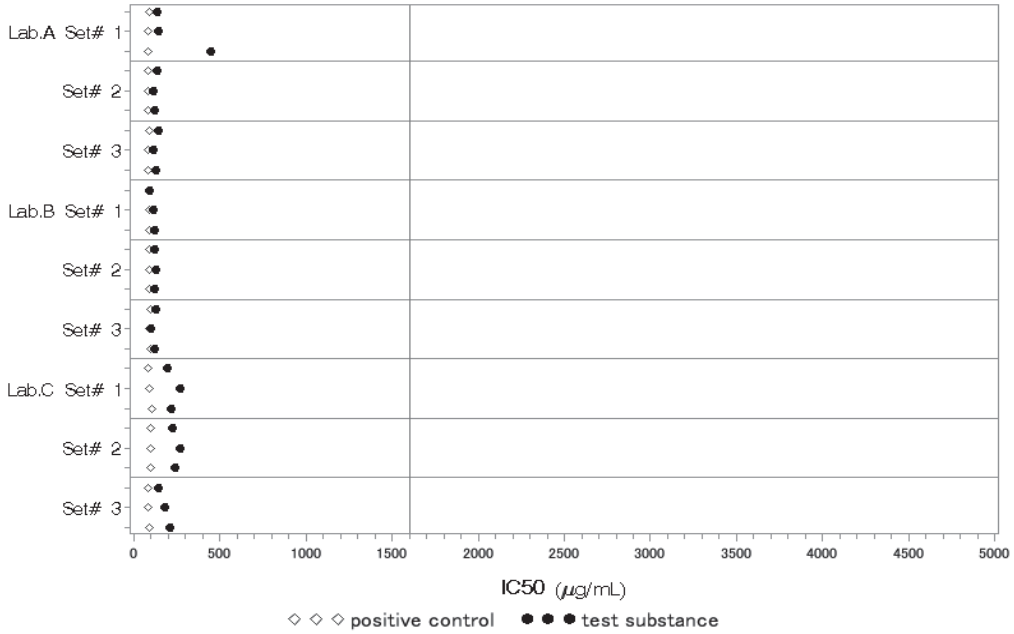


Fig.31. IC_{50}s of the test substance (P2-011) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

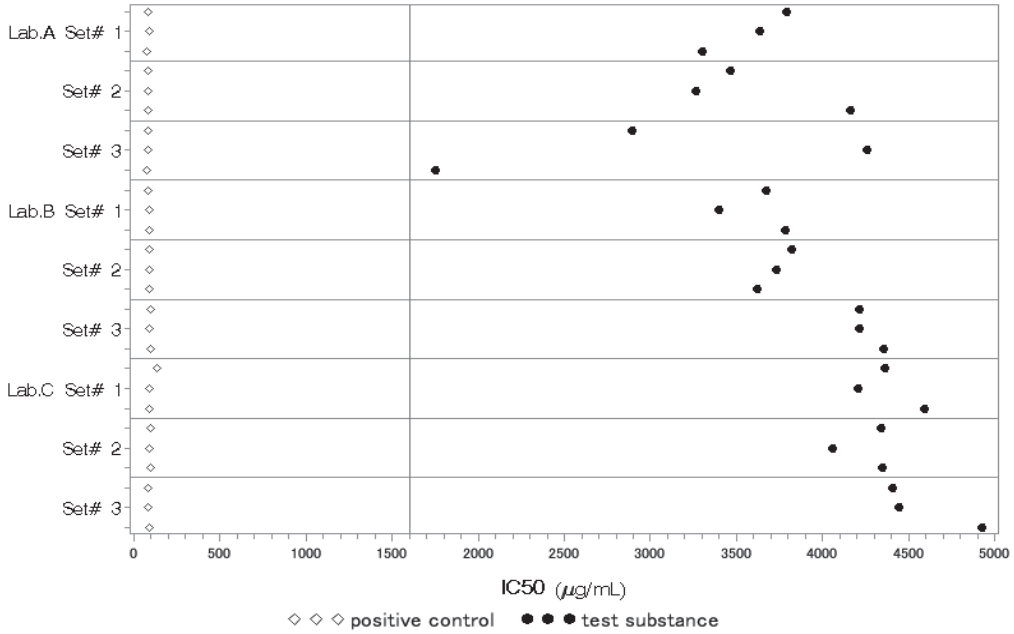


Fig.32. IC_{50}s of the test substance (P2-012) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

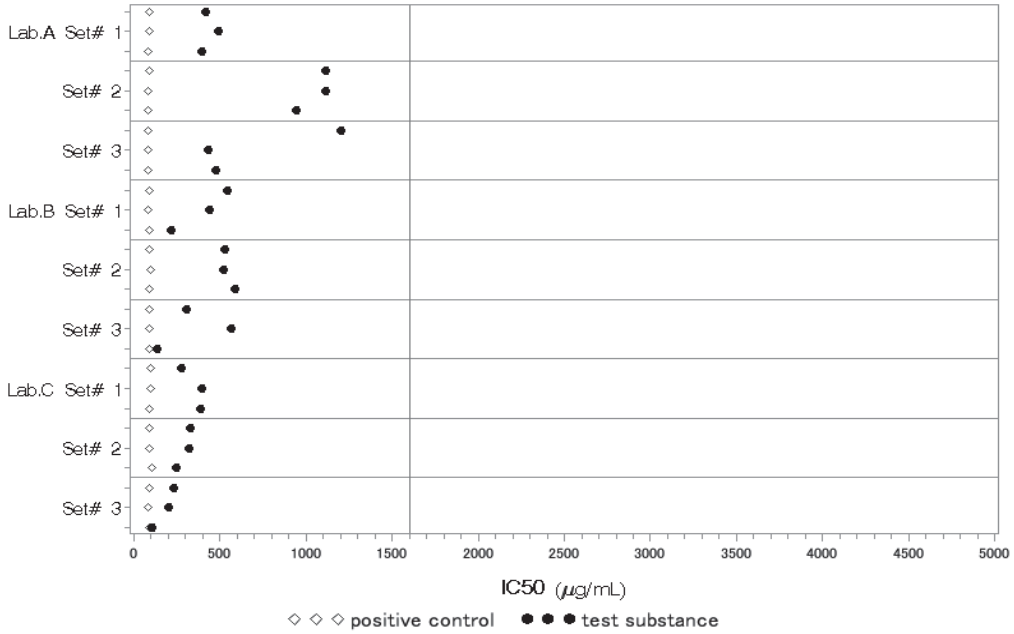


Fig.33. IC_{50}s of the test substance (P2-013) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

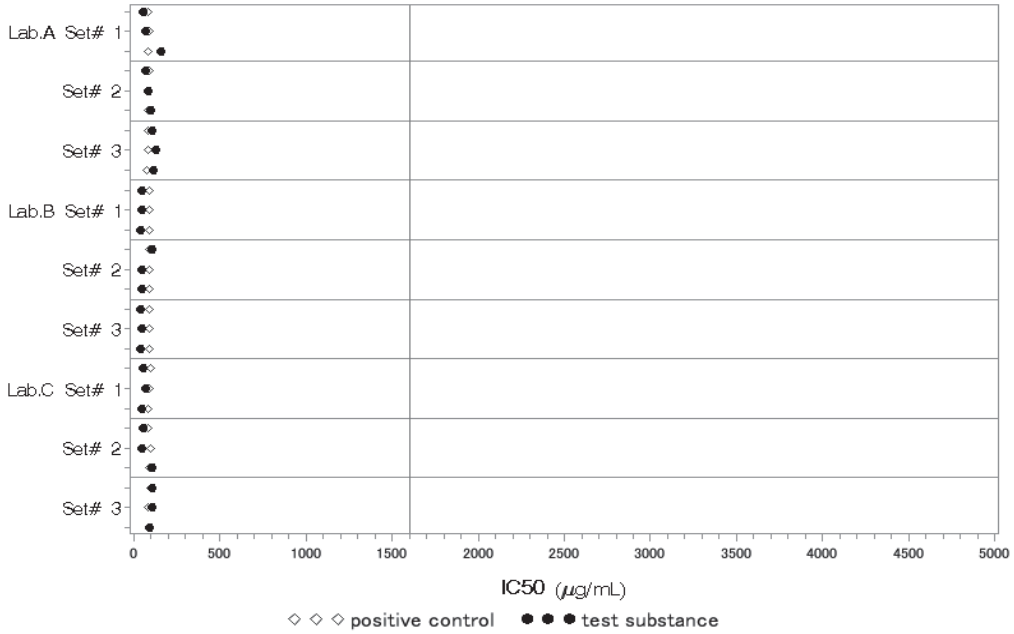


Fig.34. IC_{50}s of the test substance (P2-014) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

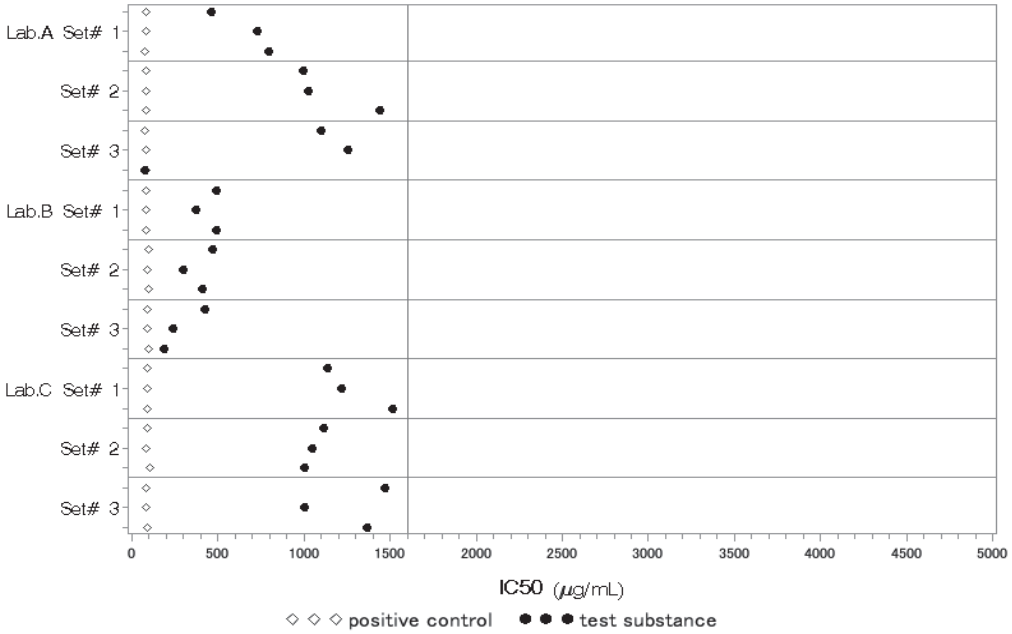


Fig.35. IC_{50}s of the test substance (P2-015) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

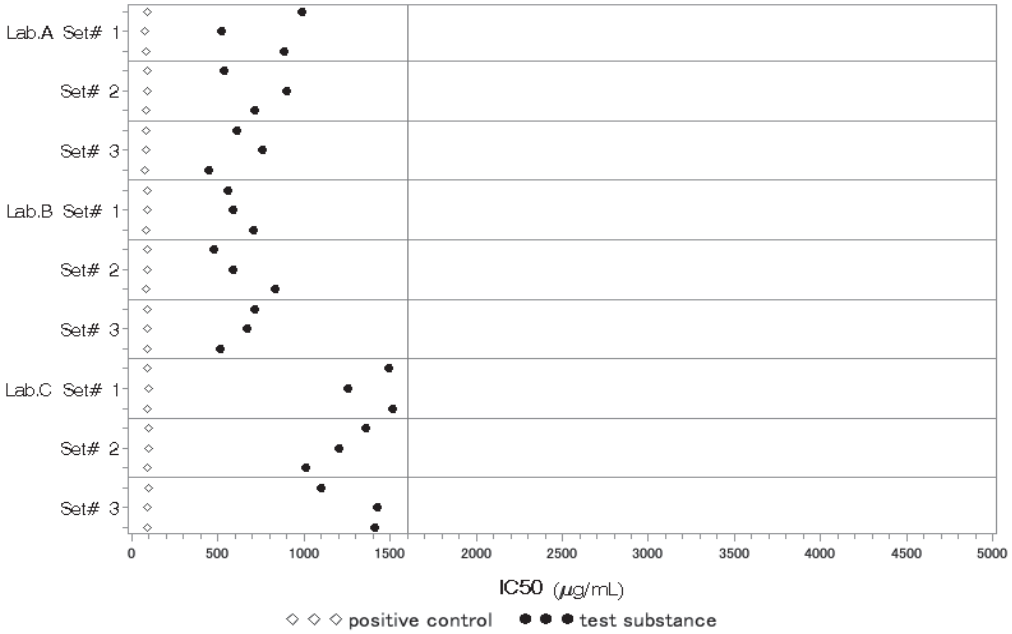


Fig.36. IC_{50}s of the test substance (P2-016) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

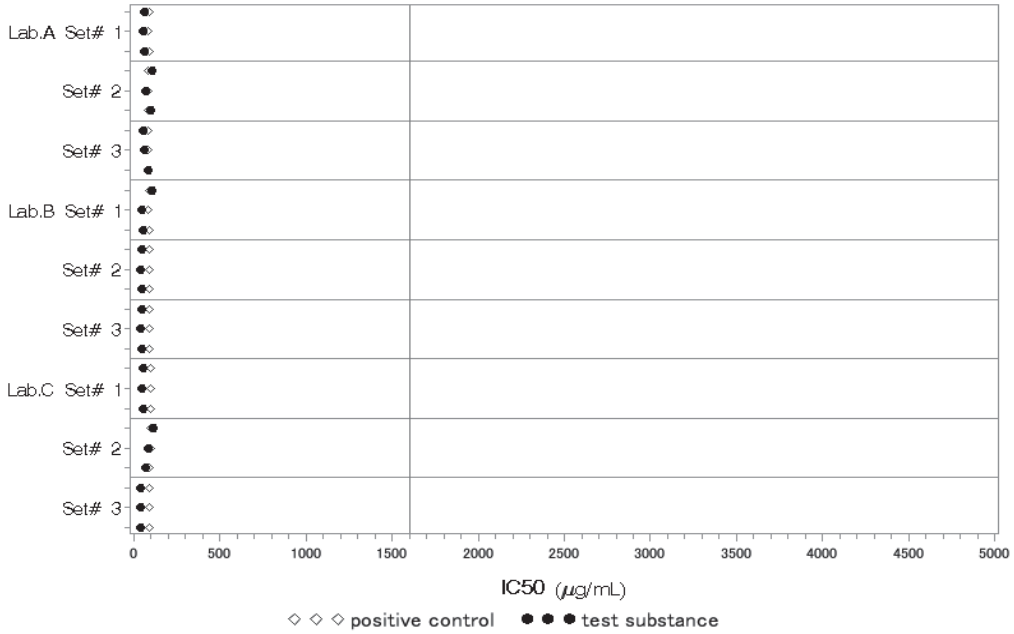


Fig.37. IC_{50}s of the test substance (P2-017) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

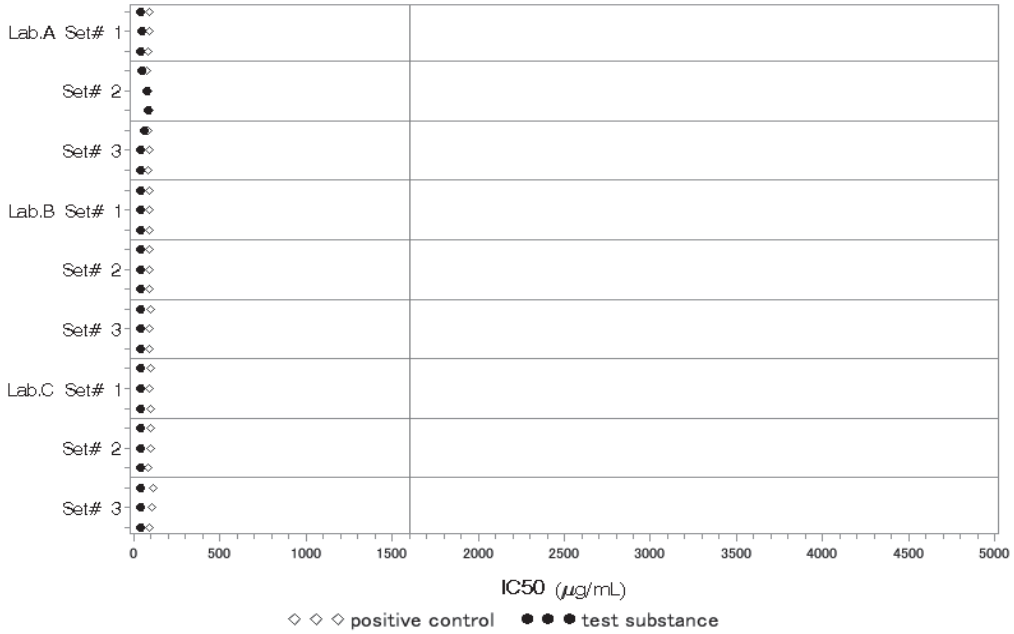


Fig.38. IC_{50}s of the test substance (P2-018) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

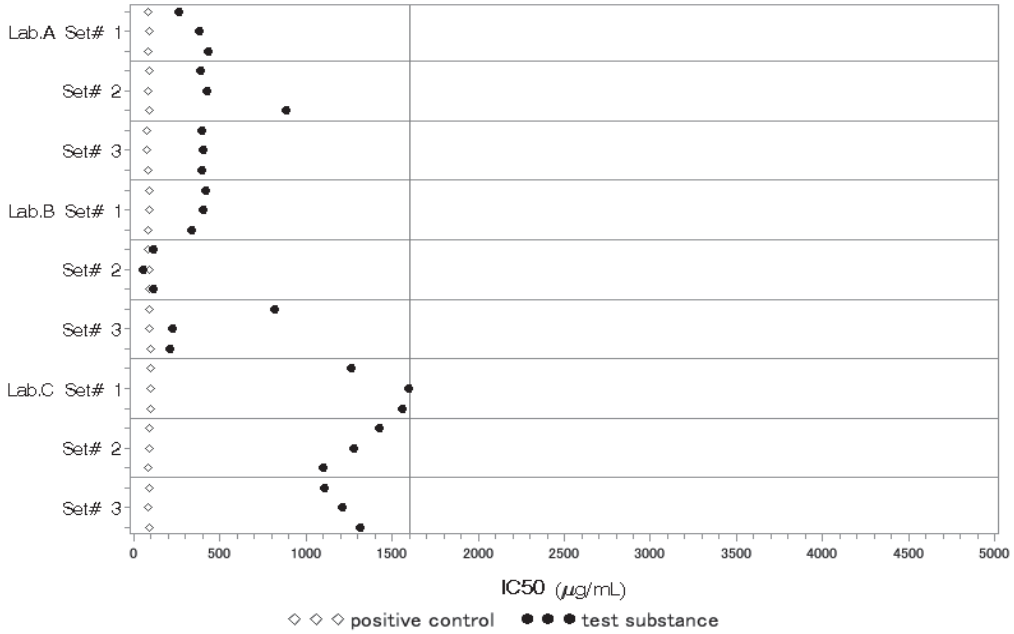


Fig.39. IC_{50}s of the test substance (P2-019) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

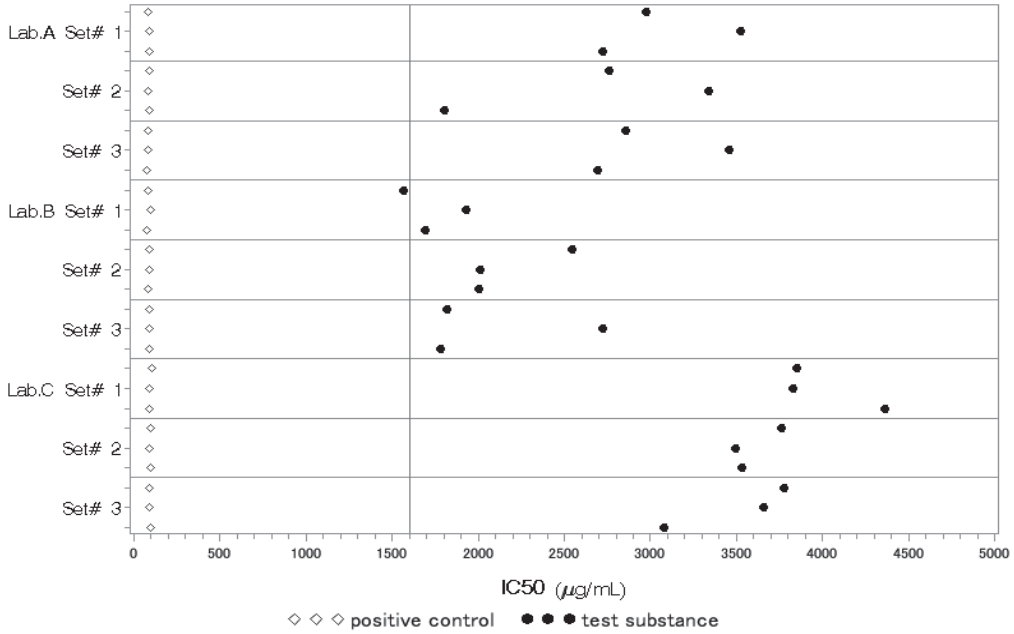


Fig.40. IC_{50}s of the test substance (P2-020) and positive controls, and IC_{50} of 1600 μg/mL as a cut-off value within each laboratory.

September 11, 2009

Informational materials for peer review of the SIRC cytotoxicity test and the three dimensional dermal model (MATREX^{TM}) test

Shiseido Research Center
Shigenobu Hagino, Ph.D.

Table 1 Information obtained from the Draize eye test and the alternative methods

Information from the Draize test	CAM	Erythrocyte	Skin model	SIRC	Human cultured cells	Animal cultured cells	EYTEX
<1> Corneal opacity							
a Degeneration of membrane parenchyma (collagen)	▲	×	○	×	×	×	○
b Swelling of collagen (depending on the intensity of epithelial/endothelial disorders)	▲	×	○	×	×	×	○
c Degeneration/exfoliation of epithelial cells (due to cytotoxicity)	▲	▲	○	○	○	○	×
<2> Iris							
a Transcorneal absorption and damage to the iris	×	×	×	×	×	×	×
b Light reflex	×	×	×	×	×	×	×
<3> Conjunctiva							
a Redness (inflammatory vascular dilation)	○	×	▲	▲	▲	▲	×
b Edema (inflammatory edema)	▲	×	▲	▲	▲	▲	×
c Secretion (excessive lacrimation/inflammatory infiltrating reaction)	×	×	×	×	×	×	×
<4> Information from follow-up							
a Repair	▲	×	▲	▲	▲	▲	×
b Presence of delayed onset	▲	▲	▲	▲	▲	▲	×
<5> Information about observation items excluded from the Draize test							
a Corneal ulcer (damage to/lack of corneal epithelium)	×	×	×	×	×	×	×
b Irregularity of cornea (dryness/concave formation)	×	×	×	×	×	×	×
c Improvement in disorders following eye irrigation	○	×	○	▲	▲	▲	×
d Evaluation of pain (observation of behavior/No. of nictitations/closed eye)	×	×	×	×	×	×	×
e Detection of disorders due to physical stimulation (insoluble substance)	×	×	×	×	×	×	×

Note: Evaluations based on the literature before starting validation: ○: possible introduction, ▲: investigation needed to establish introduction, ×: impossible to introduce

The table that reported by Kaneko et al. (1996) is translated into English.

Table 2 Scale for scoring ocular lesions in the Draize eye test

(1) Cornea	
(A) Opacity-degree of density (area most dense taken for reading)	
No Opacity	0
Scattered or diffuse area, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
(B) Area of cornea involved	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
A × B × 5	Total maximum = 80
(2) Iris	
(A) Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
A × 5	Total maximum = 10
(3) Conjunctivae	
(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
(B) Chemosis	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
(C) Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
Score (A + B + C) × 2	Total maximum = 20

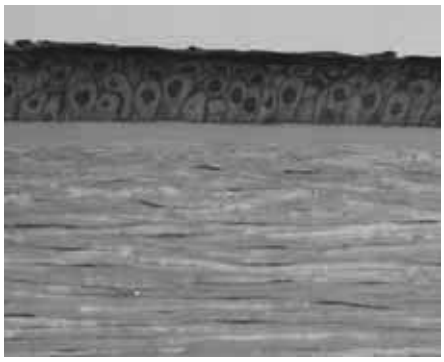
The table is the same as that reported by Draize et al. (1959)

Fig. 1 Construction of cornea

Epithelial layer
Bowman's layer

Stroma

Descemet's membrane
Endothelial layer



The figure from Hirano (2008) is translated into English.

Table 3 List of the methods evaluated and the participation of each organization

Methods	Organizations participating in the validation study																					Number of participants*		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	3rd	2nd	1st
Chorioallantoic membrane																									
HET–CAM	●	●			●		●					●											5	5	5
CAM–TB	●	●			●		●					●											5	5	5
Red blood cells																									
RBC		●	●		●						●	●								●			6	9	7
Haemoglobin																									
HD#	●		●									●	●	●						●			6	8	8
Artificial skin models																									
SKIN^{2TM} (ZK1100)	●		●	●							●					●	●			●		●	8	6	8
MATREX™					●	●				●												●	4	7	3
Normal cells from rabbit cornea																									
CornePack*#										●			●				●	●				●	5	6	7
Cell lines from rabbit cornea																									
SIRC–CVS	●	●					●									●					●		5	6	6
SIRC–NRU	●	●		●			●									●					●		6	7	7
Cell lines from the other mammals																									
HeLa–MTT						●				●	●	●	●			●			●				6	8	8
CHL–CVS							●		●			●											4	7	7
EYTEX™			●		●			●							●			●		●		●	7	5	5
Sum	6	4	4	3	4	3	5	1	2	2	3	6	3	1	1	4	2	1	2	4	2	4	67	79	76

V: suppliers of test kits. *: number of participants to each validation.
HET–CAM: hen's egg–chorioallantoic membrane method; CAM–TB: chorioallantoic membrane-trypan blue staining method; RBC: haemolysis method; HD: haemoglobin denaturation method; SIRC–CVS: cytotoxicity tests on SIRC cells using crystal violet staining; HeLa–MTT: cytotoxicity tests on HeLa cells using MTT reduction; CHL–CVS: cytotoxicity test on CHL cells using crystal violet staining; #: major changes in protocols were made before the second validation.

The table is the same as that reported by Ohno et al.(1999).

Table 4
List of the co-operating organizations for the Japanese validaton

Administrative organizations
Ministry of Health and Welfare
National Institute of Health Sciences
(Div. Pharmacol. Div. Toxicol.
and Div. Genetics Mutagen.)

Universities
Yokohama-City University
Showa University

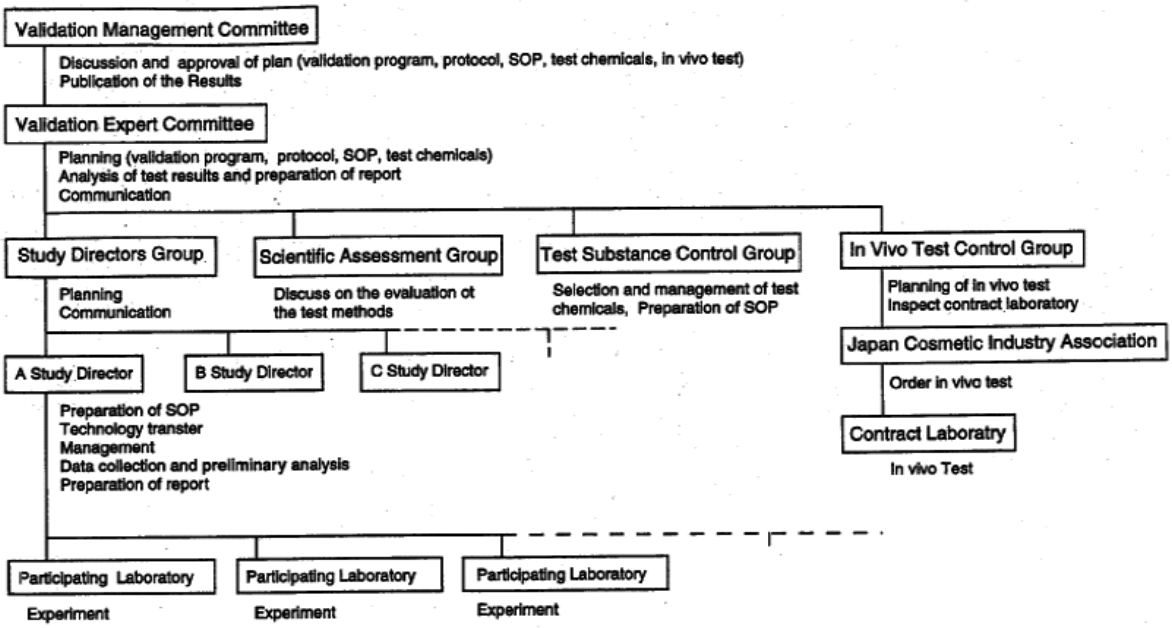
Kit suppliers
Oriental Yeast Co. Ltd
Kurabo Industries, Ltd
Invitro International Japan, Ltd
Toyobo Co., Ltd

Others
RIKEN Gene Bank
Japan Seigiken Research Centre Co. Ltd

Japan Cosmetic Industry Corporation
Shiseido Safety & Analytical Res. Center
POLA Corp.
Kanebo Ltd
KOSE Corp.
Lion Corp.
KAO Corp.
SUNSTAR Inc.
OPPEN Cosmetic Co. Ltd
NOEVIR Co. Ltd
Kaminomoto Co. Ltd
Procter & Gamble Far East, Inc.
Nippon Menard Cosmetic Co. Ltd
Yakult Central Institute for Microbiological Res.
Ajinomoto Co. Inc.
Cow Brand Soap Kyoshinsha Co., Ltd
Hoyu Co. Ltd
CLUB COSMETICS Co. Ltd
Nippon Shikizai Inc.

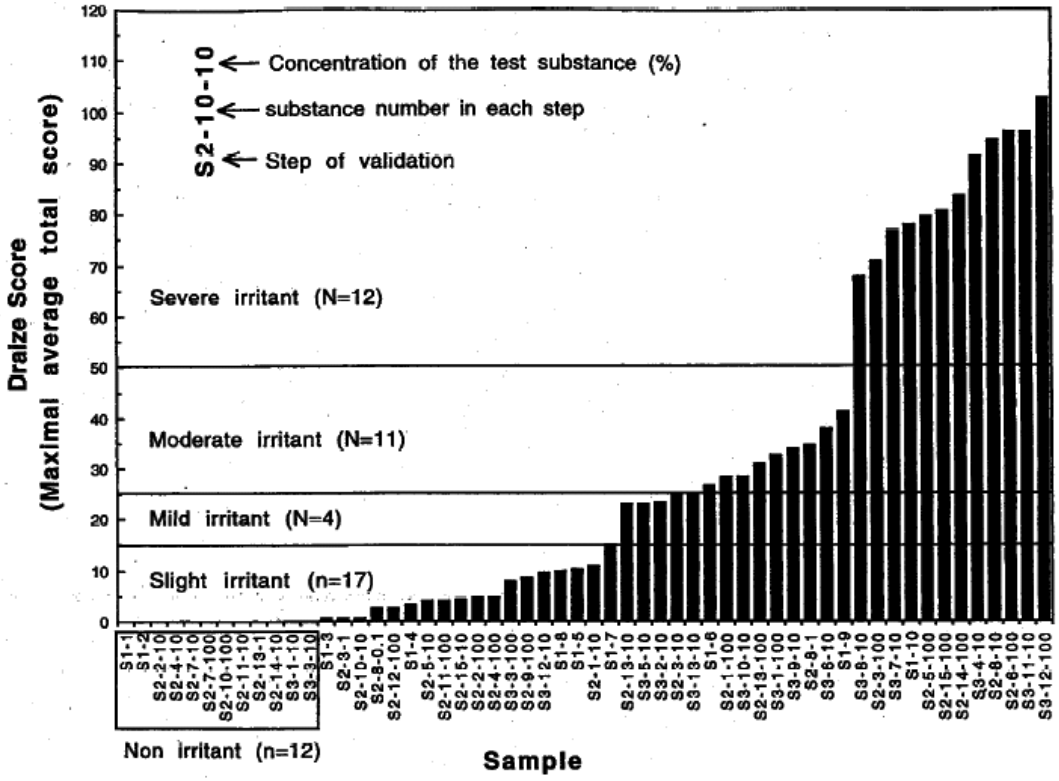
The table is the same as that reported by Ohno et al.(1999).

Fig. 2 Organization of the second and third validations



The figure is the same as that reported by Ohno et al.(1999).

Fig. # Eye irritation potential of test samples used in the Japanese validaton study



Abscissa indicates the sample number and ordinate indicates the maximal average scores (MAS). The first two characters of the sample number indicate the stage of the validation, the next two numerals indicate the identification number of the test substance in each validation, and the last numerals indicate the concentration of the test substance which was applied to the eyes of the rabbits. Chemical names corresponding to each number of the test substances are indicated separately.

Sample no. and chemical name	
S1-1	Isotonic sodium chloride solution
S1-2	Polyoxyethylene hydrogenated caster oil (60 E.O.)
S1-3	Polyoxyethylene sorbitan monolaurate (20 E.O.)
S1-4	Polyethyleneglycol monolaurate (10 E.O.)
S1-5	Sodium N-lauryl sarcosinate (30% solution)
S1-6	Sodium hydrogenated tallow L-glutamate
S1-7	Sodium lauryl sulfate
S1-8	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)
S1-9	Polyoxyethylene octylphenylether (10 E.O.)
S1-10	Benzalkonium chloride
S2-1	Sucrose fatty scid ester
S2-2	Glycerin
S2-3	Acid red 92
S2-4	Polyoxyethylene sorbitan monooleate (20E.O.)
S2-5	Calcium thioglycolate
S2-6	Distearyldimethylammonium chloride
S2-7	2-Ethylhexyl p-dimethylamino benzonate
S2-8	Cetylpyridinium chloride
S2-9	Methyl p-hydroxybenzoate
S2-10	Isopropyl myristate
S2-11	Polyethylene glycol 400
S2-12	Silicic anhydride
S2-13	Benzyl alcohol
S2-14	Sodium salicylate
S2-15	m-Phenylenediamine
S3-1	Ethanol
S3-2	Monoethanolamine
S3-3	Triethanolamine
S3-4	Stearyltrimethylammonium chloride
S3-5	Diisopropanolamine
S3-6	Potassium laurate
S3-7	Cetyltrimethylammonium bromide
S3-8	Acetic acid
S3-9	Butanol
S3-10	Chlorhexidine gluconate (20% solution)
S3-11	Domiphen bromide
S3-12	Lactic acid
S3-13	Glycolic acid
S3-14	Di (2-ethylhexyl) sodium sulfosuccinate

Table 5 List of the test substances and their characteristics

Substance no.	Name	Test substances		10% Aqueous solutions	
		Class	Nature	Nature	pH
S1-1	Isotonic sodium chloride solution	–	Solution	Solution	5.71
S1-2	Polyoxyethylene hydrogenated castor oil (60 E.O.)	Surfactants (nonionic)	White wax	Solution	4.17
S1-3	Polyoxyethylene sorbitan monolaurate (20 E.O.) (Tween 80)	Surfactants (nonionic)	Yellow liquid	Solution	6.79
S1-4	Polyethyleneglycol monolaurate (10 E.O.)	Surfactants (nonionic)	Liquid	Solution	3.86
S1-5	Sodium *N*-lauroyl sarcosinate (30% solution)	Surfactants (anionic)	Liquid	Solution	7.57
S1-6	Sodium *N*-hydrogenated tallow L-glutamate	Surfactants (anionic)	White powder	Suspension	6.85
S1-7	Sodium lauryl sulfate	Surfactants (anionic)	White flake	Solution	5.98
S1-8	Sodium polyoxyethylene laurylether sulfate (2E.O.) (27% solution)	Surfactants (anionic)	Liquid	Solution	6.65
S1-9	Polyoxyethylene octylphenylether (10 E.O.) (Triton X-100)	Surfactants (nonionic)	Liquid	Solution	6.35
S1-10	Benzalkonium chloride	Surfactants (cationic)	White powder	Suspension	4.97
S2-1	Sucrose fatty acid ester	Surfactants (nonionic)	White powder	Suspension	6.86
S2-2	Glycerin	Polyols	Liquid	Solution	5.96
S2-3	Acid Red 92	Colour additives	Red powder	Red solution	8.27
S2-4	Polyoxyethylene sorbitan monooleate (20 E.O.)	Surfactants (nonionic)	Liquid	Solution	6.23
S2-5	Calcium thioglycolate	Organic salts	White powder	turbid sol.	11.57
S2-6	Distearyldimethylammonium chloride	Surfactants (cationic)	White flake	turbid sol.	5.51
S2-7	2-Ethylhexyl *p*-dimethylamino benzoate	PABA derivatives	Liquid	Suspension	4.74
S2-8	Cetylpyridinium chloride	Surfactants (cationic)	White powder	Solution	4.41
S2-9	Methyl *p*-hydroxybenzoate	Esters	White powder	Suspension	4.99
S2-10	Isopropyl myristate	Esters	Liquid	Suspension	6.72
S2-11	Polyethylene glycol 400	Polyols	Liquid	Solution	5.05
S2-12	Silicic acid	Inorganics	White powder	Suspension	5.74
S2-13	Benzyl alcohol	Alcohols	Liquid	Suspension	6.44
S2-14	Sodium salicylate	Organic salts	Particle	Solution	6.50
S2-15	*m*-Phenylene diamine	Amines	Black pellet	Solution	8.56
S3-1	Ethanol	Alcohols	Volatile liquid	Solution	5.90
S3-2	Monoethanolamine	Alkanolamines	Liquid	Solution	12.58
S3-3	Triethanolamine	Alkanolamines	Liquid	Solution	11.26
S3-4	Stearyltrimethylammonium chloride	Surfactants (cationic)	Solid, Liquid	Solution	4.24
S3-5	Diisopropanolamine	Alkanolamines	White powder	Solution	11.89
S3-6	Potassium laurate	Surfactants (anionic)	White powder	Solution	10.49
S3-7	Cetyltrimethylammonium bromide	Surfactants (cationic)	Wax	Solution	5.89
S3-8	Acetic acid	Carboxilic acids	Liquid	Solution	2.40
S3-9	Butanol	Alcohols	Volatile liquid	Suspension	7.31
S3-10	Chlorhexidine gluconate solution (20% solution)	Organic salts	Liquid	Solution	6.56
S3-11	Domiphen bromide	Surfactants (cationic)	White powder	Solution	6.22
S3-12	Lactic acid	Carboxilic acids	Liquid	Solution	1.94
S3-13	Glycolic acid	Carboxilic acids	White powder	Solution	1.76
S3-14	Di(2-ethylhexyl) sodium sulfosuccinate	Surfactants (anionic)	White powder	Suspension	6.54

Table 6 GHS classification of serious eye damage / eye irritation

Caterory of GHS	Decision by in vivo test (Draize test) result	Decision by existing classification
1	・At least in one animal, effects on the cornea, iris or conjunctiva that are not expected to reverse or have not fully reversed within an observation period of 21 days after installation of the test material ・At least in 2 of 3 tested animals, the average values of the scores following grading at 24, 48 and 72 hours after installation of the test material are 3 or more in corneal opacity, and more than 1.5 in iritis	・The substance which is classified as Severe or Corrosive (very strong irritation or corrosiveness corresponding to AOI 80 or more) is classified as Category 1 (however, when irreversible lesion is not observed, the substance is determined as irritating to the eye (Category 2A)).
2A	・In the Draize test conducted using 3 animals, the average values of the scores following grading at 24, 48 and 72 hours after installation of the test material in two or more animals are 1 or more in corneal opacity, 1 or more in iritis, 2 or more in conjunctival redness and 2 or more in conjunctival edema. ・The effects are fully reversed within an observation period of 21 days.	・The substance which is classified as Moderate (strong irritation corresponding to AOI 30–80) is classified as Category 2A.
2B	・In the Draize test conducted using 3 animals, the average values of the scores following grading at 24, 48 and 72 hours after installation of the test material in two or more animals are 1 or more in corneal opacity, 1 or more in iritis, 2 or more in conjunctival redness and 2 or more in conjunctival edema. ・The substance is classified as mildly irritating to the eye (Category 2B) when the above description applies to the substance and the effect reverses within 7 days.	・The substance which is classified as Mild is classified as Category 2B.

Decision by physico-chemical properties: In the case of pH ≤ 2, ≥ 11.5, the substance is classified as Category 1 (determined with buffer capacity taken into consideration (Booman et al. (1989) proposed 0.2 meq HCL/g in eye irritation).

The table is the same as technical guidance document on the GHS classification.

Table 7 Grading of eye irritation by Kay and Calandra method

Scoring Index (Maximal average score)	Grading
0.0 - 5.0	None irritant
5.1 - 15.0	Minimal irritant
15.1 - 30.0	Mild irritant
30.1 - 60.0	Moderate irritant
60.1 - 80.0	Severe irritant
80.1 - 110.0	Extreme irritant

The grading was reported by Kay and Calandra (1962).

Table 8 Grading of eye irritation reported by Ohno et al.(1999)

Scoring Index (Maximal average score)	Grading
0.0 - 15.0	Slight
15.1 - 25.0	Mild irritant
25.1 - 50.0	Moderate irritant
50.1 - 110.0	Severe irritant

The grading was reported by Ohno et al. (1999).

Table 9 Grading of eye irritation reported by Ohno et al.(2004).

Scoring Index (Maximal average score)	Grading
0.0 - 5.0	Slight
5.1 - 25.0	Mild irritant
25.1 - 50.0	Moderate irritant
50.1 - 110.0	Severe irritant

The grading was reported by Ohno (2004).

Table 10 Draize eye test results in the Japanese validation study（Concentration ：10%）

Substance （Concentration ：10%）	MAS	GHS
Ethanol	0.0	NI
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI
Glycerin	0.0	NI
Polyethylene glycol 400	0.0	NI
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI
Sodium salicylate	0.0	NI
Triethanolamine	0.0	NI
Isopropyl myristate	0.7	NI
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.7	NI
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI
Calcium thioglycolate	4.0	NI
m-Phenylenediamine <lack of stability>	4.3	NI
Lactic acid	9.7	NI
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI
Sucrose fatty acid ester	11.0	NI
Diisopropanolamine	23.0	NI
Sodium lauryl sulfate	15.0[$]	1or2A
Benzyl alcohol	23.0	1or2A
Monoethanolamine	23.3	2B
Acid red 92	25.0	1or2A
Glycolic acid	25.0	2B
Sodium hydrogenated tallow L-glutamate	26.7	1or2A
Chlorhexidine gluconate (20% solution)	28.3	2A
Butanol	34.0	1or2A
Potassium laurate	38.0	1or2A
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A
Acetic acid	68.0	1or2A
Cetyltrimethylammonium bromide	76.7	1or2A
Benzalkonium chloride	78.0	1or2A
Stearyltrimethylammonium chloride	91.3	1or2A
Cetylpyridinium chloride	94.7	1
Domiphen bromide	96.3	1

MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.

Table 11 Draize eye test results in the Japanese validation study（as is）

Substance （as is）	Physical state	MAS	GHS
2-Ethylhexyl p-dimethylamino benzonate	Liquid	0.0	NI
Isopropyl myristate	Liquid	0.0	NI
Isotonic sodium chloride solution	Liquid	0.0	NI
Silicic anhydride	Powder	2.7	NI
Polyethylene glycol 400	Liquid	4.0	NI
Glycerin	Liquid	4.7	NI
Polyoxyethylene sorbitan monooleate (20 E.O.)	Liquid	4.7	NI
Triethanolamine	Liquid	8.0	NI
Methyl p-hydroxybenzoate	Powder	8.7	NI
Sucrose fatty acid ester	Powder	28.3	1or2A
Benzyl alcohol	Liquid	31.0	1or2A
Ethanol	Liquid	32.7	1or2A
Acid red 92	Powder	71.0	1or2A
Calcium thioglycolate	Powder	79.7	1
m-Phenylenediamine	Powder	80.7	1or2A
Sodium salicylate	Powder	83.7	1or2A
Distearyldimethylammonium chloride	Powder	96.3	1
Lactic acid	Liquid	102.7	1

Table 12 Draize eye test results in the Japanese validation study（Concentration ：1%）

Substace (Concentration:1%)	MAS	GHS
Benzyl alcohol	0	NI
Acid Red 92	0.7	NI
Cetylpyridinium chloride	34.7	1or2A

Table 13 Draize eye test results in the Japanese validation study（Concentration ：0.1%）

Substance (Concentration:0.1%)	MAS	GHS
Cetylpyridinium chloride	2.7	NI

Table 14 Draize eye test results in the Japanese validation study

Substance （Concentration ：10%）	Concentration	MAS	GHS
2-Ethylhexyl p-dimethylamino benzonate	as is	0.0	NI
Isopropyl myristate	as is	0.0	NI
Isotonic sodium chloride solution	as is	0.0	NI
Silicic anhydride	as is	2.7	NI
Polyethylene glycol 400	as is	4.0	NI
Glycerin	as is	4.7	NI
Polyoxyethylene sorbitan monooleate (20 E.O.)	as is	4.7	NI
Triethanolamine	as is	8.0	NI
Methyl p-hydroxybenzoate	as is	8.7	NI
Sucrose fatty acid ester	as is	28.3	1or2A
Benzyl alcohol	as is	31.0	1or2A
Ethanol	as is	32.7	1or2A
Acid red 92	as is	71.0	1or2A
Calcium thioglycolate	as is	79.7	1
Sodium salicylate	as is	83.7	1or2A
Distearyldimethylammonium chloride	as is	96.3	1
Lactic acid	as is	102.7	1
Ethanol	10	0.0	NI
2-Ethylhexyl p-dimethylamino benzonate	10	0.0	NI
Glycerin	10	0.0	NI
Polyethylene glycol 400	10	0.0	NI
Polyoxyethylene hydrogenated caster oil (60 E.O.)	10	0.0	NI
Polyoxyethylene sorbitan monooleate (20E.O.)	10	0.0	NI
Sodium salicylate	10	0.0	NI
Triethanolamine	10	0.0	NI
Isopropyl myristate	10	0.7	NI
Polyoxyethylene sorbitan monolaurate (20 E.O.)	10	0.7	NI
Polyethyleneglycol monolaurate (10 E.O.)	10	3.3	NI
Calcium thioglycolate	10	4.0	NI
Lactic acid	10	9.7	NI
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10	10.0	NI
Sodium N-lauryl sarcosinate (30% solution)	10	10.3	NI
Sucrose fatty acid ester	10	11.0	NI
Diisopropanolamine	10	23.0	NI
Sodium lauryl sulfate	10	15.0$	1or2A
Benzyl alcohol	10	23.0	1or2A
Monoethanolamine	10	23.3	2B
Acid red 92	10	25.0	1or2A
Glycolic acid	10	25.0	2B
Sodium hydrogenated tallow L-glutamate	10	26.7	1or2A
Chlorhexidine gluconate (20% solution)	10	28.3	2A
Butanol	10	34.0	1or2A
Potassium laurate	10	38.0	1or2A
Polyoxyethylene octylphenylether (10 E.O.)	10	41.3	1or2A
Di (2-ethylhexyl) sodium sulfosuccinate	10	57.0	1or2A
Acetic acid	10	68.0	1or2A
Cetyltrimethylammonium bromide	10	76.7	1or2A
Benzalkonium chloride	10	78.0	1or2A
Stearyltrimethylammonium chloride	10	91.3	1or2A
Cetylpyridinium chloride	10	94.7	1
Domiphen bromide	10	96.3	1
Benzyl alcohol	1	0	NI
Acid Red 92	1	0.7	NI
Cetylpyridinium chloride	1	34.7	1or2A
Cetylpyridinium chloride	0.1	2.7	NI

MAS: Maximal average score of the Draize eye test.

GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.

1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.

$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (at the maximal corneal score), respectively.

Table 15 Draize eye test results in the Japanese validation study
-GHS classification by considering pH-

Substance （Concentration ：10%）	pH	MAS	GHS
Ethanol	5.90	0.0	NI
2-Ethylhexyl p-dimethylamino benzonate	4.74	0.0	NI
Glycerin	5.96	0.0	NI
Polyethylene glycol 400	5.05	0.0	NI
Polyoxyethylene hydrogenated caster oil (60 E.O.)	4.17	0.0	NI
Polyoxyethylene sorbitan monooleate (20E.O.)	6.23	0.0	NI
Sodium salicylate	6.50	0.0	NI
Triethanolamine	11.26	0.0	NI
Isopropyl myristate	6.72	0.7	NI
Polyoxyethylene sorbitan monolaurate (20 E.O.)	6.79	0.7	NI
Polyethyleneglycol monolaurate (10 E.O.)	3.86	3.3	NI
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	6.65	10.0	NI
Sodium N-lauryl sarcosinate (30% solution)	7.57	10.3	NI
Sucrose fatty acid ester	6.86	11.0	NI
Calcium thioglycolate	11.57	4.0	1*
Lactic acid	1.94	9.7	1*
Sodium lauryl sulfate	5.98	15.0$	2Aor1
Benzyl alcohol	6.44	23.0	2Aor1
Diisopropanolamine	11.89	23.0	1*
Monoethanolamine	12.58	23.3	1*
Acid red 92	8.27	25.0	2Aor1
Glycolic acid	1.76	25.0	1*
Sodium hydrogenated tallow L-glutamate	6.85	26.7	2Aor1
Chlorhexidine gluconate (20% solution)	6.56	28.3	2A
Butanol	7.31	34.0	2Aor1
Potassium laurate	10.49	38.0	2Aor1
Polyoxyethylene octylphenylether (10 E.O.)	6.35	41.3	2Aor1
Di (2-ethylhexyl) sodium sulfosuccinate	6.54	57.0	2Aor1
Acetic acid	2.40	68.0	2Aor1
Cetyltrimethylammonium bromide	5.89	76.7	2Aor1
Benzalkonium chloride	4.97	78.0	2Aor1
Stearyltrimethylammonium chloride	4.24	91.3	2Aor1
Cetylpyridinium chloride	4.41	94.7	1
Domiphen bromide	6.22	96.3	1

MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (at the maximal corneal score), respectively.
: Category 1 means the classification on the basis of pH (pH≤2 or pH≥11.5: severe or corrosive irritant).

Table 16 Recovery time in the Draize eye test of the Japanese validation study
-GHS classification by considering pH-

Substance （Concentration ：10%）	pH	MAS	GHS	Recovery time (hr)
Ethanol	5.90	0.0	NI	0
2-Ethylhexyl p-dimethylamino benzonate	4.74	0.0	NI	0
Glycerin	5.96	0.0	NI	0
Polyethylene glycol 400	5.05	0.0	NI	0
Polyoxyethylene hydrogenated caster oil (60 E.O.)	4.17	0.0	NI	0
Polyoxyethylene sorbitan monooleate (20E.O.)	6.23	0.0	NI	0
Sodium salicylate	6.50	0.0	NI	0
Triethanolamine	11.26	0.0	NI	0
Isopropyl myristate	6.72	0.7	NI	4
Polyoxyethylene sorbitan monolaurate (20 E.O.)	6.79	0.7	NI	4
Polyethyleneglycol monolaurate (10 E.O.)	3.86	3.3	NI	24
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	6.65	10.0	NI	96
Sodium N-lauryl sarcosinate (30% solution)	7.57	10.3	NI	120
Sucrose fatty acid ester	6.86	11.0	NI	72
Calcium thioglycolate	11.57	4.0	1*	72
Lactic acid	1.94	9.7	1*	168<
Sodium lauryl sulfate	5.98	15.0$	1or2A	168<
Benzyl alcohol	6.44	23.0	1or2A	168<
Diisopropanolamine	11.89	23.0	1*	72
Monoethanolamine	12.58	23.3	1*	144
Acid red 92	8.27	25.0	1or2A	168<
Glycolic acid	1.76	25.0	1*	144
Sodium hydrogenated tallow L-glutamate	6.85	26.7	1or2A	168<
Chlorhexidine gluconate (20% solution)	6.56	28.3	2A	168<
Butanol	7.31	34.0	1or2A	168<
Potassium laurate	10.49	38.0	1or2A	168<
Polyoxyethylene octylphenylether (10 E.O.)	6.35	41.3	1or2A	168<
Di (2-ethylhexyl) sodium sulfosuccinate	6.54	57.0	1or2A	168<
Acetic acid	2.40	68.0	1or2A	168<
Cetyltrimethylammonium bromide	5.89	76.7	1or2A	168<
Benzalkonium chloride	4.97	78.0	1or2A	168<
Stearyltrimethylammonium chloride	4.24	91.3	1or2A	168<
Cetylpyridinium chloride	4.41	94.7	1	168<
Domiphen bromide	6.22	96.3	1	168<

MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (at the maximal corneal score), respectively.
: Category 1 means the classification on the basis of pH (pH≤2 or pH≥11.5: severe or corrosive irritant).

Table 17 Recovery time in the Draize eye test of the Japanese validation study（as is）

Substance （as is）	Physical state	MAS	GHS	Recovery time (hr)
2-Ethylhexyl p-dimethylamino benzonate	Liquid	0.0	NI	0
Isopropyl myristate	Liquid	0.0	NI	0
Isotonic sodium chloride solution	Liquid	0.0	NI	0
Silicic anhydride	Powder	2.7	NI	24
Polyethylene glycol 400	Liquid	4.0	NI	24
Glycerin	Liquid	4.7	NI	24
Polyoxyethylene sorbitan monooleate (20 E.O.)	Liquid	4.7	NI	48
Triethanolamine	Liquid	8.0	NI	72
Methyl p-hydroxybenzoate	Powder	8.7	NI	168
Sucrose fatty acid ester	Powder	28.3	1or2A	168<
Benzyl alcohol	Liquid	31.0	1or2A	168<
Ethanol	Liquid	32.7	1or2A	168<
Acid red 92	Powder	71.0	1or2A	168<
Calcium thioglycolate	Powder	79.7	1	168<
m-Phenylenediamine	Powder	80.7	1or2A	168<
Sodium salicylate	Powder	83.7	1or2A	168<
Distearyldimethylammonium chloride	Powder	96.3	1	168<
Lactic acid	Liquid	102.7	1	168<

Table 18 Recovery time in the Draize eye test of the Japanese validation study
（Concentration ：1%）

Substace (Concentration:1%)	MAS	GHS	Recovery time (hr)
Benzyl alcohol	0	NI	0
Acid Red 92	0.7	NI	24
Cetylpyridinium chloride	34.7	1or2A	168<

Table 19 Recovery time in the Draize eye test of the Japanese validation study
（Concentration ：0.1%）

Substance (Concentration:0.1%)	MAS	GHS	Recovery time (hr)
Cetylpyridinium chloride	2.7	NI	72

Table 20 Regional difference in the Draize eye test results (0<MAS<50) of the Japanese validation study

Substance	Concn (%)	MAS	GHS	Cornea	Iris	Conjunctivae	Recovery time (hr)
Acid Red 92	1	0.7	NI	0	0	0.7	24
Isopropyl myristate	10	0.7	NI	0	0	0.7	4
Polyoxyethylene sorbitan monolaurate (20 E.O.)	10	0.7	NI	0	0	0.7	4
Cetylpyridinium chloride	0.1	2.7	NI	0	0	2.7	72
Silicic anhydride	100	2.7	NI	0	0	2.7	24
Polyethyleneglycol monolaurate (10 E.O.)	10	3.3	NI	0	0	3.3	24
Polyethylene glycol 400	100	4.0	NI	0	0	4.0	24
Glycerin	100	4.7	NI	0	0	4.7	24
Polyoxyethylene sorbitan monooleate (20 E.O.)	100	4.7	NI	0	0	4.7	48
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10	10.0	NI	3.3	0	10.0	96
Sodium N-lauryl sarcosinate (30% solution)	10	10.3	NI	8.3	0	8.0	120
Sucrose fatty scid ester	10	11.0	NI	1.7	1.7	9.3	72
Calcium thioglycolate	10	4.0	I*	0	0	4.0	72
Lactic acid	10	9.7	I*	5.0	0	8.0	168<
Sodium lauryl sulfate	10	15.0$	I	8.3	0	10.0	168<
Benzyl alcohol	10	23.0	1or2A	15.0	1.7	10.0	168<
Diisopropanolamine	10	23.0	1*	16.7	1.7	4.7	72
Monoethanolamine	10	23.3	1*	13.3	1.7	10.0	144
Acid red 92	10	25.0	1or2A	20.0	1.7	10.0	168<
Glycolic acid	10	25.0	1*	15.0	0.0	14.0	144
Sodium hydrogenated tallow L-glutamate	10	26.7	1or2A	16.7	1.7	12.0	168<
Chlorhexidine gluconate (20% solution)	10	28.3	2A	18.3	1.7	12.7	168<
Sucrose fatty scid ester	100	28.3	1or2A	23.3	1.7	8.0	168<
Benzyl alcohol	100	31.0	1or2A	25.0	1.7	8.7	168<
Ethanol	100	32.7	1or2A	26.7	0.0	8.7	168<
Butanol	10	34.0	1or2A	30.0	1.7	10.0	168<
Potassium laurate	10	38.0	1or2A	30.0	1.7	10.0	168<
Cetylpyridinium chloride	1	34.7	1or2A	21.7	1.7	12.7	168<
Polyoxyethylene octylphenylether (10 E.O.)	10	41.3	1or2A	30.0	5.0	10.0	168<

MAS: Maximal average score of the Draize eye test.

GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.

1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.

$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (at the maximal corneal score), respectively.

: Category 1 means the classification on the basis of pH (pH≤2 or pH≥11.5: severe or corrosive irritant).

Table 21 Relationship between MMAS and GHS in the Draize eye test

	Test chemical	CAS No.	Supplier	Purity (%)	In vivo Draize		
					MMAS[a]	EU[b]	GHS[c]
1	3,3-Dimethylpentane	562-49-2	Aldrich	99	0.0	NI	NI
2	3-Methoxy-1,2-propanediol	623-39-2	Acros	98	0.0	NI	NI
3	Polyethylene glycol 400	25322-68-3	Aldrich	–	0.0	NI	NI
4	Glycerol	56-81-5	Sigma	99.5	1.7	NI	NI
5	Methyl cyclopentane	96-37-7	Fluka	>95	3.7	NI	NI
6	Tween 20	9005-64-5	Sigma	–	4.0	NI	NI
7	Methyl isobutyl ketone	108-10-1	Fluka	>99	4.8	NI	NI
8	Toluene	108-88-3	Acros	⩽99.5	9.0	NI	NI
9	Methyl amyl ketone	110-43-0	Aldrich	99	10.5/16.3	NI	NI
10	2-Methyl-1-pentanol	105-30-6	Acros	98.5	13.0	NI	2
11	Ethanol	64-17-5	Merck	⩽99.8	24.0	NI	2
12	Sodium hydroxide (1%)[d]	1310-73-2	Merck	⩽99	25.8	R36	2
13	Triton X-100 (5%)[d]	9002-93-1	Acros	SG	32.3	R36	2
14	1-Octanol	111-87-5	Aldrich	99	41.0	R36	2
15	2-Ethyl-1-hexanol	104-76-7	Aldrich	99	51.3	R36	2
16	*n*-Hexanol	111-27-3	Acros	98	64.8	R36	2
17	Acetone	67-64-1	Fluka	–	65.8	R36	2
18	Cyclohexanol	108-93-0	Aldrich	99	79.8	R41	1
19	Cetylpyridinium bromide (6%)[d]	140-72-7	Sigma	>99	85.8	R41	1
20	Benzalkonium chloride (10%)[d]	8001-54-5	Sigma	Ultra	108.0	R41	1

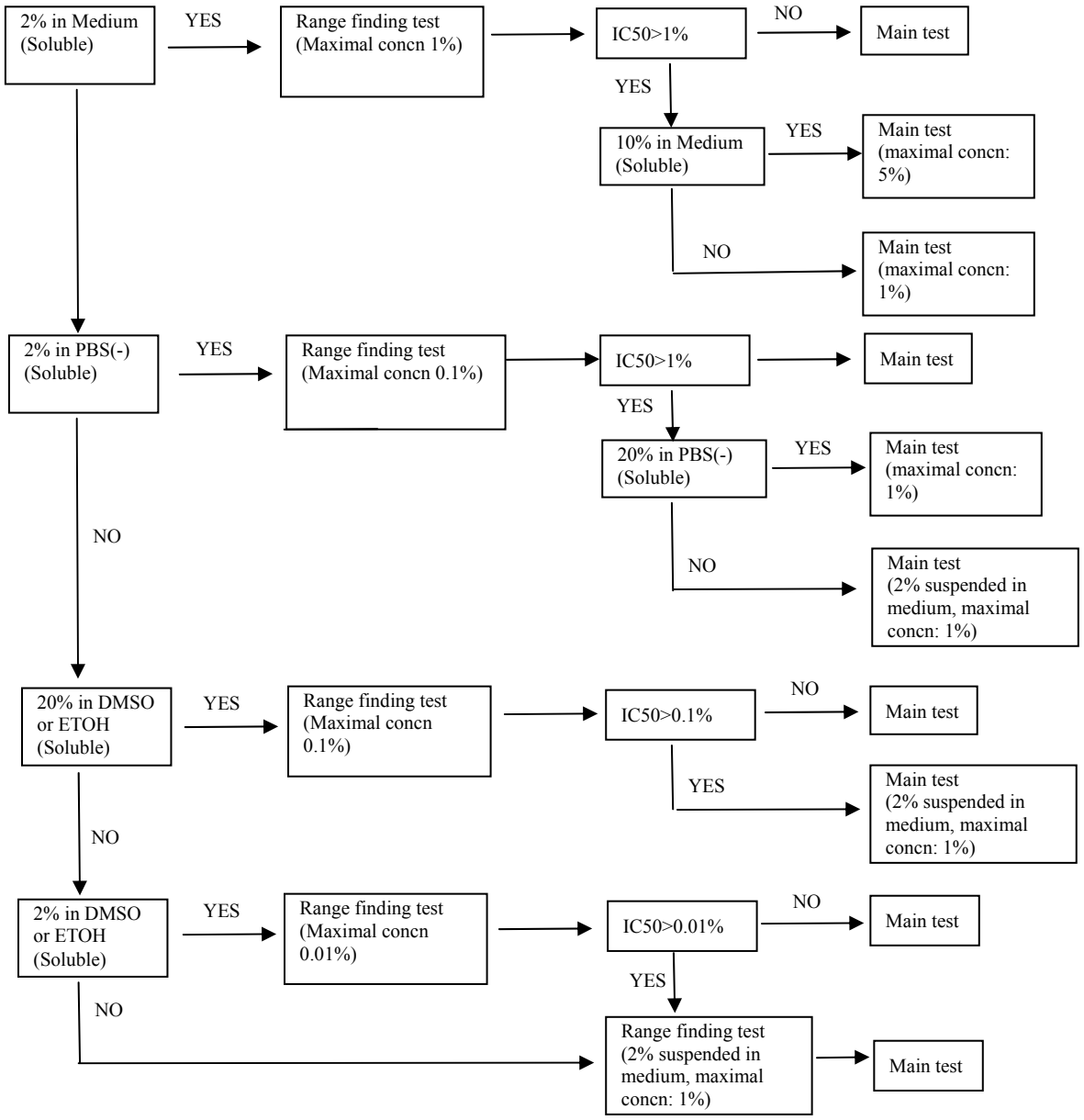
The data are the same as that reported by Goethem et al. (2006)

Table 22 Methods of the SIRC-NRU assay and the SIRC-CVS assay

Cell and culture conditions	SIRC cells derived from rabbit cornea were obtained from the American Type Culture Collection. These were cultured and distributed by the Japanese Cancer Research Resources Bank (JCRB) to each laboratory. The passage numbers of the cells used in each laboratory ranged from 417 to 425. The absence of contamination by mycoplasma was confirmed before each experiment by the JCRB and afterwards by several laboratories. SIRC cells were cultured in Eagle's MEM supplemented with 10% calf serum in a CO_2 incubator (5% in air) at 37°C. The mean (± SD) doubling time of the cells, determined in every laboratory was 20 hr (± 3.8 hr, n = 17).	
Cytotoxicity assay	*SIRC–NRU method* Various concentrations of each test substance were made up by dissolving the substance in appropriate solvent or suspending in culture medium. The solutions (100 μl) were then poured into each well of a 96-well microplate. SIRC cells were harvested from preculture bottles by trypsinization, washed once, and then resuspended (2×10^5 cells/ml) in the culture medium. A 100-μl aliquot of the cell suspension was introduced gradually into each well. The plates were maintained at room temperature for 20 min to allow the cells to settle on the bottom of the well. The cells were then cultured for 3 days. The culture medium was replaced with 250 μl fresh medium that contained neutral red (NR) (50 μg/ml) and incubated for another 3 hr. The medium was then removed and the cells were rapidly washed with an aqueous solution of 1% formaldehyde and 1% $CaCl_2$. NR that was incorporated into viable cells was extracted with 100 μl 1% acetic acid in 50% ethanol. After 15 min at room temperature, the microplates were gently agitated by a microplate shaker and the absorbance at 540 nm was measured by an automatic microplate reader. The mean absorbance of 10 wells that contained no test substance was regarded as the control value. The absorbance of the other wells was calculated as a percentage of the control absorbance. Five wells were used for each concentration of test substance. The concentration of each substance which inhibits cell viability to 50% of control (EC_{50}) was obtained from the dose–response curve.	*SIRC–CVS method* SIRC–CVS in the first phase of the validation study was performed according to the procedure for SIRC–NRU. After incubation for 3 days, dead cells were washed off with Ca^{++}-, Mg^{++}-free phosphate buffered saline (PBS (–)). The cells attached to the bottom of the plate were fixed and stained with 0.4% crystal violet solution in methanol for 30 min. The plate was washed with water, and the absorbance at 590 nm was measured by an automatic microplate reader. The SIRC–CVS method was modified for the second and third phases of validation for application to the plates used for SIRC–NRU investigation. The plates used for SIRC–NRU were washed off twice with PBS (–) and stained again with 0.4% crystal violet solution in methanol for 30 min. The other procedures were the same as these listed previously.
Minor Modifications of experimental procedure	At the time of the second and the third phases of the validation, the participating laboratories were asked to select the solvent for dissolving the test substances according to the detailed scheme described in the SOP. In addition, the SIRC–CVS was modified as described above.	

The contents are the same as those reported by Tani et al. (1999).

Fig. 3 Flow chart for preparation of test sample in cytotoxicity test



The figure that reported by Kojima (1999) is translated into English.

Table 23 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)

Substance	MAS	GHS	IC50 of the SIRC-CVS assay (μg/mL)						
(Draize eye test was performed at 10% concentration)	at 10%	at 10%	Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Ethanol	0.0	NI	10000<	10000<	10000<	10000<	10000<	NT	10000<
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	381	1193	570	97.5	484	120	474±400
Glycerin	0.0	NI	12746	5347.5	5350	6750	12500	27000	11600±8260
Polyethylene glycol 400	0.0	NI	6854.5	50000<	47500	32750	34500	40000	35300<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	2945	2792	3487	2375	3687	3350	3110±490
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	745	762	1075	1075	710	1400	963±272
Sodium salicylate	0.0	NI	840	559	1195	950	635	1525	952±364
Triethanolamine	0.0	NI	1440	1430	1750	1993	3850	NT	2090±1010
Isopropyl myristate	0.7	NI	10000<	10000<	10000<	6000	10000<	10000<	9330<
Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20	0.7	NI	541	794	737	675	1228	625	767±243
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	330	406	245	305	574	123	348±128
Calcium thioglycolate	4.0	NI	300	660	287.5	420	292.5	600< (Retest)	392±159 (Data from 5 labs)
m-Phenylenediamine•• (Lack of stability)	4.3	NI	167	73	290	255	167	355	218±102
Lactic acid	9.7	NI	994	982	1315	1285	1575	NT	1230±248
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	686	662	865	765	773	735	747±72.3
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	454	490	338	425	495	430	439±57.5
Sucrose fatty acid ester	11.0	NI	250	304	292.5	315	294.5	257.5	286±26
Diisopropanolamine	23.0	NI	455	901	720	170	1250	NT	699±414
Sodium lauryl sulfate	15.0$	1or2A	182	172	117	190	198	149	168±30.1
Benzyl alcohol	23.0	1or2A	1148	888.5	1485	1100	830	1675	1190±335
Monoethanolamine	23.3	2B	4.46	9.8	5.9	10.5	17.5	NT	9.62±5.08
Acid red 92	25.0	1or2A	230	231	340	332.5	268.5	380	297±62.7
Glycolic acid	25.0	2B	914	682	890	778	1075	NT	868±148
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	143	118	113	90.8	235	1115	140±56.1
Chlorhexidine gluconate (20% solution)	28.3	2A	67.2	44.8	67.5	45.8	112.5	NT	67.6±27.4
Butanol	34.0	1or2A	10000<	4395	10000<	10000<	10000<	NT	8880<
Potassium laurate	38.0	1or2A	103	117	73 #	110	150	NT	120±20.9 (Data from 4 labs)
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	26.7	38.0	23.3	32.3	51.0	59.5	38.4±14.2
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	210	182	181	156	175	NT	181±19.5
Acetic acid	68.0	1or2A	681	691	690	795 #	820	NT	721±66.5 (Data from 4labs)
Cetyltrimethylammonium bromide	76.7	1or2A	2.95	3.21	1.72	2.3> #	2.50	NT	2.59±0.654 (Data from 4labs)
Benzalkonium chloride	78.0	1or2A	16.2	25.2	13.2	15.5	29.0	15.0	19.0±6.50
Stearyltrimethylammonium chloride	91.3	1or2A	1.07	1.47	1.31	1.17	2.90	NT	1.58±0.752
Cetylpyridinium chloride	94.7	1	0.53	0.96	2.55	0.88	2.25	2.85	1.67±0.99
Domiphen bromide	96.3	1	13.4	11.4	7.55	13.4	14.8	NT	12.1±2.81

The data were taken from Tani et al. (1999). The classification of positive or negative using MAS was based on 15 as a cut-off point As reported by Tani et al.(1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
#: Derail from SOP
SD: Standard deviation
NT: Not tested

Table 24 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Sucrose fatty acid ester)

Substance	MAS	GHS	IC50 of the SIRC-CVS assay (μg/mL)						
(Draize eye test was performed at 10% concentration)	at 10%	at 10%	Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Ethanol	0.0	NI	10000<	10000<	10000<	10000<	10000<	NT	10000<
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	381	1193	570	97.5	484	120	474±400
Glycerin	0.0	NI	12746	5347.5	5350	6750	12500	27000	11600±8260
Polyethylene glycol 400	0.0	NI	6854.5	50000<	47500	32750	34500	40000	35300<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	2945	2792	3487	2375	3687	3350	3110±490
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	745	762	1075	1075	710	1400	963±272
Sodium salicylate	0.0	NI	840	559	1195	950	635	1525	952±364
Triethanolamine	0.0	NI	1440	1430	1750	1993	3850	NT	2090±1010
Isopropyl myristate	0.7	NI	10000<	10000<	10000<	6000	10000<	10000<	9330<
Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20	0.7	NI	541	794	737	675	1228	625	767±243
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	330	406	245	305	574	123	348±128
Calcium thioglycolate	4.0	NI	300	660	287.5	420	292.5	600< (Retest)	392±159 (Data from 5 labs)
m-Phenylenediamine•• (Lack of stability)	4.3	NI	167	73	290	255	167	355	218±102
Lactic acid	9.7	NI	994	982	1315	1285	1575	NT	1230±248
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	686	662	865	765	773	735	747±72.3
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	454	490	338	425	495	430	439±57.5
Sucrose fatty acid ester	11.0	NI	250	304	292.5	315	294.5	257.5	286±26
Diisopropanolamine	23.0	NI	455	901	720	170	1250	NT	699±414
Sodium lauryl sulfate	15.0$	1or2A	182	172	117	190	198	149	168±30.1
Benzyl alcohol	23.0	1or2A	1148	888.5	1485	1100	830	1675	1190±335
Monoethanolamine	23.3	2B	4.46	9.8	5.9	10.5	17.5	NT	9.62±5.08
Acid red 92	25.0	1or2A	230	231	340	332.5	268.5	380	297±62.7
Glycolic acid	25.0	2B	914	682	890	778	1075	NT	868±148
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	143	118	113	90.8	235	1115	140±56.1
Chlorhexidine gluconate (20% solution)	28.3	2A	67.2	44.8	67.5	45.8	112.5	NT	67.6±27.4
Butanol	34.0	1or2A	10000<	4395	10000<	10000<	10000<	NT	8880<
Potassium laurate	38.0	1or2A	103	117	73 #	110	150	NT	120±20.9 (Data from 4 labs)
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	26.7	38.0	23.3	32.3	51.0	59.5	38.4±14.2
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	210	182	181	156	175	NT	181±19.5
Acetic acid	68.0	1or2A	681	691	690	795 #	820	NT	721±66.5 (Data from 4labs)
Cetyltrimethylammonium bromide	76.7	1or2A	2.95	3.21	1.72	2.3> #	2.50	NT	2.59±0.654 (Data from 4labs)
Benzalkonium chloride	78.0	1or2A	16.2	25.2	13.2	15.5	29.0	15.0	19.0±6.50
Stearyltrimethylammonium chloride	91.3	1or2A	1.07	1.47	1.31	1.17	2.90	NT	1.58±0.752
Cetylpyridinium chloride	94.7	1	0.53	0.96	2.55	0.88	2.25	2.85	1.67±0.99
Domiphen bromide	96.3	1	13.4	11.4	7.55	13.4	14.8	NT	12.1±2.81

The data were taken from Tani et al. (1999). The classification of positive or negative using MAS was based on 15 as a cut-off point As reported by Tani et al.(1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium laury sulfate was evaluated as positive because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
#: Derail from SOP
SD: Standard deviation
NT: Not tested

Table 25 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Remaining substances)

Substance (Draize eye test was not performed at 10% concentration)	MAS as is	GHS as is	IC50 of the SIRC-CVS assay (μg/mL)						
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Isotonic sodium chloride solution	0.0	NI	10000<	10000<	10000<	10000<	10000<	10000<	10000<
Silicic anhydride	2.7	NI	10000<	10000<	10000<	38750	10000<	10000<	14800<
Methyl p-hydroxybenzoate	8.7	NI	103	214	257	195	215.5	255	207±56.4
Distearyldimethylammonium chloride	96.3	1	18.5	43.8	57	35.5	32.1	39.7	37.8±12.8

Table 26 Results of interlaboratory reproducibility on the SIRC-CVS assay (as is)

Substance (Application was as is in the Draize eye test.)	MAS as is	GHS as is	IC50 of the SIRC-CVS assay (μg/mL)						
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	381	1193	570	97.5	484	120	474±400
Isopropyl myristate	0.0	NI	10000<	10000<	10000<	6000	10000<	10000<	9330<
Isotonic sodium chloride solution	0.0	NI	10000<	10000<	10000<	10000<	10000<	10000<	10000<
Silicic anhydride	2.7	NI	10000<	10000<	10000<	38750	10000<	10000<	14800<
Polyethylene glycol 400	4.0	NI	6854.5	50000<	47500	32750	34500	40000	35300<
Glycerin	4.7	NI	12746	5347.5	5350	6750	12500	27000	11600±8260
Polyoxyethylene sorbitan monooleate (20E.O.)	4.7	NI	745	762	1075	1075	710	1400	963±272
Triethanolamine	8.0	NI	1440	1430	1750	1993	3850	NT	2090±1010
Methyl p-hydroxybenzoate	8.7	NI	103	214	257	195	215.5	255	207±56.4
Sucrose fatty acid ester	28.3	1or2A	250	304	292.5	315	294.5	257.5	286±26
Benzyl alcohol	31.0	1or2A	1148	888.5	1485	1100	830	1675	1190±335
Ethanol	32.7	1or2A	10000<	10000<	10000<	10000<	10000<	NT	10000<
Acid red 92	71.0	1or2A	230	231	340	332.5	268.5	380	297±62.7
Calcium thioglycolate	79.7	1	300	660	287.5	420	292.5	600< (Retest)	392±159 (Data from 5 labs)
m-Phenylenediamine•• (Lack of stability)	80.7	1or2A	167	73	290	255	167	355	218±102
Sodium salicylate	83.7	1or2A	840	559	1195	950	635	1525	952±364
Distearyldimethylammonium chloride	96.3	1	18.5	43.8	57	35.5	32.1	39.7	37.8±12.8
Lactic acid	102.7	1	994	982	1315	1285	1575	NT	1230±248

NT : Not tested

Table 27 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Concentration: 1%)

Substance (Draize eye test was performed at 1% concentration)	MAS at 1%	GHS at 1%	IC50 of the SIRC-CVS assay (μg/mL)						
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Benzyl alcohol	0	NI	1148	888.5	1485	1100	830	1675	1190±335
Acid red 92	0.7	NI	230	231	340	332.5	268.5	380	297±62.7
Cetylpyridinium chloride	34.7	1or2A	0.53	0.96	2.55	0.88	2.245	2.85	1.67±0.99

Table 28 Results of interlaboratory reproducibility on the SIRC-CVS cytotoxicity test
(Concentration: 0.1%)

Substance (Draize eye test was performed at 0.1% concentration)	MAS at 10%	GHS at 10%	IC50 of the SIRC-CVS assay (μg/mL)						
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Cetylpyridinium chloride	2.7	NI	0.53	0.96	2.55	0.88	2.245	2.85	1.67±0.99

Table 29 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)
-GHS classification by considering pH-

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the SIRC-CVS assay (μg/mL) Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Ethanol	0.0	NI	10000<	10000<	10000<	10000<	10000<	NT	10000<
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	381	1193	570	97.5	484	120	474±400
Glycerin	0.0	NI	12746	5347.5	5350	6750	12500	27000	11600±8260
Polyethylene glycol 400	0.0	NI	6854.5	50000<	47500	32750	34500	40000	35300<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	2945	2792	3487	2375	3687	3350	3110±490
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	745	762	1075	1075	710	1400	963±272
Sodium salicylate	0.0	NI	840	559	1195	950	635	1525	952±364
Triethanolamine	0.0	NI	1440	1430	1750	1993	3850	NT	2090±1010
Isopropyl myristate	0.7	NI	10000<	10000<	10000<	6000	10000<	10000<	9330<
Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20	0.7	NI	541	794	737	675	1228	625	767±243
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	330	406	245	305	574	123	348±128
m-Phenylenediamine•• (Lack of stability)	4.3	NI	167	73	290	255	167	355	218±102
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	686	662	865	765	773	735	747±72.3
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	454	490	338	425	495	430	439±57.5
Sucrose fatty acid ester	11.0	NI	250	304	292.5	315	294.5	257.5	286±26
Calcium thioglycolate	4.0	1*	300	660	287.5	420	292.5	600< (Retest)	392±159 (Data from 5 labs)
Lactic acid	9.7	1*	994	982	1315	1285	1575	NT	1230±248
Sodium lauryl sulfate	15.0[§]	1or2A	182	172	117	190	198	149	168±30.1
Benzyl alcohol	23.0	1or2A	1148	888.5	1485	1100	830	1675	1190±335
Diisopropanolamine	23.0	1*	455	901	720	170	1250	NT	699±414
Monoethanolamine	23.3	1*	4.46	9.8	5.9	10.5	17.5	NT	9.62±5.08
Acid red 92	25.0	1or2A	230	231	340	332.5	268.5	380	297±62.7
Glycolic acid	25.0	1*	914	682	890	778	1075	NT	868±148
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	143	118	113	90.8	235	1115	140±56.1
Chlorhexidine gluconate (20% solution)	28.3	2A	67.2	44.8	67.5	45.8	112.5	NT	67.6±27.4
Butanol	34.0	1or2A	10000<	4395	10000<	10000<	10000<	NT	8880<
Potassium laurate	38.0	1or2A	103	117	73 #	110	150	NT	120±20.9 (Data from 4 labs)
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	26.7	38.0	23.3	32.3	51.0	59.5	38.4±14.2
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	210	182	181	156	175	NT	181±19.5
Acetic acid	68.0	1or2A	681	691	690	795 #	820	NT	721±66.5 (Data from 4labs)
Cetyltrimethylammonium bromide	76.7	1or2A	2.95	3.21	1.72	2.3> #	2.50	NT	2.59±0.654 (Data from 4labs)
Benzalkonium chloride	78.0	1or2A	16.2	25.2	13.2	15.5	29.0	15.0	19.0±6.50
Stearyltrimethylammonium chloride	91.3	1or2A	1.07	1.47	1.31	1.17	2.90	NT	1.58±0.752
Cetylpyridinium chloride	94.7	1	0.53	0.96	2.55	0.88	2.245	2.85	1.67±0.99
Domiphen bromide	96.3	1	13.4	11.4	7.55	13.4	14.8	NT	12.1±2.81

NT : Not tested
#:Derail from SOP

Table 30 Results of interlaboratory reproducibility on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Sucrose fatty acid ester)
-GHS classification by considering pH-

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the SIRC-CVS assay (μg/mL) Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Average±SD
Ethanol	0.0	NI	10000<	10000<	10000<	10000<	10000<	NT	10000<
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	381	1193	570	97.5	484	120	474±400
Glycerin	0.0	NI	12746	5347.5	5350	6750	12500	27000	11600±8260
Polyethylene glycol 400	0.0	NI	6854.5	50000<	47500	32750	34500	40000	35300<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	2945	2792	3487	2375	3687	3350	3110±490
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	745	762	1075	1075	710	1400	963±272
Sodium salicylate	0.0	NI	840	559	1195	950	635	1525	952±364
Triethanolamine	0.0	NI	1440	1430	1750	1993	3850	NT	2090±1010
Isopropyl myristate	0.7	NI	10000<	10000<	10000<	6000	10000<	10000<	9330<
Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20	0.7	NI	541	794	737	675	1228	625	767±243
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	330	406	245	305	574	123	348±128
m-Phenylenediamine•• (Lack of stability)	4.3	NI	167	73	290	255	167	355	218±102
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	686	662	865	765	773	735	747±72.3
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	454	490	338	425	495	430	439±57.5
Sucrose fatty acid ester	11.0	NI	250	304	292.5	315	294.5	257.5	286±26
Calcium thioglycolate	4.0	1*	300	660	287.5	420	292.5	600< (Retest)	392±159 (Data from 5 labs)
Lactic acid	9.7	1*	994	982	1315	1285	1575	NT	1230±248
Sodium lauryl sulfate	15.0$	1or2A	182	172	117	190	198	149	168±30.1
Benzyl alcohol	23.0	1or2A	1148	888.5	1485	1100	830	1675	1190±335
Diisopropanolamine	23.0	1*	455	901	720	170	1250	NT	699±414
Monoethanolamine	23.3	1*	4.46	9.8	5.9	10.5	17.5	NT	9.62±5.08
Acid red 92	25.0	1or2A	230	231	340	332.5	268.5	380	297±62.7
Glycolic acid	25.0	1*	914	682	890	778	1075	NT	868±148
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	143	118	113	90.8	235	1115	140±56.1
Chlorhexidine gluconate (20% solution)	28.3	2A	67.2	44.8	67.5	45.8	112.5	NT	67.6±27.4
Butanol	34.0	1or2A	10000<	4395	10000<	10000<	10000<	NT	8880<
Potassium laurate	38.0	1or2A	103	117	73 #	110	150	NT	120±20.9 (Data from 4 labs)
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	26.7	38.0	23.3	32.3	51.0	59.5	38.4±14.2
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	210	182	181	156	175	NT	181±19.5
Acetic acid	68.0	1or2A	681	691	690	795 #	820	NT	721±66.5 (Data from 4labs)
Cetyltrimethylammonium bromide	76.7	1or2A	2.95	3.21	1.72	2.3> #	2.50	NT	2.59±0.654 (Data from 4labs)
Benzalkonium chloride	78.0	1or2A	16.2	25.2	13.2	15.5	29.0	15.0	19.0±6.50
Stearyltrimethylammonium chloride	91.3	1or2A	1.07	1.47	1.31	1.17	2.90	NT	1.58±0.752
Cetylpyridinium chloride	94.7	1	0.53	0.96	2.55	0.88	2.245	2.85	1.67±0.99
Domiphen bromide	96.3	1	13.4	11.4	7.55	13.4	14.8	NT	12.1±2.81

NT : Not tested
#:Derail from SOP

Table 31 Results of the SIRC-NRU assay and the SIRC-CVS assay in the Japanese validation study

	SIRC-NRU						SIRC-CVS					
Substance no.	n*	Solvent†	EC_{50}‡ (μg/ml)	SD	CV	Rank	n	Solvent	EC_{50} (μg/ml)	SD	CV	Rank
S1-1	7	–	10000<	–	–	34	6	–	10000<	–	–	32
S1-2	7	–	2910	1600	55.1%	31	6	–	3110	490	15.8%	31
S1-3	7	–	946	230	24.3%	27	6	–	767	243	31.6%	24
S1-4	7	–	428	107	25.0%	19	6	–	348	128	36.8%	17
S1-5	7	–	444	157	35.4%	20	6	–	439	57.5	13.1%	19
S1-6	7	–	147	34.5	23.6%	11	6	–	140	56.1	40.2%	11
S1-7	7	–	171	25.2	14.8%	12	6	–	168	30.1	17.9%	12
S1-8	7	–	675	134	19.8%	23	6	–	747	72.3	9.7%	23
S1-9	7	–	41.8	16.8	40.2%	7	6	–	38.4	14.2	36.9%	8
S1-10	7	–	18.0	6.40	35.4%	6	6	–	19.0	6.50	34.0%	6
S2-1	1	M	320	–	–		1	M	315	–	–	
	2	MS	230	28.3	12.3%		1	MS	250	–	–	
	2	E	290	2.1	0.7%		2	E	298	7.99	2.7%	
	2	D	266	51.6	19.3%		2	D	276	26.2	9.5%	
	7	M + MS + E + D	271	41.0	15.1%	15	6	M + MS + E + D	286	26	9.1%	15
S2-2	7	M	9760	5060	51.8%	33	6	M	11600	8260	71.2%	32
S2-3	7	M	316	57.0	18.1%	17	6	M	297	62.7	21.1%	16
S2-4	7	M	1250	257	20.4%	28	6	M	963	272	28.2%	27
S2-5	6	MS	475	134	31.7%		4	MS	325	63.4	19.5%	
							1	MS	600<	–	–	
	1	D	589	–	–		1	D	660	–	–	
	7	MS + D	484	121	25.1%	21	5	MS + D	392	159	40.6%	18
S2-6	6	E	46.8	21	47.3%		5	E	36.6	13.9	38.0%	
	1	P	50.2	–	–		1	P	43.8	–	–	
	7	E + P	47.4	17.6	37.1%	8	6	E + P	37.8	12.8	33.9%	7
S2-7	2	MS	194	112	58.0%		3	MS	240	200	83.3%	
	5	D	412	220	53.4%		3	D	591	444	75.1%	
	7	MS + D	350	210	60.1%	18	6	MS + D	474	400	84.4%	20
S2-8	5	M	1.40	0.91	60.7%		4	M	2	1.2	60.0%	
	2	P	2.74	2.06	75.2%		2	P	1.56	0.97	62.2%	
	7	M + P	2.00	1.00	50.0%	2	6	M + P	1.67	0.99	59.3%	2
S2-9	7	D	173	37.0	21.5%	14	6	D	207	56.4	27.2%	14
S2-10	6	MS	10000<	–	–		5	MS	10000<	–	–	
	1	MS	8400	–	–		1	MS	6000	–	–	
	7	MS	9770<	–	–	34	6	MS	9330<	–	–	32
S2-11	6	M	30900	13900	45.0%		5	M	32300	15400	47.5%	
	1	M	50000<	–	–		1	M	50000<	–	–	
	7	M	33600<	–	–	34	6	M	35300<	–	–	32
S2-12	6	MS	10000<	–	–		5	MS	10000<	–	–	
	1	MS	43000	–	–		1	MS	38800	–	–	
	7	MS	20400<	–	–	34	6	MS	14800<	–	–	32
S2-13	7	M	1370	543	39.6%	29	6	M	1190	335	28.2%	28
S2-14	7	M	856	439	51.2%	25	6	M	952	364	38.2%	26
S2-15§	6	M	255	–	–		5	M	190	–	–	
	1	E	365	–	–		1	E	355	–	–	
	7	M + E	271	–	–	–	6	M + E	218	–	–	–
S3-1	6	M	10000<	–	–	34	5	M	10000<	–	–	32
S3-2	6	M	12.6	6.31	50.1%	5	5	M	9.62	5.08	52.9%	4
S3-3	6	M	3140	820	26.1%	32	5	M	2090	1010	48.3%	30
S3-4	2	M	1.93	0.534	27.6%		2	M	1.27	0.283	22.4%	
	2	P	2.22	0.375	16.9%		1	P	1.17	–	–	
	2	MS	1.74	0.933	53.6%		2	MS	2.11	1.12	53.4%	
	6	M + P + MS	1.96	0.552	28.1%	1	5	M + P + MS	1.58	0.752	47.6%	1
S3-5	6	M	1370	391	28.6%	30	5	M	699	414	59.2%	21
S3-6	6	P	126	16.4	13.1%	10	4	P	120	20.9	17.4%	10
S3-7	2	M	3.30	0.633	19.2%		1	M	3.21	–	–	
	2	P	3.50	0.361	10.3%		1	P	2.95	–	–	
	1	E	1.80	–	–		1	E	2.50	–	–	
	1	MS	1.15	–	–		1	MS	1.72	–	–	
	6	M + P + E + MS	2.76	1.07	38.8%	3	4	M + P + E + MS	2.59	0.654	25.2%	3
S3-8	6	M	620	131	21.1%	22	4	M	721	66.5	9.2%	22
S3-9	2	M	3620	244	6.7%		1	M	4400	–	–	
	4	M	10000<	–	–		4	M	10000<	–	–	
	6	M	7870<	–	–	34	6	M	8880<	–	–	32
S3-10	6	D	92.2	37.3	40.5%	9	5	D	67.6	27.4	40.6%	9
S3-11	6	P	10.8	3.42	31.6%	4	5	P	12.1	2.81	23.3%	5
S3-12	6	M	938	289	30.8%	26	5	M	1230	248	20.2%	29
S3-13	6	M	774	197	25.4%	24	5	M	868	148	17.1%	25
S3-14	5	D	175	17.3	9.9%		5	D	181	19.5	10.8%	13
	1	MS	160	–	–							
	6	D + MS	172	16.5	9.6%	13						
S1-SLS	7	M	170	25.3	14.9%	–	6	M	162	33.9	20.9%	–
S2-SLS	7	M	170	15.0	8.8%	–	7	M	176	13.4	7.6%	–
S3-SLS	6	M	165	17.8	10.8%	–	5	M	167	23.2	13.8%	–

* = no. of data.
† = solvents were selected under a common SOP. (second and third phases of validation).
M = culture medium, MS: suspension in culture medium, P = PBS(–), D = DMSO, E = ethanol.
‡ = mean value of EC_{50} which was average of two EC_{50} results obtained in each laboratory.
§ = S2-15 was excluded from analysis due to instability.

Substance no. and substance name

S1-1	Isotonic sodium chloride solution
S1-2	Polyoxyethylene hydrogenated caster oil (60 E.O.)
S1-3	Polyoxyethylene sorbitan monolaurate (20 E.O.)
S1-4	Polyethyleneglycol monolaurate (10 E.O.)
S1-5	Sodium N-lauryl sarcosinate (30% solution)
S1-6	Sodium hydrogenated tallow L-glutamate
S1-7	Sodium lauryl sulfate
S1-8	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)
S1-9	Polyoxyethylene octylphenylether (10 E.O.)
S1-10	Benzalkonium chloride
S2-1	Sucrose fatty scid ester
S2-2	Glycerin
S2-3	Acid red 92
S2-4	Polyoxyethylene sorbitan monooleate (20E.O.)
S2-5	Calcium thioglycolate
S2-7	2-Ethylhexyl p-dimethylamino benzonate
S2-8	Cetylpyridinium chloride
S2-10	Isopropyl myristate
S2-11	Polyethylene glycol 400
S2-13	Benzyl alcohol
S2-14	Sodium salicylate
S2-15	m-Phenylenediamine
S3-1	Ethanol
S3-2	Monoethanolamine
S3-3	Triethanolamine
S3-4	Stearyltrimethylammonium chloride
S3-5	Diisopropanolamine
S3-6	Potassium laurate
S3-7	Cetyltrimethylammonium bromide
S3-8	Acetic acid
S3-9	Butanol
S3-10	Chlorhexidine gluconate (20% solution)
S3-11	Domiphen bromide
S3-12	Lactic acid
S3-13	Glycolic acid
S3-14	Di (2-ethylhexyl) sodium sulfosuccinate

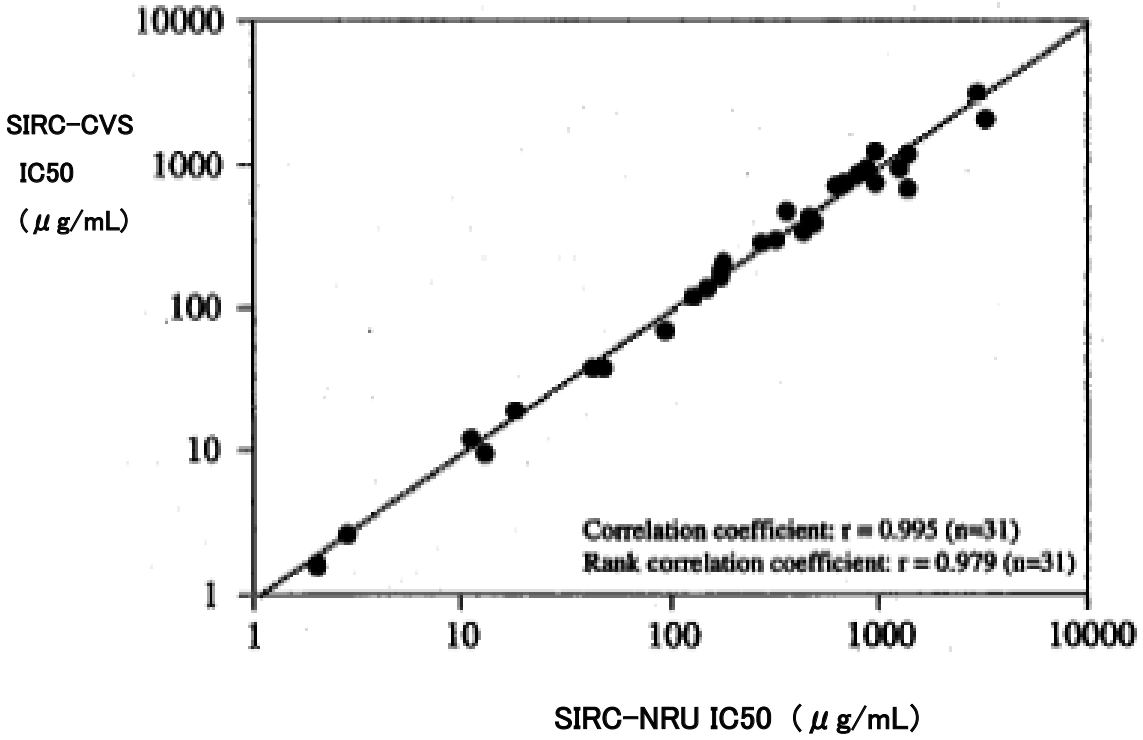
Data are the same as those reported by Tani et al.(1999).

Table 32 Correlation of the results between alternative methods in the Japanese validation study

	HET-CAM	CAM-TB	RBC	SKIN^{2TM}	MATREX^{TM}	CornePack*	SIRC-CVS	SIRC-NRU	HeLa-MTT	CHL-CVS	EYTEX^{TM}
HET-CAM	1.000										
CAM-TB	0.679	1.000									
RBC	−0.653	−0.743	1.000								
SKIN^{2TM}	−0.479	−0.811	**0.953**	1.000							
MATREX^{TM}	−0.628	−0.733	**0.936**	**0.931**	1.000						
CornePack*	−0.480	−0.690	0.892	0.846	**0.913**	1.000					
SIRC-CVS	−0.582	−0.820	0.809	0.838	0.813	0.773	1.000				
SIRC-NRU	−0.589	−0.823	0.814	0.821	0.814	0.768	**0.997**	1.000			
HeLa-MTT	−0.580	−0.831	0.838	0.872	0.840	0.812	**0.985**	**0.985**	1.000		
CHL-CVS	−0.545	−0.765	0.811	0.841	0.820	0.798	**0.972**	**0.968**	**0.969**	1.000	
EYTEX^{TM}	0.751	0.313	−0.542	−0.188	−0.389	−0.202	−0.397	−0.391	−0.370	−0.331	1.000

#: Non-irritants for which EC_{50} values are not determined are given maximum values.

Fig.4 Correlation between IC50 values obtained for SIRC-NRU assay and those of SIRC-CVS assay



Data are the same as those reported by Tani et al. (1999).

Table 33 Correlation of the results obtained by alternative methods and Draize eye test

Methods	Analysis using all data			Analysis excluding specific classes of chemicals			
		Correlation coefficients				Correlation coefficients	
	N	Pearson's linear	Spearman's rank	class###	N	Pearson's linear	Spearman's rank
Chorioallantoic membranc							
HET–CAM	52	0.688	0.802	1	46	0.702	0.831
				2	6	0.779	0.714
CAM–TB	55	0.718	0.838	1	48	0.801	0.863
				2	7	0.926	0.964
Red blood cells							
RBC	17	–0.631	0.643	3	16	–0.651	0.674
Haemoglobin							
RDC_{50}	8##	0.906	0.714				
1%RDR	23##	0.671	0.579				
1% λ max	31##	0.791	0.697				
Artificial skin models							
SKIN™ (ZK1100)#	30	–0.694	0.680	4	20	–0.842	
MATREX™#	30	–0.672	0.832	4	20	–0.754	
Normal cells from rabbit cornea							
CornePack*#	28	–0.538	0.588	4	21	–0.731	0.787
Cell lines from rabbit cornea							
SIRC–CVS#	29	–0.805	0.779	4	22	–0.924	0.945
SIRC–NRU#	30	–0.816	0.787	4	23	–0.916	0.931
Cell lines from the other mammals							
HeLa–MTT#	29	–0.799	0.745	4	22	–0.922	0.926
CHL–CVS#	29	–0.729	0.703	4	22	–0.864	0.880
EYTEX™	38	0.313					

#: log (EC_{50}) were correlated with Draize scores (maximal average total score). ##: include the data of substances of the first validation, for which the experiments were conducted afterwards, during the second and the third validations. ###: 1: liquid sample only, 2: powder sample only; 3: excluded strong alkali and acid samples; 4: excluded alcohol (lower mono-ol), strong acids and strong alkalis.

Data are the same as those of Ohno et al.(1999).

Table 34 Predictability of the alternative tests – Classification into positive and negative irritants

Methods	Analysis by using all data: No. of samples	Type of errors#	No. of errors	% of errors	Sample number falsely predicted	Analysis excluding specific chemical class##: No. of samples	Type of errors#	No. of errors	% of errors
Chorioallantoic membrane									
HET-CAM	52	FP	8	17.3	S1-4-10, S1-5-10, S1-8-10, S2-5-10, S2-9-100, S2-14-10, S3-3-100, S3-12-10	38	FP	7	18.4
		FN	1		S2-1-100		FN	0	
CAM-TB	55	FP	9	16.4	S1-4-10, S1-5-10, S1-8-10, S2-1-10, S2-3-1, S2-5-10, S2-8-0.1, S3-3-100, S3-12-10	41	FP	7	19.5
		FN	0				FN	1	
Red blood cells									
RBC	30	FP	4	30.0	S1-4, S1-5, S1-8, S2-1	24	FP	3	16.7
		FN	5		S2-13, S3-2, S3-5, S3-9, S3-10		FN	1	
Haemoglobin									
RDC_{50}	23###	FP	0####	26.1					
		FN	6####		S1-9, S2-13, S3-2, S3-5, S3-8, S3-9				
1% RDR	23###	FP	2####	34.7	S1-5, S1-8				
		FN	6####		S1-9, S2-13, S3-2, S3-5, S3-8, S3-9				
1% λmax	31###	FP	2####	29.0	S1-5, S1-8				
		FN	7####		S1-9, S2-13, S3-2, S3-5, S3-8, S3-9, S3-10				
Artificial dermal models									
SKIN^{2TM} (ZK1100)	33	FP	6	30.3	S1-3, S1-4, S1-5, S1-7, S1-8, S2-1	24	FP	5	25.0
		FN	4		S2-13, S3-5, S3-8, S3-9		FN	1	
MATREX^{TM}	34	FP	6	23.4	S1-3, S1-4, S1-5, S1-8, S2-1, S2-12	25	FP	5	20.0
		FN	2		S2-13, S3-9		FN	0	
Normal cells from rabbit cornea									
CornePack^{+}	35	FP	8	40.0	S1-2, S1-3, S1-4, S1-5, S1-8, S2-1, S2-4, S2-5	26	FP	4	15.4
		FN	6		S2-13, S3-2, S3-5, S3-8, S3-13, S3-9		FN	0	
Cell lines from rabbit cornea									
SIRC-CVS	34	FP	5	29.4	S1-4, S1-5, S2-1, S2-5, S2-7	25	FP	2	8.0
		FN	5		S2-13, S3-5, S3-8, S3-9, S3-13		FN	0	
SIRC-NRU	34	FP	6	29.4	S1-4, S1-5, S1-8, S2-1, S2-5, S2-7	25	FP	4	16.0
		FN	4		S2-13, S3-5,S3-9, S3-13		FN	0	
Cell lines from the other mammals									
HeLa-MTT	34	FP	5	29.4	S1-3, S1-4, S1-5, S1-8, S2-1	25	FP	3	16.0
		FN	5		S2-13, S3-5, S3-8, S3-9, S3-13		FN	1	
CHL-CVS	34	FP	6	29.4	S1-3, S1-4, S1-5, S2-1, S2-5, S2-7	25	FP	4	20.0
		FN	4		S2-13, S3-8, S3-9, S3-13		FN	1	
EYTEX^{TM}	54	FP	11	27.7	S1-7-10, S2-3-1, S2-4-100, S2-5-10, S2-9-100, S2-11-100	46	FP	15	37.0
					S2-13-10, S3-2-10, S3-3-10, S3-3-100, S3-4-10, S3-12-10				
		FN	5		S1-6-10, S1-9-10, S2-1-100, S2-6-100, S2-8-1, S3-9-10, S3-14-10		FN	2	

Eye irritation potentials were classified into two classes according to the Draize scores (0–15 and >15) and the number of false predictions was calculated from linear regression lines. #: FP: false positive, FN: false negative. ##: Regression lines were made excluding the data of powder sample in the case of HET-CAM and CAM-TB. The data for acids, alkalis, and alcohols (lower mono-ol) were excluded in the case of the other methods. ###: include the data of the test substances of the first validation, for which the experiments were conducted afterwards, during the second and the third validations. ####: estimated from the prediction according to their own potocols without using regression lines.

False positive: Polyethyleneglycol monolaurate (10 E.O.) , Sodium N-lauryl sarcosinate (30% solution), Sucrose fatty acid ester, Calcium thioglycolate, 2-Ethylhexyl p-dimethylamino benzonate
False negative: Benzyl alcohol, Diisopropanolamine, Acetic acid, Butanol, Glycolic acid

Substance no.	and substance name
S1-1	Isotonic sodium chloride solution
S1-2	Polyoxyethylene hydrogenated caster oil (60 E.O.)
S1-3	Polyoxyethylene sorbitan monolaurate (20 E.O.)
S1-4	Polyethyleneglycol monolaurate (10 E.O.)
S1-5	Sodium N-lauryl sarcosinate (30% solution)
S1-6	Sodium hydrogenated tallow L-glutamate
S1-7	Sodium lauryl sulfate
S1-8	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)
S1-9	Polyoxyethylene octylphenylether (10 E.O.)
S1-10	Benzalkonium chloride
S2-1	Sucrose fatty scid ester
S2-2	Glycerin
S2-3	Acid red 92
S2-4	Polyoxyethylene sorbitan monooleate (20E.O.)
S2-5	Calcium thioglycolate
S2-6	Distearyldimethylammonium chloride
S2-7	2-Ethylhexyl p-dimethylamino benzonate
S2-8	Cetylpyridinium chloride
S2-9	Methyl p-hydroxybenzoate
S2-10	Isopropyl myristate
S2-11	Polyethylene glycol 400
S2-12	Silicic anhydride
S2-13	Benzyl alcohol
S2-14	Sodium salicylate
S2-15	m-Phenylenediamine
S3-1	Ethanol
S3-2	Monoethanolamine
S3-3	Triethanolamine
S3-4	Stearyltrimethylammonium chloride
S3-5	Diisopropanolamine
S3-6	Potassium laurate
S3-7	Cetyltrimethylammonium bromide
S3-8	Acetic acid
S3-9	Butanol
S3-10	Chlorhexidine gluconate (20% solution)
S3-11	Domiphen bromide
S3-12	Lactic acid
S3-13	Glycolic acid
S3-14	Di (2-ethylhexyl) sodium sulfosuccinate

For example, "S2-3-1"means the application of substance "S2-3" at 1% concentration.

Fig. 5 Relationship between the SIRC-CVS assay and the Draize eye test

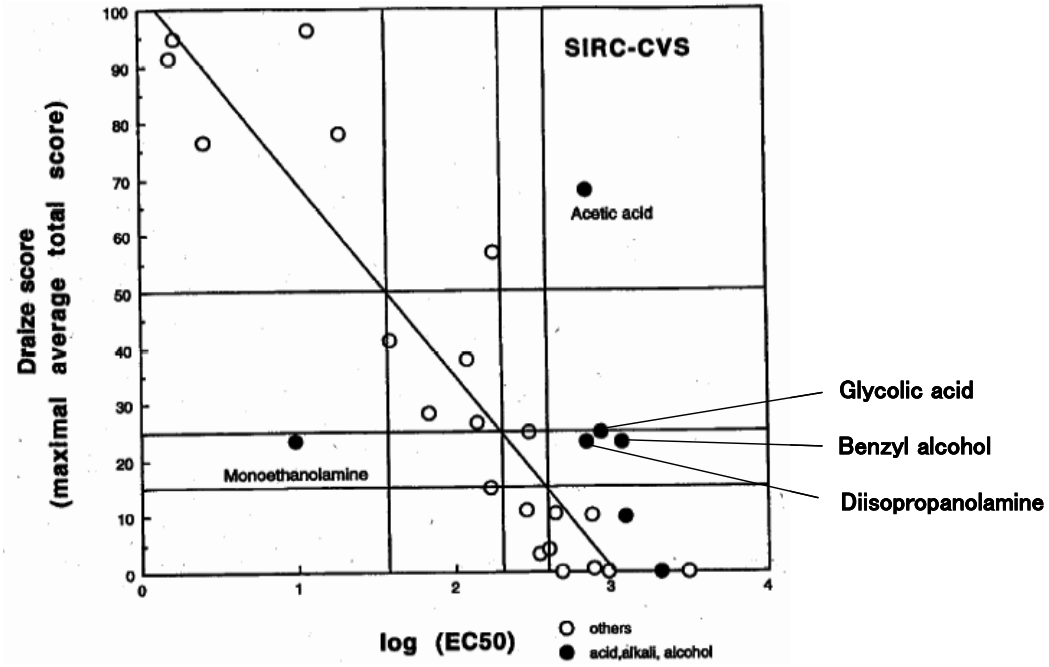


Fig. 6 Relationship between the SIRC-NRU assay and the Draize eye test

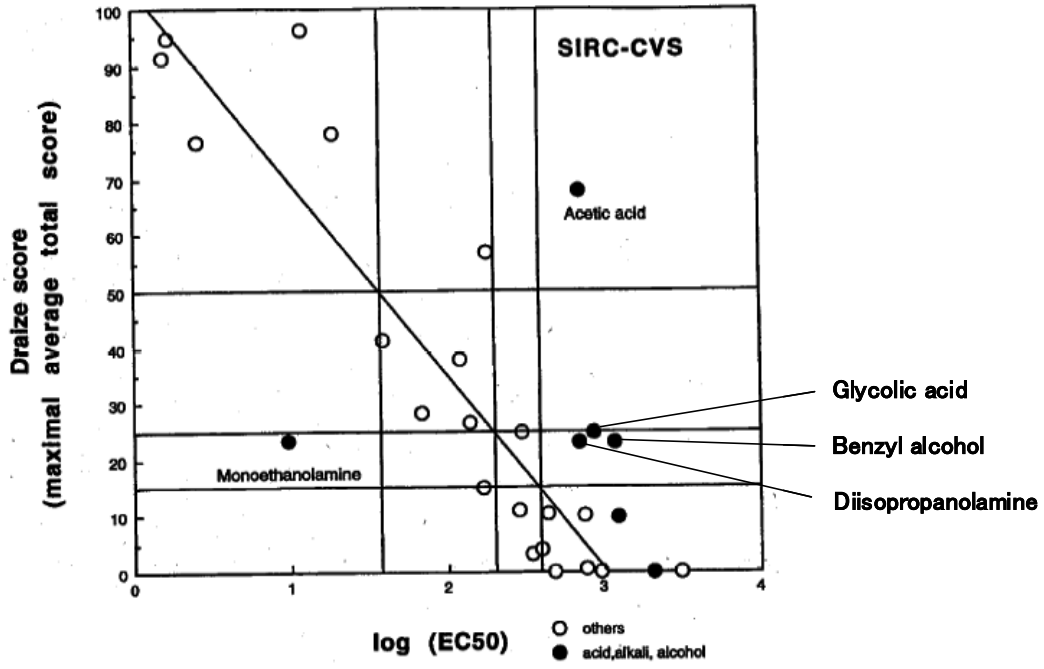


Fig. 7 Relationship between the HeLa-MTT assay and the Draize eye test

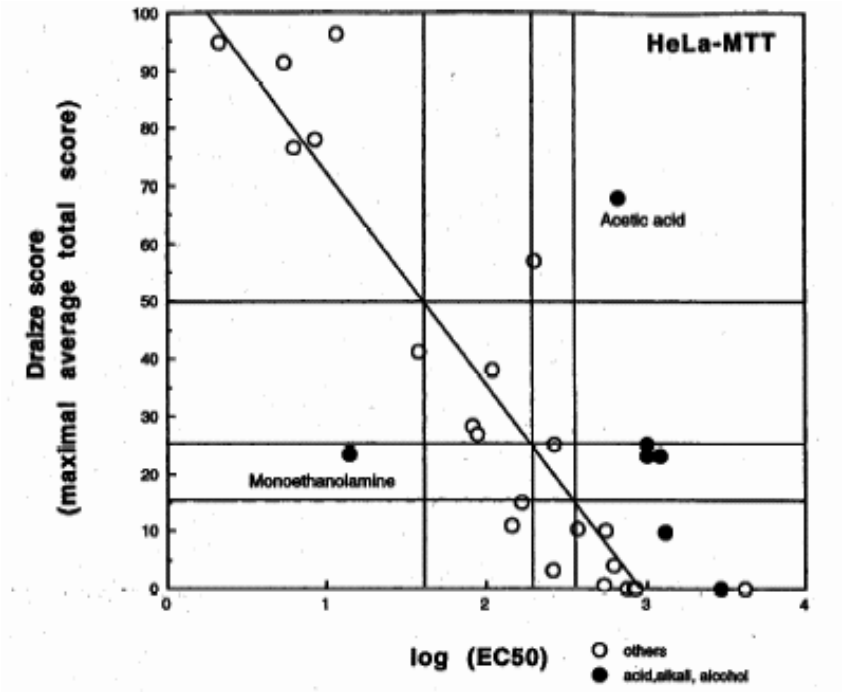


Fig. 8 Relationship between the CHL-CVS assay and the Draize eye test

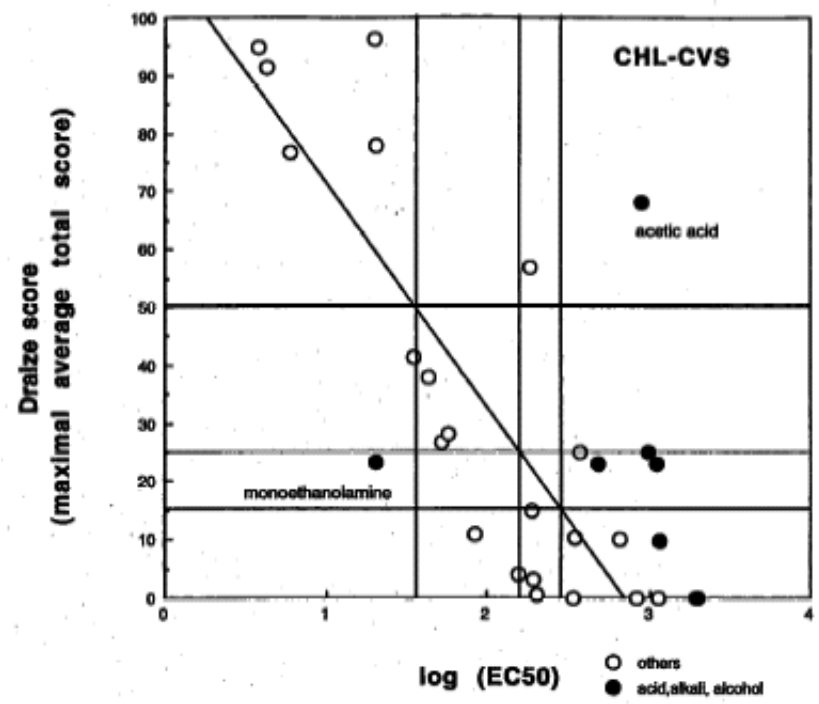


Table 35 Predicted irritancy of test samples based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 14	Benzyl alcohol Glycolic acid Butanol 3
	NI	2-Ethylhexyl p-dimethylamino benzonate Polyethyleneglycol monolaurate (10 E.O.) Calcium thioglycolate Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester Diisopropanolamine 7	Ethanol Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Polyoxyethylene sorbitan monooleate (20E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Polyoxyethylene sorbitan monolaurate (20 E.O.)=Tween 20 Lactic acid 10

Table 36 Predicted irritancy of test samples based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Sucrose fatty acid ester)

		In vitro (Classification by SIRC-CVS assay using Sucrose fatty acid ester as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 12	Benzyl alcohol Acid red 92 Glycolic acid Butanol Acetic acid 5
	NI	0	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Polyoxyethylene sorbitan monooleate (20E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Polyoxyethylene sorbitan monolaurate (20 E.O.)=Tween 20 Polyethyleneglycol monolaurate (10 E.O.) Calcium thioglycolate Lactic acid Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester Diisopropanolamine 17

Table 37 Predicted irritancy of test samples based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)
-GHS classification by considering pH-

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Calcium thioglycolate Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 16	Lactic acid Benzyl alcohol Glycolic acid Butanol 4
	NI	2-Ethylhexyl p-dimethylamino benzonate Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester 5	Ethanol Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Polyoxyethylene sorbitan monooleate (20E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20 9

Table 38 Predicted irritancy of test samples based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Sucrose fatty acid ester)
-GHS classification by considering pH-

		In vitro (Classification by SIRC-CVS assay using Sucrose fatty acid ester as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 12	Benzyl alcohol Acid red 92 Glycolic acid Butanol Acetic acid 5
	NI	0	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Polyoxyethylene sorbitan monooleate (20E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20 Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester 14

Table 39 Forty-eight substances

No	Substance	CAS	Supplier	*in vivo* data reported previously		GHS	Reference
			(*in vitro* test)	Classification at 10% concn	Classification at the applied concn	at 10% concn	
1	2-Bromo-2-nitropropane-1,3-diol	52-51-7	Fluorochem	Positive	Positive: 100, 20, 10, 5% Negative: 2, 0.5%	1, 2A or 2B	JACT 3(3):139-155,1984. JEPT 4(4):47-61, 1980.
2	Benzalkonium chloride	8001-54-5	Wako	Positive	Positive: 2, 1, 0.5% Negative: 0.1, 0.01%	1, 2A or 2B	JACT 8(4):589-625, 1989.
3	Cetrimonium chloride	112-02-7	Wako	Positive	Positive: 2.5, 1.2, 0.5% Negative: 0.1%	1, 2A or 2B	IJT 16(S3):195-220,1997.
4	Chlorhexidine digluconate	18472-51-0	Wako	Positive	Positive: 20, 2% Negative: 0.05%	1, 2A or 2B	JACT 12(3):201-23, 1993.
5	Chlorophene	120-32-1	Wako	Positive	Positive: 100, 3% Negative: 1, 0.3%	1, 2A or 2B	IJT 23(S1):1-27 2004.
6	Dioctyl sodium sulfosuccinate	577-11-7	Alfa Aesar	Positive	Positive: 10% Negative: 2, 0.5%	1, 2A or 2B	IJT 17(S4):1-20, 1998.
7	Lauramide DEA	120-40-1	Wako	Positive	Positive: 20, 10%	1, 2A or 2B	JACT 5(5):415-54, 1986.
8	Phenethyl alcohol	60-12-8	Wako	Positive	Positive: 100, 15, 5% Negative: 0.3%	1, 2A or 2B	JACT 9(2):165-83, 1990.
9	Stearalkonium chloride	122-19-0	Wako	Positive	Positive: 25, 4, 2.5% N egative: 0.5%	1, 2A or 2B	JACT 1(2):57-69, 1982.
10	TEA-Lauryl sulfate	139-96-8	Wako	Positive	Positive: 20, 10, 5, 2.5, 1.25%	1, 2A or 2B	JACT 1(4):143-67, 1982.
11	Acetyl tributyl citrate	77-90-7	Wako	Negative	Negative: 100%	NI	IJT 21(S2):1-17, 2002.
12	Benzophenone-1	131-56-6	Wako	Negative	Positive: 100% Negative: 16, 8, 4%	NI	JACT 2(5):35-77, 1983.
13	Benzophenone-2	131-55-5	Wako	Negative	Positive: 100% Negative: 16, 8, 4%	NI	JACT 2(5):79-84, 1983.
14	Butylene glycol	107-88-0	Wako	Negative	Negative: 100, 10%	NI	Hifu 26(5):1065-1074, 1984.
15	Carnauba wax	8015-86-9	Wako	Negative	Negative: 50%	NI	JACT 3(3):1-41, 1984.
16	Cetyl alcohol	36653-82-4	Wako	Negative	Negative: 100%	NI	JACT 7(3):359-413, 1988.
17	Cetyl palmitate	540-10-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
18	Decyl oleate	3687-46-5	Wako	Negative	Negative: 100%	NI	JACT 1(2):85-95, 1982.
19	Diazolidinyl urea	78491-02-8	MP Biomedicals	Negative	Negative: 30%	NI	JACT 9(2):229-45, 1990.
20	Diethylhexyl adipate	103-23-1	Wako	Negative	Negative: 100%	NI	JACT 3(3):101-30, 1984.
21	Diisopropyl adipate	6938-94-9	Wako	Negative	Negative: 100%	NI	JACT 3(3):101-30, 1984.
22	Ethylhexyl palmitate	29806-73-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
23	Ethylhexyl stearate	22047-49-0	Wako	Negative	Negative: 100%	NI	JACT 4(5):107-46, 1985.
24	Glyceryl stearate	11099-07-3	Wako	Negative	Negative: 100%	NI	JACT 1(4):169-192, 1982.
25	Hexylene glycol	107-41-5	Wako	Negative	Positive: 100% Negative: 25%	NI	JACT 4(5):223-48, 1985.
26	Isocetyl stearate	25339-09-7	Wako	Negative	Negative: 100%	NI	JACT 4(5):107-46, 1985.
27	Isopropyl myristate	110-27-0	TCI	Negative	Negative: 100%	NI	JACT 1(4):55-80, 1982.
28	Isopropyl palmitate	142-91-6	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
29	Oleyl alcohol	143-28-2	Wako	Negative	Negative: 100%	NI	JACT 4(5):1-29, 1985.
30	PEG-2 stearate	106-11-6	Wako	Negative	Negative: 100%	NI	JACT 2(7):17-60, 1983.
31	PEG-40 stearate	9004-99-4	Wako	Negative	Negative: 100%	NI	JACT 2(7):17-60, 1983.
32	Phytantriol	74563-64-7	Wako	Negative	Positive: 100, 23% Negative: 10, 3%	NI	IJT 26(Suppl. 1):107-117, 2007.
33	Propylene carbonate	108-32-7	Wako	Negative	Negative: 100, 17.5, 10.5%	NI	JACT 6(1):23-51, 1987.
34	Castor seed oil	8001-79-4	Wako	Negative	Negative: 100%	NI	JACT 7(6):721-739, 1988.
35	Safflower oil	8001-23-8	Wako	Negative	Negative: 100%	NI	JACT 4(5):171-97, 1985.
36	Sesame (Sesamum indicum) oil	8008-74-0	Wako	Negative	Negative: 100%	NI	JACT 12(3):261-77, 1993.
37	Sodium dehydroacetate	4418-26-2	Wako	Negative	Negative: 100%	NI	JACT 4(3):123-159, 1985.
38	Sodium stearate	822-16-2	Wako	Negative	Negative: 100%	NI	JACT 1(2):143-77, 1982.
39	Sorbitan oleate	1338-43-8	Wako	Negative	Negative: 100%	NI	JACT 4(3):65-121, 1985.
40	Sorbitan sesquioleate	8007-43-0	Wako	Negative	Negative: 100, 30%	NI	JACT 4(3):65-121, 1985.
41	Sorbitan stearate	1338-41-6	Wako	Negative	Negative: 30%	NI	JACT 4(3):65-121, 1985.
42	Squalane	111-01-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):37-56, 1982.
43	Steareth-2	9005-00-9	Wako	Negative	Negative: 60%	NI	JACT 7(6):881-910, 1988.
44	Steareth-20	9005-00-9	Wako	Negative	Negative: 60%	NI	JACT 7(6):881-910, 1988.
45	Stearyl alcohol	112-92-5	Wako	Negative	Negative: 100%	NI	JACT 4(5):1-29, 1985.
46	Triacetin	102-76-1	Wako	Negative	Negative: 100%	NI	IJT 22(S2):1-10, 2003.
47	Triethylene glycol	112-27-6	Wako	Negative	Negative: 100%	NI	IJT 25(5) :121-138,2006.
48	Zinc stearate	557-05-1	Wako	Negative	Negative: 100%	NI	JACT 1(2):143-77, 1982.

Supplier means manufacturer of the material used in this study. The *in vivo* classification of positive or negative was based on the appearance or not of corneal damage, or an MAS value of 15 as a cut-off point, where reported MAS values are available. The classification was essentially based on whether or not corneal damage appeared after the application of 0.1 mL to rabbit eye without irrigation. However, where there were differences of test conditions, these were considered individually. For example, a case where corneal damage appeared after the application of 0.05 mL was judged as positive. In cases without data at 10% concentration, the assessment of positive or negative at the concentration of 10% was made on the basis of dose-response analysis of each ingredient.

Table 40 Results of 48 substances on the SIRC-CVS assay

No	Substance	Physical state of 2% in medium	Range finding test				Main test				Judgement
			Medium	Starting concn (μg/mL)	Physical state	Range of IC50 (μg/mL)	Medium	Starting concn (μg/mL)	Physical state	IC50 (μg/mL) ±SD	
1	2-Bromo-2-Nitropropane-1,3-Diol	Solution	Medium	10000	Solution	1<IC50<10	Medium	10	Solution	6.42±0.85	Positive
2	Benzalkonium chloride	Suspension	DMSO /Medium	1000	Solution	1<IC50<10	DMSO /Medium	10	Solution	3.47±0.47	Positive
3	Cetrimonium chloride	Solution	Medium	10000	Solution	IC50<1	Medium	10	Solution	0.56±0.16	Positive
4	Chlorhexidine digluconate (20% Solution)	Not suspended	DMSO /Medium	200 【1000】	Solution	2<IC50<20 【10<IC50<100】	DMSO /Medium	200 【1000】	Solution	7.92±3.92 【39.6±19.6】	Positive
5	Chlorophene	Not suspended	DMSO /Medium	100	Solution	10<IC50<100	DMSO /Medium	100	Suspension	25.6±9.1	Positive
6	Dioctyl sodium sulfosuccinate	Suspension	DMSO /Medium	1000	Solution	10<IC50<100	DMSO/Medium	100	Solution	81.3±4.8	Positive
7	Lauramide DEA	Suspension	DMSO/Medium	1000	Suspension	10<IC50<100	DMSO /Medium	100	Solution	18.3±4.1	Positive
8	Phenethyl alcohol	Suspension	DMSO/Medium	1000	Solution	1000<IC50	Medium	10000	Suspension	1830±1360	False negative
9	Stearalkonium chloride	Not suspended	Ethanol/Medium	100	Solution	1<IC50<10	Ethanol /Medium	10	Solution	2.66±0.56	Positive
10	TEA-Lauryl sulfate 【40% Solution】	Solution	Medium	4000 【10000】	Solution	40<IC50<400 【100<IC50<1000】	Medium	400 【1000】	Solution	117±3 【290±4】	Positive
11	Acetyl tributyl citrate	Not suspended	Ethanol /Medium	100	Solution	100<IC50	Medium	-	Not suspended	Could not be tested	NE
12	Benzophenone-1	Not suspended	DMSO/Medium	100	Suspension	10<IC50<100	DMSO /Medium	100	Suspension	29.3±8.0	False positive
13	Benzophenone-2	Not suspended	DMSO /Medium	100	Suspension	10<IC50<100	DMSO/Medium	100	Suspension	53.4±6.4	False positive
14	Butylene glycol	Solution	Medium	10000	Solution	10000<IC50	Medium	10000	Solution	10000<	Negative
15	Carnauba (Copernicia cerifera) wax	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
16	Cetyl alcohol	Not suspended	DMSO /Medium	100	Suspension	10<IC50<100	DMSO /Medium	100	Suspension	25.1±12.1	False positive
17	Cetyl palmitate	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
18	Decyl oleate	Not suspended	Ethanol /Medium	100	Suspension	100<IC50	Medium	-	Not suspended	Could not be tested	NE
19	Diazolidinyl urea	Solution	Medium	10000	Solution	1<IC50<10	Medium	100	Solution	11.5±7.7	False positive
20	Diethylhexyl adipate(=Octyl)	Not suspended	Ethanol /Medium	1000	Suspension	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
21	Diisopropyl adipate	Not suspended	DMSO/Medium	1000	Suspension	100<IC50<1000	DMSO /Medium	1000	Suspension	633±16	Negative
22	Ethylhexyl palmitate (=Octyl)	Suspension	Ethanol /Medium	1000	Suspension	1000<IC50	Medium	10000	Suspension	10000<	Negative
23	Ethylhexyl stearate (=Octyl)	Not suspended	Ethanol /Medium	100	Suspension	100<IC50	Medium	-	Not suspended	Could not be tested	NE
24	Glyceryl stearate	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
25	Hexylene glycol	Solution	Medium	10000	Solution	1000<IC50<10000	Medium	10000	Suspension	7500±600	Negative
26	Isocetyl stearate	Not suspended	Ethanol /Medium	1000	Suspension	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
27	Isopropyl Myristate	Not suspended	Ethanol/Medium	1000	Suspension	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
28	Isopropyl Palmitate	Not suspended	Ethanol /Medium	1000	Suspension	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
29	Oleyl alcohol	Not suspended	Ethanol /Medium	100	Suspension	10<IC50<100	Ethanol /Medium	100	Suspension	41.9±13.3	False positive
30	PEG-2 stearate	Not suspended	DMSO /Medium	100	Solution	100<IC50	Medium	10000	Not suspended	Could not be tested	NE
31	PEG-40 stearate	Suspension	Medium	10000 〔5000〕	Suspension	100<IC50<1000	Medium	1000	Solution	230±79	False positive
32	Phytantriol	Not suspended	DMSO /Medium	1000	Suspension	10<IC50<100	DMSO /Medium	100	Suspension	37.2±11.8	False positive
33	Propylene carbonate	Solution	Medium	10000	Solution	1000<IC50<10000	Medium	10000	Solution	6050±490	Negative
34	Ricinus communis (Castor) seed oil	Not suspended	DMSO /Medium	100	Solution	100<IC50	Medium	-	Not suspended	Could not be tested	NE
35	Safflower (Carthamus tinctorius) oil	Not suspended	DMSO /Medium	1000	Solution	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
36	Sesame (Sesamum indicum) oil	Not suspended	DMSO /Medium	1000	Solution	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
37	Sodium dehydroacetate	Solution	Medium	10000	Solution	100<IC50<1000	Medium	1000	Solution	860±224	Negative
38	Sodium stearate	Suspension	Medium	10000 〔2500〕	Suspension	10<IC50<100	Medium	1000	Suspension	56.5±8.2	False positive

39	Sorbitan oleate	Suspension	DMSO /Medium	1000	Solution	1000<IC50	Medium	10000	Suspension	5170±1560	Negative
40	Sorbitan sesquioleate	Suspension	DMSO /Medium	1000	Solution	1000<IC50	Medium	10000	Suspension	10000<	Negative
41	Sorbitan stearate	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
42	Squalane	Not suspended	DMSO /Medium	1000	Solution	1000<IC50	Medium	-	Not suspended	Could not be tested	Negative #
43	Steareth-2	Not suspended	Ethanol /Medium	100	Solution	10<IC50<100	Ethanol /Medium	100	Solution	22.4±5.4	False positive
44	Steareth-20	Solution	Medium	10000	Solution	10<IC50<100	Medium	100	Solution	16.5±8.3	False positive
45	Stearyl alcohol	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
46	Triacetin	Solution	Medium	10000	Solution	1000<IC50<1000 0	Medium	10000	Solution	1780±720	Negative
47	Triethylene glycol	Solution	Medium	10000	Solution	10000<IC50	Medium	10000	Solution	10000<	Negative
48	Zinc stearate	Not suspended	-	-	Not suspended	Could not be tested	-	-	-	Could not be tested	NE
Negative reference	Tween 20	-	-	-	-	-	Medium	1000	Solution	501±33	Negative

#: It was judged as negative from results of range finding assay.
NE: It could not be evaluated.
【 】: The data was obtained from diluted agent.
〔 〕: The precipitation was appear at the concentration of 10000μg/mL in the culture of 72hr. The maximal concentrations without the precipitation were 5000ug/mL and 2500ug/mL in No31 and No38, respectively.

Table 41 Predicted irritancy of 48 substances based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)		
		Positive	Negative	Could not be tested
In vivo (Classification by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive	2-Bromo-2-nitropropane-1,3-diol (6.42 ± 0.85) Benzalkonium chloride (3.47 ± 0.47) Cetrimonium chloride (0.56 ± 0.16) Chlorhexidine digluconate (7.92 ± 3.92) Chlorophene (25.6 ± 9.1) Dioctyl sodium sulfosuccinate (81.3 ± 4.8) Lauramide DEA (18.3 ± 4.1) Stearalkonium chloride (2.66 ± 0.56) TEA-Lauryl sulphate (117 ± 3) 9	Phenethyl alcohol (1830 ± 1360) 1	0
	Negative NI for GHS	Benzophenone-1 (29.3 ± 8.0) Benzophenone-2 (53.4 ± 6.4) Cetyl alcohol (25.1 ± 12.1) Diazolidinyl urea (11.5 ± 7.7) Oleyl alcohol (41.9 ± 13.3) PEG-40 stearate (230 ± 79) Phytantriol (37.2 ± 11.8) Sodium stearate (56.5 ± 8.2) Steareth-2 (22.4 ± 5.4) Steareth-20 (16.5 ± 8.3) 10	Butylene glycol (10000<) Diethylhexyl adipate (1000<) Diisopropyl adipate (633 ± 16) Ethylhexyl palmitate (10000<) Hexylene glycol (7500 ± 600) Isocetyl stearate (1000<) Isopropyl myristate (1000<) Isopropyl palmitate (1000<) Propylene carbonate (6050 ± 490) Safflower oil (1000<) Sesame oil (1000<) Sodium dehydroacetate (860 ± 224) Sorbitan oleate (5170 ± 1560) Sorbitan sesquioleate (10000<) Squalane (1000<) Triacetin (1780 ± 720) Triethylene glycol (10000<) 17	Acetyl tributyl citrate Carnauba wax Castor seed oil Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate PEG-2 stearate Sorbitan stearate Stearyl alcohol Zinc stearate 11

The results of SIRC-CVS assay are shown as average ± standard deviation (n=3) of IC50 value in parenthesis. Tween 20 (IC50=501 ± 33 □g/ml) was used as a reference substance for non-irritancy. The 11 substances that were insufficiently soluble to be tested are also shown in this table.

Table 42 Predicted irritancy of 48 substances based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Sucrose fatty acid ester)

		In vitro (Classification by SIRC-CVS assay using Sucrose fatty acid ester as a reference substance for non-irritancy)		
		Positive	Negative	Could not be tested
In vivo (Classification by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive	2-Bromo-2-nitropropane-1,3-diol (6.42 ± 0.85) Benzalkonium chloride (3.47 ± 0.47) Cetrimonium chloride (0.56 ± 0.16) Chlorhexidine digluconate (7.92 ± 3.92) Chlorophene (25.6 ± 9.1) Dioctyl sodium sulfosuccinate (81.3 ± 4.8) Lauramide DEA (18.3 ± 4.1) Stearalkonium chloride (2.66 ± 0.56) TEA-Lauryl sulphate (117 ± 3) 9	Phenethyl alcohol (1830 ± 1360) 1	0
	Negative NI for GHS	Benzophenone-1 (29.3 ± 8.0) Benzophenone-2 (53.4 ± 6.4) Cetyl alcohol (25.1 ± 12.1) Diazolidinyl urea (11.5 ± 7.7) Oleyl alcohol (41.9 ± 13.3) PEG-40 stearate (230 ± 79) Phytantriol (37.2 ± 11.8) Sodium stearate (56.5 ± 8.2) Steareth-2 (22.4 ± 5.4) Steareth-20 (16.5 ± 8.3) 10	Butylene glycol (10000<) Diethylhexyl adipate (1000<) Diisopropyl adipate (633 ± 16) Ethylhexyl palmitate (10000<) Hexylene glycol (7500 ± 600) Isocetyl stearate (1000<) Isopropyl myristate (1000<) Isopropyl palmitate (1000<) Propylene carbonate (6050 ± 490) Safflower oil (1000<) Sesame oil (1000<) Sodium dehydroacetate (860 ± 224) Sorbitan oleate (5170 ± 1560) Sorbitan sesquioleate (10000<) Squalane (1000<) Triacetin (1780 ± 720) Triethylene glycol (10000<) 17	Acetyl tributyl citrate Carnauba wax Castor seed oil Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate PEG-2 stearate Sorbitan stearate Stearyl alcohol Zinc stearate 11

The results of SIRC-CVS assay are shown as average ± standard deviation (n=3) of IC50 value in parenthesis. Sucrose fatty acid ester (IC50=250 □g/ml) was used as a reference substance for non-irritancy. The 11 substances that were insufficiently soluble to be tested are also shown in this table.

Table 43 Predicted irritancy of 48 substances based on the SIRC-CVS assay
(Concentration: 10%, Negative reference: Tween 20)
-GHS classification by considering pH-

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)		
		Positive	Negative	Could not be tested
In vivo (Classification by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive	Calcium thioglycolate Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 2-Bromo-2-nitropropane-1,3-diol ~~Benzalkonium chloride~~ Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulphate 24	Lactic acid Benzyl alcohol Glycolic acid Butanol Phenethyl alcohol 5	0
	Negative NI for GHS	2-Ethylhexyl p-dimethylamino benzonate Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester Benzophenone-1 Benzophenone-2 Cetyl alcohol Diazolidinyl urea Oleyl alcohol PEG-40 stearate Phytantriol Sodium stearate Steareth-2 Steareth-20 15	Ethanol Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Polyoxyethylene sorbitan monooleate (20E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20 Butylene glycol Diethylhexyl adipate Diisopropyl adipate Ethylhexyl palmitate Hexylene glycol Isocetyl stearate ~~Isopropyl myristate~~ Isopropyl palmitate Propylene carbonate Safflower oil Sesame oil Sodium dehydroacetate Sorbitan oleate Sorbitan sesquioleate Squalane Triacetin Triethylene glycol 25	Acetyl tributyl citrate Carnauba wax Castor seed oil Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate PEG-2 stearate Sorbitan stearate Stearyl alcohol Zinc stearate 11

Table 44 Methods of the LDM-MTT assay

Test substance preparation	The MATREX kit was donated by Organogenesis Inc. for the first phase of the validation study, and by Toyobo Co. Ltd for the second and third phases. The kit consisted of LDMs, polyethylene ring, silicon sealant and assay medium, and included all requirements for the test. The solvents for diluting test substances were distilled water, 50% dimethyl sulfoxide and ethylene glycol in EC_{50} value measurement, while in the MATREX scoring method the solvent was distilled water only. In this case, if a substance was not soluble or could not be dispersed in water, it was carried out at only one dose level–100%.
Test kit and procedures	The MATREX kit was donated by Organogenesis Inc. for the first phase of the validation study, and by Toyobo Co. Ltd for the second and third phases. The kit consisted of LDMs, polyethylene ring, silicon sealant and assay medium, and included all requirements for the test. The solvents for diluting test substances were distilled water, 50% dimethyl sulfoxide and ethylene glycol in EC_{50} value measurement, while in the MATREX scoring method the solvent was distilled water only. In this case, if a substance was not soluble or could not be dispersed in water, it was carried out at only one dose level–100%. plate and 5 ml assay medium was added to the surface of the LDM for 30 min at room temperature to remove any residual conditioned medium from the LDM. Then, 5 ml the assay medium was aspirated and 1.5 ml of fresh assay medium was added underneath each LDM. The polyethylene ring was applied to the surface of the LDM using silicon sealant around the area of exposure. Then, 80 μl (or 80 mg in the case of a solid) test substance was applied to the surface. The LDM was exposed to the test substance for 24 hr at 37°C in a 5% CO_2 incubator. After incubation, the test substance was removed from the LDM by washing with the assay medium. The LDM was dipped in 1.5 ml MTT solution (0.333 mg MTT/1 ml assay medium) for 3–4 hr at 37°C. After exposure to MTT, the centre of the LDM tissue was excised using an 8 mm diameter skin biopsy punch. As an indicator of cell viability, MTT formazan formed by the reaction of MTT was extracted by exposure to 0.3 ml isopropanol containing 0.04 N HCl for 2 hr. The absorbance at 570 nm was measured after calibrating with the extraction solvent as a blank. Untreated controls were handled in the same manner, except that they were treated without the test substance.
EC50 value measurement	A preliminary range-finding test was performed with several concentrations of each test substance. The cell survival rate was calculated against untreated control value. According to the results of the preliminary test, the definitive test was carried out using five doses to obtain the EC_{50} value. The EC_{50} value for each test substance was estimated from a dose–response curve obtained.

The contents are the same as those reported by Ohuchi et al. (1999).

Table 45 Results of interlaboratory reproducibility on the LDM-MTT assay
(Concentration: 10%, Cut-off value: 4.15%)

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the LDM-MTT assay (%)							
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab. G	Average±SD (%)
Ethanol	0.0	NI	36	41	37.5	56	NT	NT	NT	43±9
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Glycerin	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyethylene glycol 400	0.0	NI	100<	100<	85	78	100	67	82	67<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	36	26.5	21.5	NT	NT	NT	NT	28.0±7.4
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	4.8	2.53	1.65	1.4	1.93	3.2	1.55	2.4±1.2
Sodium salicylate	0.0	NI	9.2	9.8	8.5	6.0	5.47	11.5	11.5	8.9±2.4
Triethanolamine	0.0	NI	7.6	4.1	6.2	8.4	NT	NT	NT	6.6±1.9
Isopropyl myristate	0.7	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.7	NI	0.072	0.057	0.061	NT	NT	NT	NT	0.063±0.008
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	0.064	0.06	0.058	NT	NT	NT	NT	0.061±0.003
Calcium thioglycolate	4.0	NI	1.4	6.4	6.0	7.7	2.15	7.0	1.5	4.6±2.8
m-Phenylenediamine (Lack of stability)	4.3	NI	0.56	3.4	0.145	0.72	0.47	0.45	0.4	0.88±1.13
Lactic acid	9.7	NI	0.31	0.27	0.285	0.26	NT	NT	NT	0.28±0.02
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	0.060	0.047	0.06	NT	NT	NT	NT	0.056±0.008
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	0.22	0.25	0.32	NT	NT	NT	NT	0.26±0.05
Sucrose fatty scid ester	11.0	NI	0.027	0.014	0.024	0.009	0.013	0.02	0.033	0.020±0.009
Diisopropanolamine	23.0	NI	1.2	1.1	0.92	0.88	NT	NT	NT	1.0±0.2
Sodium lauryl sulfate	15.0[$]	1or2A	0.017	0.015	0.018	NT	NT	NT	NT	0.017±0.002
Benzyl alcohol	23.0	1or2A	7.4	7.0	8.6	6.2	8.2	7.15	6.4	7.3±0.9
Monoethanolamine	23.3	2B	0.34	0.38	0.33	0.53	NT	NT	NT	0.40±0.09
Acid red 92	25.0	1or2A	0.0086	0.0062	0.0074	0.0038	0.0073	0.0008	0.018	0.0074±0.0054
Glycolic acid	25.0	2B	0.22	0.21	0.155	0.16	NT	NT	NT	0.19±0.03
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	0.0018	0.00385	0.0041	NT	NT	NT	NT	0.0033±0.0013
Chlorhexidine gluconate (20% solution)	28.3	2A	0.061	0.037	0.0195	0.042	NT	NT	NT	0.040±0.017
Butanol	34.0	1or2A	8.6	6.0	12.3	9.6	NT	NT	NT	9.1±2.6
Potassium laurate	38.0	1or2A	0.17	0.23	0.13	0.13	NT	NT	NT	0.17±0.05
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	0.034	0.0285	0.060	NT	NT	NT	NT	0.041±0.017
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	0.0066	0.0083	0.0068	0.0074	NT	NT	NT	0.0073±0.0008
Acetic acid	68.0	1or2A	0.23	0.24	0.215	0.96	NT	NT	NT	0.41±0.37
Cetyltrimethylammonium bromide	76.7	1or2A	0.0015	0.0014	0.0018	0.0022	NT	NT	NT	0.0017±0.0004
Benzalkonium chloride	78.0	1or2A	0.0018	0.0023	0.0016	NT	NT	NT	NT	0.0019±0.0004
Stearyltrimethylammonium chloride	91.3	1or2A	0.0030	0.0012	0.0013	0.0014	NT	NT	NT	0.0017±0.0009
Cetylpyridinium chloride	94.7	1	0.0027	0.0013	0.00265	0.0013	0.00124	0.00165	0.0026	0.0019±0.0007
Domiphen bromide	96.3	1	0.0018	0.0021	0.0070	0.0019	NT	NT	NT	0.0032±0.0025

The data were taken from Ohuchi et al. (1999). The cut off value of 4.15% was used for the classification in the LDM-MTT assay. As reported by Ohuchi et al. (1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium lauryl sulfate was evaluated as positive in the evaluation on the basis of MAS, because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
SD: Standard deviation
NT: Not tested

Table 46 Results of interlaboratory reproducibility on the LDM-MTT assay
(Concentration: 10%, Cut-off value: 4.15%)
-GHS classification by considering pH-

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the LDM-MTT assay (%)							
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab. G	Average±SD (%)
Ethanol	0.0	NI	36	41	37.5	56	NT	NT	NT	43±9
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Glycerin	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyethylene glycol 400	0.0	NI	100<	100<	85	78	100	67	82	67<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	36	26.5	21.5	NT	NT	NT	NT	28.0±7.4
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	4.8	2.53	1.65	1.4	1.93	3.2	1.55	2.4±1.2
Sodium salicylate	0.0	NI	9.2	9.8	8.5	6.0	5.47	11.5	11.5	8.9±2.4
Triethanolamine	0.0	NI	7.6	4.1	6.2	8.4	NT	NT	NT	6.6±1.9
Isopropyl myristate	0.7	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.7	NI	0.072	0.057	0.061	NT	NT	NT	NT	0.063±0.008
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	0.064	0.06	0.058	NT	NT	NT	NT	0.061±0.003
m-Phenylenediamine (Lack of stability)	4.3	NI	0.56	3.4	0.145	0.72	0.47	0.45	0.4	0.88±1.13
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	0.060	0.047	0.06	NT	NT	NT	NT	0.056±0.008
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	0.22	0.25	0.32	NT	NT	NT	NT	0.26±0.05
Sucrose fatty scid ester	11.0	NI	0.027	0.014	0.024	0.009	0.013	0.02	0.033	0.020±0.009
Calcium thioglycolate	4.0	1*	1.4	6.4	6.0	7.7	2.15	7.0	1.5	4.6±2.8
Lactic acid	9.7	1*	0.31	0.27	0.285	0.26	NT	NT	NT	0.28±0.02
Sodium lauryl sulfate	15.0$	1or2A	0.017	0.015	0.018	NT	NT	NT	NT	0.017±0.002
Benzyl alcohol	23.0	1or2A	7.4	7.0	8.6	6.2	8.2	7.15	6.4	7.3±0.9
Diisopropanolamine	23.0	1*	1.2	1.1	0.92	0.88	NT	NT	NT	1.0±0.2
Monoethanolamine	23.3	1*	0.34	0.38	0.33	0.53	NT	NT	NT	0.40±0.09
Acid red 92	25.0	1or2A	0.0086	0.0062	0.0074	0.0038	0.0073	0.0008	0.018	0.0074±0.0054
Glycolic acid	25.0	1*	0.22	0.21	0.155	0.16	NT	NT	NT	0.19±0.03
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	0.0018	0.00385	0.0041	NT	NT	NT	NT	0.0033±0.0013
Chlorhexidine gluconate (20% solution)	28.3	2A	0.061	0.037	0.0195	0.042	NT	NT	NT	0.040±0.017
Butanol	34.0	1or2A	8.6	6.0	12.3	9.6	NT	NT	NT	9.1±2.6
Potassium laurate	38.0	1or2A	0.17	0.23	0.13	0.13	NT	NT	NT	0.17±0.05
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	0.034	0.0285	0.060	NT	NT	NT	NT	0.041±0.017
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	0.0066	0.0083	0.0068	0.0074	NT	NT	NT	0.0073±0.0008
Acetic acid	68.0	1or2A	0.23	0.24	0.215	0.96	NT	NT	NT	0.41±0.37
Cetyltrimethylammonium bromide	76.7	1or2A	0.0015	0.0014	0.0018	0.0022	NT	NT	NT	0.0017±0.0004
Benzalkonium chloride	78.0	1or2A	0.0018	0.0023	0.0016	NT	NT	NT	NT	0.0019±0.0004
Stearyltrimethylammonium chloride	91.3	1or2A	0.0030	0.0012	0.0013	0.0014	NT	NT	NT	0.0017±0.0009
Cetylpyridinium chloride	94.7	1	0.0027	0.0013	0.00265	0.0013	0.00124	0.00165	0.0026	0.0019±0.0007
Domiphen bromide	96.3	1	0.0018	0.0021	0.0070	0.0019	NT	NT	NT	0.0032±0.0025

The data were taken from Ohuchi et al. (1999). The cut off value of 4.15% was used for the classification in the LDM-MTT assay. As reported by Ohuchi et al. (1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium lauryl sulfate was evaluated as positive in the evaluation on the basis of MAS, because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
SD: Standard deviation
NT: Not tested

Table 47 Results of interlaboratory reproducibility on the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the LDM-MTT assay (%)							
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab. G	Average±SD (%)
Ethanol	0.0	NI	36	41	37.5	56	NT	NT	NT	43±9
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Glycerin	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyethylene glycol 400	0.0	NI	100<	100<	85	78	100	67	82	67<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	36	26.5	21.5	NT	NT	NT	NT	28.0±7.4
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	4.8	2.53	1.65	1.4	1.93	3.2	1.55	2.4±1.2
Sodium salicylate	0.0	NI	9.2	9.8	8.5	6.0	5.47	11.5	11.5	8.9±2.4
Triethanolamine	0.0	NI	7.6	4.1	6.2	8.4	NT	NT	NT	6.6±1.9
Isopropyl myristate	0.7	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.7	NI	0.072	0.057	0.061	NT	NT	NT	NT	0.063±0.008
Polyethyleneglycol monolaurate (10 E.O.)	3.3	NI	0.064	0.06	0.058	NT	NT	NT	NT	0.061±0.003
Calcium thioglycolate	4.0	NI	1.4	6.4	6.0	7.7	2.15	7.0	1.5	4.6±2.8
m-Phenylenediamine (Lack of stability)	4.3	NI	0.56	3.4	0.145	0.72	0.47	0.45	0.4	0.88±1.13
Lactic acid	9.7	NI	0.31	0.27	0.285	0.26	NT	NT	NT	0.28±0.02
Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)	10.0	NI	0.060	0.047	0.06	NT	NT	NT	NT	0.056±0.008
Sodium N-lauryl sarcosinate (30% solution)	10.3	NI	0.22	0.25	0.32	NT	NT	NT	NT	0.26±0.05
Sucrose fatty scid ester	11.0	NI	0.027	0.014	0.024	0.009	0.013	0.02	0.033	0.020±0.009
Diisopropanolamine	23.0	NI	1.2	1.1	0.92	0.88	NT	NT	NT	1.0±0.2
Sodium lauryl sulfate	15.0$	1or2A	0.017	0.015	0.018	NT	NT	NT	NT	0.017±0.002
Benzyl alcohol	23.0	1or2A	7.4	7.0	8.6	6.2	8.2	7.15	6.4	7.3±0.9
Monoethanolamine	23.3	2B	0.34	0.38	0.33	0.53	NT	NT	NT	0.40±0.09
Acid red 92	25.0	1or2A	0.0086	0.0062	0.0074	0.0038	0.0073	0.0008	0.018	0.0074±0.0054
Glycolic acid	25.0	2B	0.22	0.21	0.155	0.16	NT	NT	NT	0.19±0.03
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	0.0018	0.00385	0.0041	NT	NT	NT	NT	0.0033±0.0013
Chlorhexidine gluconate (20% solution)	28.3	2A	0.061	0.037	0.0195	0.042	NT	NT	NT	0.040±0.017
Butanol	34.0	1or2A	8.6	6.0	12.3	9.6	NT	NT	NT	9.1±2.6
Potassium laurate	38.0	1or2A	0.17	0.23	0.13	0.13	NT	NT	NT	0.17±0.05
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	0.034	0.0285	0.060	NT	NT	NT	NT	0.041±0.017
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	0.0066	0.0083	0.0068	0.0074	NT	NT	NT	0.0073±0.0008
Acetic acid	68.0	1or2A	0.23	0.24	0.215	0.96	NT	NT	NT	0.41±0.37
Cetyltrimethylammonium bromide	76.7	1or2A	0.0015	0.0014	0.0018	0.0022	NT	NT	NT	0.0017±0.0004
Benzalkonium chloride	78.0	1or2A	0.0018	0.0023	0.0016	NT	NT	NT	NT	0.0019±0.0004
Stearyltrimethylammonium chloride	91.3	1or2A	0.0030	0.0012	0.0013	0.0014	NT	NT	NT	0.0017±0.0009
Cetylpyridinium chloride	94.7	1	0.0027	0.0013	0.00265	0.0013	0.00124	0.00165	0.0026	0.0019±0.0007
Domiphen bromide	96.3	1	0.0018	0.0021	0.0070	0.0019	NT	NT	NT	0.0032±0.0025

The data were taken from Ohuchi et al. (1999). Triethanolamine was used as negative reference. In Lab. E-G that triethanolamine was not tested, 6.6% was used as the cut-off value. As reported by Ohuchi et al. (1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium lauryl sulfate was evaluated as positive in the evaluation on the basis of MAS, because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
SD: Standard deviation
NT: Not tested

Table 48 Results of interlaboratory reproducibility on the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)
-GHS classification by considering pH-

Substance (Draize eye test was performed at 10% concentration)	MAS at 10%	GHS at 10%	IC50 of the LDM-MTT assay (%)							
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab. G	Average±SD (%)
Ethanol	0.0	NI	36	41	37.5	56	NT	NT	NT	43±9
2-Ethylhexyl p-dimethylamino benzonate	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Glycerin	0.0	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyethylene glycol 400	0.0	NI	100<	100<	85	78	100	67	82	67<
Polyoxyethylene hydrogenated caster oil (60 E.O.)	0.0	NI	36	26.5	21.5	NT	NT	NT	NT	28.0±7.4
Polyoxyethylene sorbitan monooleate (20E.O.)	0.0	NI	4.8	2.53	1.65	1.4	1.93	3.2	1.55	2.4±1.2
Sodium salicylate	0.0	NI	9.2	9.8	8.5	6.0	5.47	11.5	11.5	8.9±2.4
Triethanolamine	0.0	NI	7.6	4.1	6.2	8.4	NT	NT	NT	6.6±1.9
Isopropyl myristate	0.7	NI	100<	100<	100<	100<	100<	100<	100<	100<
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.7	NI	0.072	0.057	0.061	NT	NT	NT	NT	0.063±0.008
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Calcium thioglycolate	4.0	1*	1.4	6.4	6.0	7.7	2.15	7.0	1.5	4.6±2.8
Lactic acid	9.7	1*	0.31	0.27	0.285	0.26	NT	NT	NT	0.28±0.02
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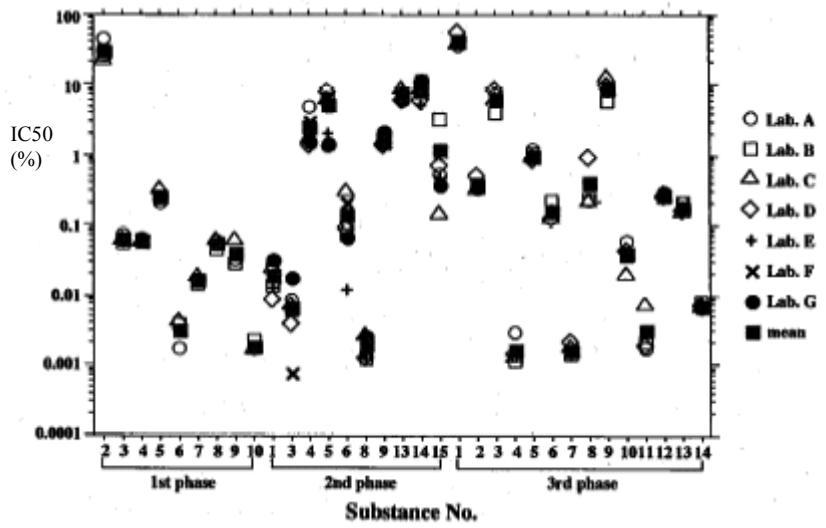
Monoethanolamine	23.3	1*	0.34	0.38	0.33	0.53	NT	NT	NT	0.40±0.09
Acid red 92	25.0	1or2A	0.0086	0.0062	0.0074	0.0038	0.0073	0.0008	0.018	0.0074±0.0054
Glycolic acid	25.0	1*	0.22	0.21	0.155	0.16	NT	NT	NT	0.19±0.03
Sodium hydrogenated tallow L-glutamate	26.7	1or2A	0.0018	0.00385	0.0041	NT	NT	NT	NT	0.0033±0.0013
Chlorhexidine gluconate (20% solution)	28.3	2A	0.061	0.037	0.0195	0.042	NT	NT	NT	0.040±0.017
Butanol	34.0	1or2A	8.6	6.0	12.3	9.6	NT	NT	NT	9.1±2.6
Potassium laurate	38.0	1or2A	0.17	0.23	0.13	0.13	NT	NT	NT	0.17±0.05
Polyoxyethylene octylphenylether (10 E.O.)	41.3	1or2A	0.034	0.0285	0.060	NT	NT	NT	NT	0.041±0.017
Di (2-ethylhexyl) sodium sulfosuccinate	57.0	1or2A	0.0066	0.0083	0.0068	0.0074	NT	NT	NT	0.0073±0.0008
Acetic acid	68.0	1or2A	0.23	0.24	0.215	0.96	NT	NT	NT	0.41±0.37
Cetyltrimethylammonium bromide	76.7	1or2A	0.0015	0.0014	0.0018	0.0022	NT	NT	NT	0.0017±0.0004
Benzalkonium chloride	78.0	1or2A	0.0018	0.0023	0.0016	NT	NT	NT	NT	0.0019±0.0004
Stearyltrimethylammonium chloride	91.3	1or2A	0.0030	0.0012	0.0013	0.0014	NT	NT	NT	0.0017±0.0009
Cetylpyridinium chloride	94.7	1	0.0027	0.0013	0.00265	0.0013	0.00124	0.00165	0.0026	0.0019±0.0007
Domiphen bromide	96.3	1	0.0018	0.0021	0.0070	0.0019	NT	NT	NT	0.0032±0.0025

The data were taken from Ohuchi et al. (1999). Triethanolamine was used as negative reference. In Lab. E-G that triethanolamine was not tested, 6.6% was used as the cut-off value. As reported by Ohuchi et al. (1999), m-phenylenediamine was excluded from the subsequent analysis due to instability.
MAS: Maximal average score of the Draize eye test.
GHS category 1: Severe or corrosive irritant, 2A: Irritant, 2B: Irritant, NI: Non irritant.
1or2A: The Draize eye test results couldn't discriminate between 1 and 2A for no observation data on day 21. The observation was performed to day 14.
$: Sodium lauryl sulfate was evaluated as positive in the evaluation on the basis of MAS, because 2 of 3 individuals had the corneal damage of 15 and 10 (for the maximal corneal score), respectively.
SD: Standard deviation
NT: Not tested

Table 49 Results of interlaboratory reproducibility on the LDM-MTT assay
(Remainging substances)

Substance (Draize eye test was not performed at 10% concentration)	MAS as is	GHS as is	IC50 of the LDM-MTT assay (%)							
			Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab.G	Average±SD
Isotonic sodium chloride solution	0.0	NI	100<	100<	100<	NT	NT	NT	NT	10000<
Silicic anhydride	2.7	NI	100<	100<	100<	100<	000<	100<	100<	100<
Methyl p-hydroxybenzoate	8.7	NI	1.6	1.66	1.6	1.4	1.36	1.75	2.2	1.7±0.3
Distearyldimethylammonium chloride	96.3	1	0.11	0.072	0.295	0.092	0.0125	0.22	0.07	0.12±0.10

Fig. 9 Interlaboratory variability in the LDM-MTT assay



Substance no.	and substance name
1st phase -2	Polyoxyethylene hydrogenated caster oil (60 E.O.)
1st phase -3	Polyoxyethylene sorbitan monolaurate (20 E.O.)
1st phase -4	Polyethyleneglycol monolaurate (10 E.O.)
1st phase -5	Sodium N-lauryl sarcosinate (30% solution)
1st phase-6	Sodium hydrogenated tallow L-glutamate
1st phase-7	Sodium lauryl sulfate
1st phase-8	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)
1st phase-9	Polyoxyethylene octylphenylether (10 E.O.)
1st phase-10	Benzalkonium chloride
2nd phase-1	Sucrose fatty scid ester
2nd phase-3	Acid red 92
2nd phase-4	Polyoxyethylene sorbitan monooleate (20E.O.)
2nd phase-5	Calcium thioglycolate
2nd phase-6	Distearyldimethylammonium chloride
2nd phase-8	Cetylpyridinium chloride
2nd phase-9	Methyl p-hydroxybenzoate
2nd phase-13	Benzyl alcohol
2nd phase-14	Sodium salicylate
2nd phase-15	m-Phenylenediamine
3rd phase-1	Ethanol
3rd phase-2	Monoethanolamine
3rd phase-3	Triethanolamine
3rd phase-4	Stearyltrimethylammonium chloride
3rd phase-5	Diisopropanolamine
3rd phase-6	Potassium laurate
3rd phase-7	Cetyltrimethylammonium bromide
3rd phase-8	Acetic acid
3rd phase-9	Butanol
3rd phase-10	Chlorhexidine gluconate (20% solution)
3rd phase-11	Domiphen bromide
3rd phase-12	Lactic acid
3rd phase-13	Glycolic acid
3rd phase-14	Di (2-ethylhexyl) sodium sulfosuccinate

The figure is the same as that reported by Ohuchi et al (1999). IC50 values obtained were plotted on the figure. The following substances which did not inhibit MTT conversion by 50% when tested at full strength were excluded: S1-1, S2-2, S2-7, S2-10, S2-11, S2-12. Participation: first phase-three laboratories; second phase-seven laboratories; third phase-four laboratories.

Table 50 Rank correlation coefficient between the average IC50 of all laboratories and the IC50 of each laboratory in the LDM-MTT assay

	Lab. A	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F	Lab. G
Rank correlation coefficent	0.996	0.997	0.993	0.995	0.995	0.998	0.991

The data are extracted from the table reported by Ohuchi et al (1999).

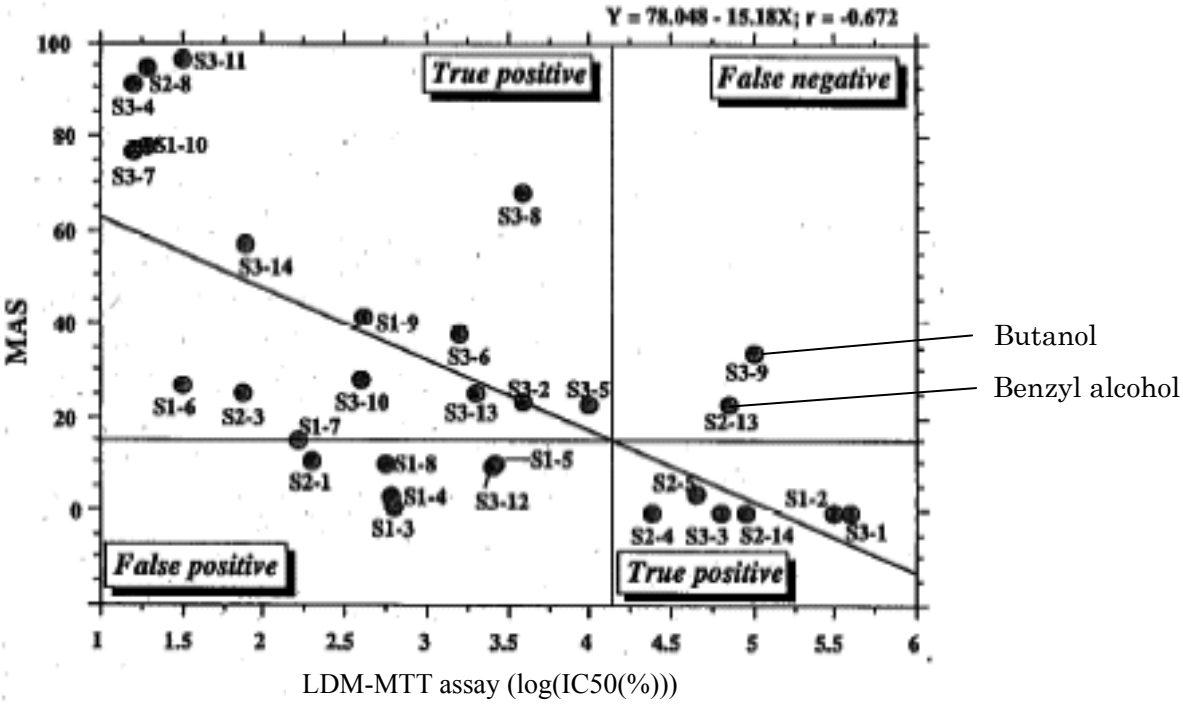
Table 51 Correlation of the results obtained by alternative methods and Draize eye test

Methods	Analysis using all data			Analysis excluding specific classes of chemicals			
		Correlation coefficients				Correlation coefficients	
	N	Pearson's linear	Spearman's rank	class###	N	Pearson's linear	Spearman's rank
Chorioallantoic membranc							
HET–CAM	52	0.688	0.802	1	46	0.702	0.831
				2	6	0.779	0.714
CAM–TB	55	0.718	0.838	1	48	0.801	0.863
				2	7	0.926	0.964
Red blood cells							
RBC	17	–0.631	0.643	3	16	–0.651	0.674
Haemoglobin							
RDC_{50}	8##	0.906	0.714				
1%RDR	23##	0.671	0.579				
1% λ max	31##	0.791	0.697				
Artificial skin models							
SKIN™ (ZK 1100)#	30	–0.694	0.680	4	20	–0.842	
MATREX™#	30	–0.672	0.832	4	20	–0.754	
Normal cells from rabbit cornea							
CornePack*#	28	–0.538	0.588	4	21	–0.731	0.787
Cell lines from rabbit cornea							
SIRC–CVS#	29	–0.805	0.779	4	22	–0.924	0.945
SIRC–NRU#	30	–0.816	0.787	4	23	–0.916	0.931
Cell lines from the other mammals							
HeLa–MTT#	29	–0.799	0.745	4	22	–0.922	0.926
CHL–CVS#	29	–0.729	0.703	4	22	–0.864	0.880
EYTEX™	38	0.313					

#: log (EC_{50}) were correlated with Draize scores (maximal average total score). ##: include the data of substances of the first validation, for which the experiments were conducted afterwards, during the second and the third validations. ###: 1: liquid sample only, 2: powder sample only; 3: excluded strong alkali and acid samples; 4: excluded alcohol (lower mono-ol), strong acids and strong alkalis.

The data are the same as those of Ohno et al. (1999). The LDM-MTT assay is shown as "MATREX™" in the figure.

Fig. 10 Relationship between the LDM-MTT assay and the Draize eye test



The figure is the same as that reported by Ohuchi et al (1999).

Substance no. and substance name	
S1-1	Isotonic sodium chloride solution
S1-2	Polyoxyethylene hydrogenated caster oil (60 E.O.)
S1-3	Polyoxyethylene sorbitan monolaurate (20 E.O.)
S1-4	Polyethyleneglycol monolaurate (10 E.O.)
S1-5	Sodium N-lauryl sarcosinate (30% solution)
S1-6	Sodium hydrogenated tallow L-glutamate
S1-7	Sodium lauryl sulfate
S1-8	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution)
S1-9	Polyoxyethylene octylphenylether (10 E.O.)
S1-10	Benzalkonium chloride
S2-1	Sucrose fatty scid ester
S2-2	Glycerin
S2-3	Acid red 92
S2-4	Polyoxyethylene sorbitan monooleate (20E.O.)
S2-5	Calcium thioglycolate
S2-6	Distearyldimethylammonium chloride
S2-7	2-Ethylhexyl p-dimethylamino benzonate
S2-8	Cetylpyridinium chloride
S2-9	Methyl p-hydroxybenzoate
S2-10	Isopropyl myristate
S2-11	Polyethylene glycol 400
S2-12	Silicic anhydride
S2-13	Benzyl alcohol
S2-14	Sodium salicylate
S2-15	m-Phenylenediamine
S3-1	Ethanol
S3-2	Monoethanolamine
S3-3	Triethanolamine
S3-4	Stearyltrimethylammonium chloride
S3-5	Diisopropanolamine
S3-6	Potassium laurate
S3-7	Cetyltrimethylammonium bromide
S3-8	Acetic acid
S3-9	Butanol
S3-10	Chlorhexidine gluconate (20% solution)
S3-11	Domiphen bromide
S3-12	Lactic acid
S3-13	Glycolic acid
S3-14	Di (2-ethylhexyl) sodium sulfosuccinate

Table 52 Predicted irritancy of test samples based on the LDM-MTT assay
(Concentration: 10%, Cut-off value: 4.15%)

		In vitro (Classification by the LDM-MTT assay)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Acid red 92 Glycolic acid Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 15	Benzyl alcohol Butanol 2
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) Polyoxyethylene sorbitan monolaurate (20 E.O.) Polyethyleneglycol monolaurate (10 E.O.) Lactic acid Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty scid ester Diopropanolamine 8	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Calcium thioglycolate 9

Table 53 Predicted irritancy of test samples based on the LDM-MTT assay
(Concentration: 10%, Cut-off value: 4.15%)
-GHS classification by considering pH-

		In vitro (Classification by the LDM-MTT assay)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Lactic acid Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Glycolic acid Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 17	Calcium thioglycolate Benzyl alcohol Butanol 3
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) Polyoxyethylene sorbitan monolaurate (20 E.O.) Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty scid ester 6	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Sodium salicylate Triethanolamine Isopropyl myristate 8

Table 54 Predicted irritancy of test samples based on the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)

		In vitro (Classification by the LDM-MTT assay)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Acid red 92 Glycolic acid Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 15	Benzyl alcohol Butanol 2
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) Polyoxyethylene sorbitan monolaurate (20 E.O.) Polyethyleneglycol monolaurate (10 E.O.) Lactic acid Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty scid ester Diopropanolamine 8	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Calcium thioglycolate 9

Table 55 Predicted irritancy of test samples based on the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)
-GHS classification by considering pH-

		In vitro (Classification by the LDM-MTT assay)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Calcium thioglycolate Lactic acid Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Glycolic acid Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 18	Benzyl alcohol Butanol 2
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) Polyoxyethylene sorbitan monolaurate (20 E.O.) Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty scid ester 6	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Sodium salicylate Triethanolamine Isopropyl myristate 8

Table 56 Forty-eight substances (Concentration: 10%)

No	Substance	CAS	Supplier (*in vitro* test)	*in vivo* data reported previously		Estimated GHS at 10% concn	Reference
				Classification at 10% concn	Classification at the applied concn		
1	2-Bromo-2-nitropropane-1,3-diol	52-51-7	Fluorochem	Positive	Positive: 100, 20, 10, 5% Negative: 2, 0.5%	1, 2A or 2B	JACT 3(3):139-155,1984. JEPT 4(4):47-61, 1980.
2	Benzalkonium chloride	8001-54-5	Wako	Positive	Positive: 2, 1, 0.5% Negative: 0.1, 0.01%	1, 2A or 2B	JACT 8(4):589-625, 1989.
3	Cetrimonium chloride	112-02-7	Wako	Positive	Positive: 2.5, 1.2, 0.5% Negative: 0.1%	1, 2A or 2B	IJT 16(S3):195-220,1997.
4	Chlorhexidine digluconate	18472-51-0	Wako	Positive	Positive: 20, 2% Negative: 0.05%	1, 2A or 2B	JACT 12(3):201-23, 1993.
5	Chlorophene	120-32-1	Wako	Positive	Positive: 100, 3% Negative: 1, 0.3%	1, 2A or 2B	IJT 23(S1):1-27 2004.
6	Dioctyl sodium sulfosuccinate	577-11-7	Alfa Aesar	Positive	Positive: 10% Negative: 2, 0.5%	1, 2A or 2B	IJT 17(S4):1-20, 1998.
7	Lauramide DEA	120-40-1	Wako	Positive	Positive: 20, 10%	1, 2A or 2B	JACT 5(5):415-54, 1986.
8	Phenethyl alcohol	60-12-8	Wako	Positive	Positive: 100, 15, 5% Negative: 0.3%	1, 2A or 2B	JACT 9(2):165-83, 1990.
9	Stearalkonium chloride	122-19-0	Wako	Positive	Positive: 25, 4, 2.5% Negative: 0.5%	1, 2A or 2B	JACT 1(2):57-69, 1982.
10	TEA-Lauryl sulfate	139-96-8	Wako	Positive	Positive: 20, 10, 5, 2.5, 1.25%	1, 2A or 2B	JACT 1(4):143-67, 1982.
11	Acetyl tributyl citrate	77-90-7	Wako	Negative	Negative: 100%	NI	IJT 21(S2):1-17, 2002.
12	Benzophenone-1	131-56-6	Wako	Negative	Positive: 100% Negative: 16, 8, 4%	NI	JACT 2(5):35-77, 1983.
13	Benzophenone-2	131-55-5	Wako	Negative	Positive: 100% Negative: 16, 8, 4%	NI	JACT 2(5):79-84, 1983.
14	Butylene glycol	107-88-0	Wako	Negative	Negative: 100, 10%	NI	Hifu 26(5):1065-1074, 1984.
15	Carnauba wax	8015-86-9	Wako	Negative	Negative: 50%	NI	JACT 3(3):1-41, 1984.
16	Cetyl alcohol	36653-82-4	Wako	Negative	Negative: 100%	NI	JACT 7(3):359-413, 1988.
17	Cetyl palmitate	540-10-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
18	Decyl oleate	3687-46-5	Wako	Negative	Negative: 100%	NI	JACT 1(2):85-95, 1982.
19	Diazolidinyl urea	78491-02-8	MP Biomedicals	Negative	Negative: 30%	NI	JACT 9(2):229-45, 1990.
20	Diethylhexyl adipate	103-23-1	Wako	Negative	Negative: 100%	NI	JACT 3(3):101-30, 1984.
21	Diisopropyl adipate	6938-94-9	Wako	Negative	Negative: 100%	NI	JACT 3(3):101-30, 1984.
22	Ethylhexyl palmitate	29806-73-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
23	Ethylhexyl stearate	22047-49-0	Wako	Negative	Negative: 100%	NI	JACT 4(5):107-46, 1985.
24	Glyceryl stearate	11099-07-3	Wako	Negative	Negative: 100%	NI	JACT 1(4):169-192, 1982.
25	Hexylene glycol	107-41-5	Wako	Negative	Positive: 100% Negative: 25%	NI	JACT 4(5):223-48, 1985.
26	Isocetyl stearate	25339-09-7	Wako	Negative	Negative: 100%	NI	JACT 4(5):107-46, 1985.
27	Isopropyl myristate	110-27-0	TCI	Negative	Negative: 100%	NI	JACT 1(4):55-80, 1982.
28	Isopropyl palmitate	142-91-6	Wako	Negative	Negative: 100%	NI	JACT 1(2):13-35, 1982.
29	Oleyl alcohol	143-28-2	Wako	Negative	Negative: 100%	NI	JACT 4(5):1-29, 1985.
30	PEG-2 stearate	106-11-6	Wako	Negative	Negative: 100%	NI	JACT 2(7):17-60, 1983.
31	PEG-40 stearate	9004-99-4	Wako	Negative	Negative: 100%	NI	JACT 2(7):17-60, 1983.
32	Phytantriol	74563-64-7	Wako	Negative	Positive: 100, 23% Negative: 10, 3%	NI	IJT 26(Suppl. 1):107-117, 2007.
33	Propylene carbonate	108-32-7	Wako	Negative	Negative: 100, 17.5, 10.5%	NI	JACT 6(1):23-51, 1987.
34	Castor seed oil	8001-79-4	Wako	Negative	Negative: 100%	NI	JACT 7(6):721-739, 1988.
35	Safflower oil	8001-23-8	Wako	Negative	Negative: 100%	NI	JACT 4(5):171-97, 1985.
36	Sesame (Sesamum indicum) oil	8008-74-0	Wako	Negative	Negative: 100%	NI	JACT 12(3):261-77, 1993.
37	Sodium dehydroacetate	4418-26-2	Wako	Negative	Negative: 100%	NI	JACT 4(3):123-159, 1985.
38	Sodium stearate	822-16-2	Wako	Negative	Negative: 100%	NI	JACT 1(2):143-77, 1982.
39	Sorbitan oleate	1338-43-8	Wako	Negative	Negative: 100%	NI	JACT 4(3):65-121, 1985.
40	Sorbitan sesquioleate	8007-43-0	Wako	Negative	Negative: 100, 30%	NI	JACT 4(3):65-121, 1985.
41	Sorbitan stearate	1338-41-6	Wako	Negative	Negative: 30%	NI	JACT 4(3):65-121, 1985.
42	Squalane	111-01-3	Wako	Negative	Negative: 100%	NI	JACT 1(2):37-56, 1982.
43	Steareth-2	9005-00-9	Wako	Negative	Negative: 60%	NI	JACT 7(6):881-910, 1988.
44	Steareth-20	9005-00-9	Wako	Negative	Negative: 60%	NI	JACT 7(6):881-910, 1988.
45	Stearyl alcohol	112-92-5	Wako	Negative	Negative: 100%	NI	JACT 4(5):1-29, 1985.
46	Triacetin	102-76-1	Wako	Negative	Negative: 100%	NI	IJT 22(S2):1-10, 2003.
47	Triethylene glycol	112-27-6	Wako	Negative	Negative: 100%	NI	IJT 25(5) :121-138,2006.
48	Zinc stearate	557-05-1	Wako	Negative	Negative: 100%	NI	JACT 1(2):143-77, 1982.

Supplier means manufacturer of the material used in this study. The *in vivo* classification of positive or negative was based on the appearance or not of corneal damage, or an MAS value of 15 as a cut-off point, where reported MAS values are available. The classification was essentially based on whether or not corneal damage appeared after the application of 0.1 mL to rabbit eye without irrigation. However, where there were differences of test conditions, these were considered individually. For example, a case where corneal damage appeared after the application of 0.05 mL was judged as positive. In cases without data at 10% concentration, the assessment of positive or negative at the concentration of 10% was made on the basis of dose-response analysis of each ingredient.

Table 57 Results of 48 substances in the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)

No	Substance	Draize eye test at 10% concn	Estimated GHS at 10% concn	LDM-MTT assay		
				Medium	IC50(%)	Results
1	2-Bromo-2-Nitropropane-1,3-Diol	Positive	1, 2A or 2B	DW	<1	Positive
2	Benzalkonium chloride	Positive	1, 2A or 2B	DW	<1	Positive
3	Cetrimonium chloride	Positive	1, 2A or 2B	DW	<1	Positive
4	Chlorhexidine digluconate	Positive	1, 2A or 2B	DW	<1	Positive
5	Chlorophene	Positive	1, 2A or 2B	EG	<1	Positive
6	Dioctyl sodium sulfosuccinate	Positive	1, 2A or 2B	DW	<1	Positive
7	Lauramide DEA	Positive	1, 2A or 2B	DW	<1	Positive
8	Phenethyl alcohol	Positive	1, 2A or 2B	50%DMSO	2.7	Positive
9	Stearalkonium chloride	Positive	1, 2A or 2B	DW	<1	Positive
10	TEA-Lauryl sulfate	Positive	1, 2A or 2B	DW	<1	Positive
11	Acetyl tributyl citrate	Negative	NI	-	100	Negative
12	Benzophenone-1	Negative	NI	50%DMSO	<1	Positive
13	Benzophenone-2	Negative	NI	50%DMSO	<1	Positive
14	Butylene glycol	Negative	NI	-	100	Negative
15	Carnauba (Copernicia cerifera) wax	Negative	NI	-	100	Negative
16	Cetyl alcohol	Negative	NI	LP	10<	Negative
17	Cetyl palmitate	Negative	NI	LP	10<	Negative
18	Decyl oleate	Negative	NI	-	100	Negative
19	Diazolidinyl urea	Negative	NI	DW	<1	Positive
20	Diethylhexyl adipate(=Octyl)	Negative	NI	-	100	Negative
21	Diisopropyl adipate	Negative	NI	LP	8.6	Negative
22	Ethylhexyl palmitate (=Octyl)	Negative	NI	-	100	Negative
23	Ethylhexyl stearate (=Octyl)	Negative	NI	-	100	Negative
24	Glyceryl stearate	Negative	NI	-	100	Negative
25	Hexylene glycol	Negative	NI	DW	10<	Negative
26	Isocetyl stearate	Negative	NI	-	100	Negative
27	Isopropyl Myristate	Negative	NI	-	100	Negative
28	Isopropyl Palmitate	Negative	NI	-	100	Negative
29	Oleyl alcohol	Negative	NI	-	100	Negative
30	PEG-2 stearate	Negative	NI	-	100	Negative
31	PEG-40 stearate	Negative	NI	50%DMSO	1.3	Positive
32	Phytantriol	Negative	NI	50%DMSO	1.9	Positive
33	Propylene carbonate	Negative	NI	DW	10<	Negative
34	Ricinus communis (Castor) seed oil	Negative	NI	-	100	Negative
35	Safflower (Carthamus tinctorius) oil	Negative	NI	-	100	Negative
36	Sesame (Sesamum indicum) oil	Negative	NI	-	100	Negative
37	Sodium dehydroacetate	Negative	NI	DW	10<	Negative
38	Sodium stearate	Negative	NI	DW	2.1	Positive
39	Sorbitan oleate	Negative	NI	-	100	Negative
40	Sorbitan sesquioleate	Negative	NI	-	100	Negative
41	Sorbitan stearate	Negative	NI	-	100	Negative
42	Squalane	Negative	NI	-	100	Negative
43	Steareth-2	Negative	NI	DW	10<	Negative
44	Steareth-20	Negative	NI	DW	<1	Positive
45	Stearyl alcohol	Negative	NI	-	100	Negative
46	Triacetin	Negative	NI	DW	10<	Negative
47	Triethylene glycol	Negative	NI	-	100	Negative
48	Zinc stearate	Negative	NI	-	100	Negative
Negative 基準	Triethanolamine	Negative	NI	DW	4.6	Negative

The results of LDM-MTT assay are shown as average (n=2-3) of IC50 value.

Table 58 Predicted irritancy of 48 substances in the LDM-MTT assay

		In vitro (Classification by LDM-MTT assay using triethanolamine as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classicication by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive 1,2Aor2B in GHS	2-Bromo-2-nitropropane-1,3-diol Benzalkonium chloride Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulphate 9	Phenethyl alcohol 1
	Negative NI in GHS	Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20 7	Acetyl tributyl citrate Butylene glycol Carnauba wax Castor seed oil Cetyl alcohol Cetyl palmitate Decyl oleate Diethylhexyl adipate Diisopropyl adipate Ethylhexyl palmitate Ethylhexyl stearate Glyceryl stearate Hexylene glycol Isocetyl stearate Isopropyl Myristate Isopropyl Palmitate Oleyl alcohol PEG-2 stearate Propylene carbonate Safflower oil Sesame oil Sodium dehydroacetate Sorbitan oleate Sorbitan sesquioleate Sorbitan stearate Squalane Steareth-2 Stearyl alcohol Triacetin Triethylene glycol Zinc stearate 31

Triethanolamine (IC50=4.6%) was used as a reference substance for non-irritancy.

Table 59 Predicted irritancy of test samples based on the LDM-MTT assay
(Concentration: 10%, Negative reference: Triethanolamine)
-GHS classification by considering pH-

		In vitro (Classification by the LDM-MTT assay)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Calcium thioglycolate Lactic acid Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Glycolic acid Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 2-Bromo-2-nitropropane-1,3-diol ~~Benzalkonium chloride~~ ~~Cetrimonium chloride~~ Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulphate 25	Benzyl alcohol Butanol Phenethyl alcohol 3
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) Polyoxyethylene sorbitan monolaurate (20 E.O.) Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty scid ester Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20 13	Ethanol 2-Ethylhexyl p-dimethylamino benzonate Glycerin Polyethylene glycol 400 Polyoxyethylene hydrogenated caster oil (60 E.O.) Sodium salicylate Triethanolamine Isopropyl myristate Acetyl tributyl citrate Butylene glycol Carnauba wax Castor seed oil Cetyl alcohol Cetyl palmitate Decyl oleate Diethylhexyl adipate Diisopropyl adipate Ethylhexyl palmitate Ethylhexyl stearate Glyceryl stearate Hexylene glycol Isocetyl stearate ~~Isopropyl Myristate~~ Isopropyl Palmitate Oleyl alcohol PEG-2 stearate Propylene carbonate Safflower oil Sesame oil Sodium dehydroacetate Sorbitan oleate Sorbitan sesquioleate Sorbitan stearate Squalane Steareth-2 Stearyl alcohol Triacetin Triethylene glycol Zinc stearate 38

Table 60 Relationship between IC_{50} in the LDM-MTT assay and concentration evaluated as non irritant in the Draize eye test.

Test substance	Three-dimensional dermal model IC_{50} (%)	Draize eye irritation test results				
		Concentration evaluated as non irritant (MAS<5)	MAS at each applied concentration			
			100%	10%	1%	0.1%
Isotonic sodium chloride solution	100	100	0	NT	NT	NT
2-Ethylhexyl p-dimethylaminobenzoate	100	100	0	0	NT	NT
Isopropyl myristate	100	100	0	0.7	NT	NT
Silicic anhydride	100	100	2.7	NT	NT	NT
Glycerin	100	100	4.7	0	NT	NT
Polyethylene glycol 400	67-100	100	4	0	NT	NT
Polyoxyethylene sorbitan monooleate (20E.O.)	2.4	100	4.7	0	NT	NT
Sodium salicylate	8.9	10	83.7	0	NT	NT
Triethanolamine	6.6	10	8.0	0	NT	NT
Calcium thioglycolate	4.6	10	79.7	4.0	NT	NT
Polyoxyethylene sorbitan monolaurate (20 E.O.)	0.063	10≤	NT	0.7	NT	NT
Polyethyleneglycol monolaurate (10 E.O.)	0.061	10≤	NT	3.3	NT	NT
Acid red 92	0.0074	1	71.0	25.0	0.7	NT
Cetylpyridinium chloride	0.0019	0.1	NT	94.7	34.7	2.7
Ethanol	43	10	32.7	0	NT	NT
Polyoxyethylene hydrogenated caster oil (60E.O)	28.0	10≤	NT	0	NT	NT
Benzyl alcohol	7.3	1	31.0	23.0	0	NT

The figure is the same as that reported by Hagino et al (2008).The data were taken from Ohno et al. (1999) and Ohuchi et al. (1999). The IC_{50} in LDM-MTT assay was the mean of data from 3-7 laboratories. The result of IC_{50} for polyethylene glycol 400 was 100% in 3 laboratories and 67, 78, 82, 85% in the other 4 laboratories. “Not tested” is shown as NT. No conclusion could be reached for ethanol, polyoxyethylene hydorogenated caster oil (60 E.O.) or benzyl alcohol, because of the large concentration intervals in the Draize eye test.

Table 61 Prediction of eye irritancy at various concentrations in the LDM-MTT assay

		In vitro (Classification by LDM-MTT assay using a viability of 50% as cut-off point)	
		Positive	Negative
In vivo (Estimated classification by GHS)	1, 2A or 2B	Calcium thioglycolate (100, 10%) Lactic acid (100, 10%) Sodium lauryl sulfate (10%) Benzyl alcohol (100, 10%) Diisopropanolamine (10%) Monoethanolamine (10%) Acid red 92 (100, 10%) Glycolic acid (10%) Sodium hydrogenated tallow L-glutamate (10%) Chlorhexidine gluconate (20% solution) (10%) Butanol (10%) Potassium laurate (10%) Polyoxyethylene octylphenylether (10 E.O.) (10%) Di (2-ethylhexyl) sodium sulfosuccinate (10%) Acetic acid (10%) Cetyltrimethylammonium bromide (10%) Benzalkonium chloride (10%) Stearyltrimethylammonium chloride (10%) Cetylpyridinium chloride (10, 1%) Domiphen bromide (10%) Sucrose fatty acid ester (100%) Ethanol (100%) Sodium salicylate (100%) Distearyldimethylammonium chloride (100%) 29	0
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) (100, 10%) Sodium salicylate (10%) Triethanolamine (100, 10%) Polyoxyethylene sorbitan monolaurate (20 E.O.) (10%) Polyethyleneglycol monolaurate (10 E.O.) (10%) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) (10%) Sodium N-lauryl sarcosinate (30% solution) (10%) Sucrose fatty scid ester (10%) Methyl p-hydroxybenzoate (100%) Acid red 92 (1%) Cetylpyridinium chloride (0.1%) 13	Ethanol (10%) 2-Ethylhexyl p-dimethylamino benzonate (100, 10%) Glycerin (100, 10%) Polyethylene glycol 400 (10%) Polyoxyethylene hydrogenated caster oil (60 E.O.) (10%) Isopropyl myristate (100, 10%) Isotonic sodium chloride solution (100%) Silicic anhydride (100%) Benzyl alcohol (1%) 12

PEG 400 (100%) could not be classified (IC50=67-100%).

Table 62 Fifty-nine test substances

No	Substance	CAS	Supplier (*in vitro* test)	Estimated classification of GHS at the applied concn by using *in vivo* data reported previously	Reference
1	2-Bromo-2-nitropropane-1,3-diol	52-51-7	Fluorochem	1, 2A or 2B：100, 20, 10, 5% NI：2, 0.5%	JACT 3(3):139-155,1984. JEPT 4(4):47-61, 1980.
2	Benzalkonium chloride	8001-54-5	Wako	1, 2A or 2B：2, 1, 0.5%　NI：0.1, 0.01%	JACT 8(4):589-625, 1989.
3	Cetrimonium chloride	112-02-7	Wako	1, 2A or 2B：2.5, 1.2, 0.5%　NI：0.1%	IJT 16(S3):195-220,1997.
4	Chlorhexidine digluconate	18472-51-0	Wako	1, 2A or 2B：20, 2%　NI：0.05%	JACT 12(3):201-23, 1993.
5	Chlorophene	120-32-1	Wako	1, 2A or 2B：100, 3%　NI：1, 0.3%	IJT 23(S1):1-27 2004.
6	Dioctyl sodium sulfosuccinate	577-11-7	Alfa Aesar	1, 2A or 2B：10%　NI：2, 0.5%	IJT 17(S4):1-20, 1998.
7	Lauramide DEA	120-40-1	Wako	1, 2A or 2B：20, 10%	JACT 5(5):415-54, 1986.
8	Phenethyl alcohol	60-12-8	Wako	1, 2A or 2B：100, 15, 5%　NI：0.3%	JACT 9(2):165-83, 1990.
9	Stearalkonium chloride	122-19-0	Wako	1, 2A or 2B：25, 4, 2.5%　NI：0.5%	JACT 1(2):57-69, 1982.
10	TEA-Lauryl sulfate	139-96-8	Wako	1, 2A or 2B：20, 10, 5, 2.5, 1.25%	JACT 1(4):143-67, 1982.
11	Acetyl tributyl citrate	77-90-7	Wako	NI：100%	IJT 21(S2):1-17, 2002.
12	Benzophenone-1	131-56-6	Wako	1, 2A or 2B：100%　NI：16, 8, 4%	JACT 2(5):35-77, 1983.
13	Benzophenone-2	131-55-5	Wako	1, 2A or 2B：100%　NI：16, 8, 4%	JACT 2(5):79-84, 1983.
14	Butylene glycol	107-88-0	Wako	NI：100, 10%	Hifu 26(5):1065-1074, 1984.
15	Carnauba wax	8015-86-9	Wako	NI：50%	JACT 3(3):1-41, 1984.
16	Cetyl alcohol	36653-82-4	Wako	NI：100%	JACT 7(3):359-413, 1988.
17	Cetyl palmitate	540-10-3	Wako	NI：100%	JACT 1(2):13-35, 1982.
18	Decyl oleate	3687-46-5	Wako	NI：100%	JACT 1(2):85-95, 1982.
19	Diazolidinyl urea	78491-02-8	MP Biomedicals	NI：30%	JACT 9(2):229-45, 1990.
20	Diethylhexyl adipate	103-23-1	Wako	NI：100%	JACT 3(3):101-30, 1984.
21	Diisopropyl adipate	6938-94-9	Wako	NI：100%	JACT 3(3):101-30, 1984.
22	Ethylhexyl palmitate	29806-73-3	Wako	NI：100%	JACT 1(2):13-35, 1982.
23	Ethylhexyl stearate	22047-49-0	Wako	NI：100%	JACT 4(5):107-46, 1985.
24	Glyceryl stearate	11099-07-3	Wako	NI：100%	JACT 1(4):169-192, 1982.
25	Hexylene glycol	107-41-5	Wako	1, 2A or 2B：100%　NI：25%	JACT 4(5):223-48, 1985.
26	Isocetyl stearate	25339-09-7	Wako	NI：100%	JACT 4(5):107-46, 1985.
27	Isopropyl myristate	110-27-0	TCI	NI：100%	JACT 1(4):55-80, 1982.
28	Isopropyl palmitate	142-91-6	Wako	NI：100%	JACT 1(2):13-35, 1982.
29	Oleyl alcohol	143-28-2	Wako	NI：100%	JACT 4(5):1-29, 1985.
30	PEG-2 stearate	106-11-6	Wako	NI：100%	JACT 2(7):17-60, 1983.
31	PEG-40 stearate	9004-99-4	Wako	NI：100%	JACT 2(7):17-60, 1983.
32	Phytantriol	74563-64-7	Wako	1, 2A or 2B：100, 23%　NI：10, 3%	IJT 26(Suppl. 1):107-117, 2007.
33	Propylene carbonate	108-32-7	Wako	NI：100, 17.5, 10.5%	JACT 6(1):23-51, 1987.
34	Castor seed oil	8001-79-4	Wako	NI：100%	JACT 7(6):721-739, 1988.
35	Safflower oil	8001-23-8	Wako	NI：100%	JACT 4(5):171-97, 1985.
36	Sesame (Sesamum indicum) oil	8008-74-0	Wako	NI：100%	JACT 12(3):261-77, 1993.
37	Sodium dehydroacetate	4418-26-2	Wako	NI：100%	JACT 4(3):123-159, 1985.
38	Sodium stearate	822-16-2	Wako	NI：100%	JACT 1(2):143-77, 1982.
39	Sorbitan oleate	1338-43-8	Wako	NI：100%	JACT 4(3):65-121, 1985.
40	Sorbitan sesquioleate	8007-43-0	Wako	NI：100, 30%	JACT 4(3):65-121, 1985.
41	Sorbitan stearate	1338-41-6	Wako	NI：30%	JACT 4(3):65-121, 1985.
42	Squalane	111-01-3	Wako	NI：100%	JACT 1(2):37-56, 1982.
43	Steareth-2	9005-00-9	Wako	NI：60%	JACT 7(6):881-910, 1988.
44	Steareth-20	9005-00-9	Wako	NI：60%	JACT 7(6):881-910, 1988.
45	Stearyl alcohol	112-92-5	Wako	NI：100%	JACT 4(5):1-29, 1985.
46	Triacetin	102-76-1	Wako	NI：100%	IJT 22(S2):1-10, 2003.
47	Triethylene glycol	112-27-6	Wako	NI：100%	IJT 25(5) :121-138,2006.
48	Zinc stearate	557-05-1	Wako	NI：100%	JACT 1(2):143-77, 1982.
49	Benzethonium chloride	121-54-0	TCI	NI：0.5%	JACT 4(5):65-106, 1985.
50	Butoxyethanol	111-76-2	Wako	1, 2A or 2B：100, 15%　NI：5%	JACT 15(6):462-526, 1996.
51	Chloroxylenol	88-04-0	Wako	1, 2A or 2B：100, 30%	JACT 4(5):147-69, 1985.
52	Methoxyisopropyl acetate	108-65-6	Wako	1, 2A or 2B：100%	IJT 27(S2), 2008.
53	Phenoxyethanol	122-99-6	Wako	1, 2A or 2B：100%　NI：2.2%	JACT 9(2):259-77, 1990.
54	Phenyl methyl pyrazolone	89-25-8	Wako	NI：0.66%	JACT 11(4):475-88, 1992.
55	Resorcinol	108-46-3	Wako	1, 2A or 2B：100%	JACT 5(3):167-203, 1986.
56	Sodium hexametaphosphate	10124-56-8	Wako	NI：0.2%	IJT 20(S3):75-89, 2001.
57	Sodium lauroyl sarcosinate	137-16-6	Wako	NI：5%	IJT 20(S1):1-14, 2001.
58	Sodium naphthalenesulfonate	532-02-5	Wako	1, 2A or 2B：100%　NI：2%	IJT 22(Suppl. 2:37-44,)2003.

Supplier means manufacturer of the material used in this study. The *in vivo* classification of positive or negative was based on the appearance or not of corneal damage, or an MAS value of 15 as a cut-off point, where reported MAS values are available. The classification was essentially based on whether or not corneal damage appeared after the application of 0.1 mL to rabbit eye without irrigation. However, where there were differences of test conditions, these were considered individually. For example, a case where corneal damage appeared after the application of 0.05 mL was judged as positive. In cases without data at 10% concentration, the assessment of positive or negative at the concentration of 10% was made on the basis of dose-response analysis of each ingredient.

Table 63 Prediction of eye irritancy at various concentrations in the LDM-MTT assay

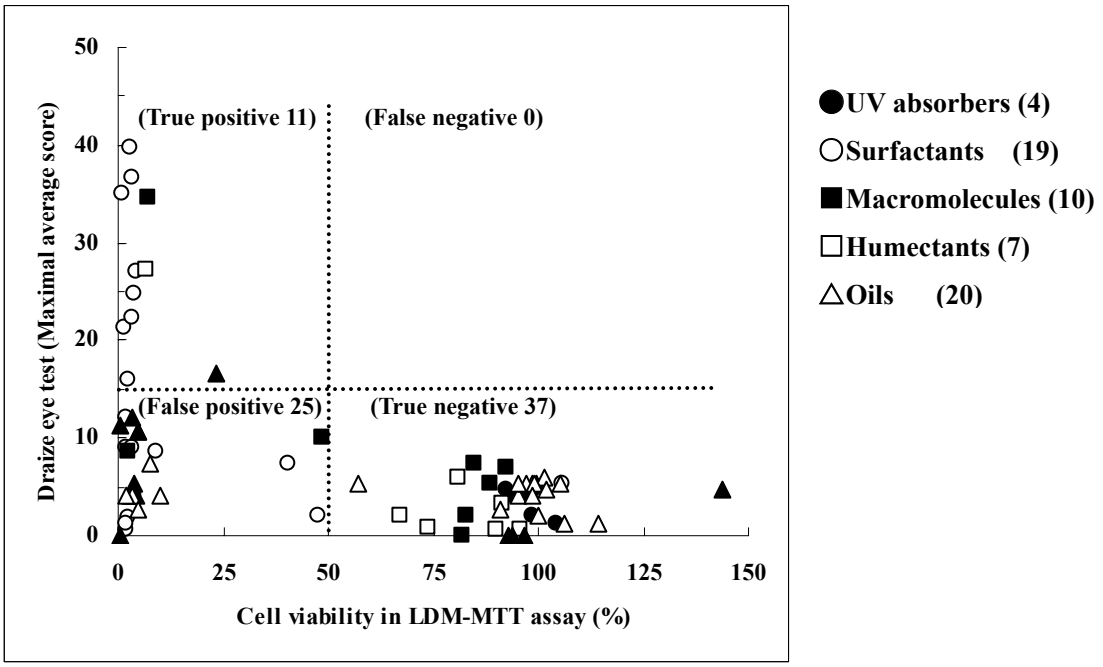
		In vitro (Classification by LDM-MTT assay using a viability of 50% as cut-off point)	
		Positive	Negative
In vivo (Estimated classification by GHS)	1, 2A or 2B	2-Bromo-2-nitropropane-1,3-diol (100, 20, 10, 5%) Benzalkonium chloride (2, 1, 0.5%) Benzophenone-1 (100%) Benzophenone-2 (100%) Butoxyethanol (100, 15%) Cetrimonium chloride (2.5, 1.2, 0.5%) Chlorhexidine digluconate (20, 2%) Chlorophene (100, 3%) Chloroxylenol (100, 30%) Dioctyl sodium sulfosuccinate (10%) Hexylene glycol (100%) Lauramide DEA (20, 10%) Methoxyisopropyl acetate (100%) Phenethyl alcohol (100%) Phenethyl alcohol (15, 5%) Phenoxyethanol (100%) Phytantriol (100, 23%) Resorcinol (100%) Sodium naphthalenesulfonate (100%) Stearalkonium chloride (25, 4, 2.5%) TEA-Lauryl sulfate (20, 10, 5, 2.5, 1.25%) Triisopropanolamine (100%) 42	0
	NI	2-Bromo-2-nitropropane-1,3-diol (2, 0.5%) Benzalkonium chloride (0.1, 0.01%) Benzethonium chloride (0.5%) Benzophenone-1 (16, 8, 4%) Benzophenone-2 (16, 8, 4%) Cetrimonium chloride (0.1%) Cetyl alcohol (100%) Cetyl palmitate (100%) Chlorhexidine digluconate (0.05%) Chlorophene (1, 0.3%) Diazolidinyl urea (30%) Diisopropyl adipate (100%) Dioctyl sodium sulfosuccinate (2, 0.5%) PEG-40 stearate (100%) Phytantriol (10, 3%) Propylene carbonate (100%) Sodium dehydroacetate (100%) Sodium lauroyl sarcosinate (5%) Sodium naphthalenesulfonate (2%) Sodium stearate (100%) Stearalkonium chloride (0.5%) Steareth-2 (60%) Steareth-20 (60%) Triacetin (100%) 33	Acetyl tributyl citrate (100%) Buthoxyethanol (5%) Butylene glycol (100, 10%) Carnauba wax (50%) Decyl oleate (100%) Diethylhexyl adipate (100%) Ethylhexyl palmitate (100%) Ethylhexyl stearate (100%) Glyceryl stearate (100%) Hexylene glycol (25%) Isocetyl stearate (100%) Isopropyl myristate (100%) Isopropyl palmitate (100%) Oleyl alcohol (100%) PEG-2 stearate (100%) Phenethyl alcohol (0.3%) Phenoxyethanol (2.2%) Phenyl methyl pyrazolone (0.66%) Propylene carbonate (17.5, 10.5%) Castor seed oil (100%) Safflower oil (100%) Sesame oil (100%) Sodium hexametaphosphate (0.2%) Sorbitan oleate (100%) Sorbitan sesquioleate (100, 30%) Sorbitan stearate (30%) Squalane (100%) Stearyl alcohol (100%) Triethylene glycol (100%) Zinc stearate (100%) 33

LDM-MTT assay was performed at the concentration at which a reported *in vivo* result was previously obtained. The concentrations of substance are shown in parenthesis, as substances are classified as true positive, true negative, false positive or false negative.

Table 64 Seventy-three substances

Category	Nos of Positive	Nos of Negative	Total
UV absorbers	0	4	4
Surfactants	8	11	19
Macromolecules	1	9	10
Oils	0	20	20
Humectants	1	6	7
Medicants	1	12	13
Total	11	62	73

Fig. 11 The relationship between LDM-MTT assay and Draize eye test results for cosmetic ingredients



The classification in the Draize eye test was based on MAS 15 as the cut-off point. That in the LDM-MTT assay was based on a viability of 50% as the cut-off point. The number of substances classified as true positive, true negative, false positive and false negative is shown in each area in the figure.

Table 65 Sixty substances

No.	Substance	CAS	GHS
1	1-Decanol	112-30-1	NI
2	2,4-Dichloro-5-sulfamoylbenzoic acid	2736-23-4	NI
3	2-Aminophenol	95-55-6	NI
4	2-Mercaptopyrimidine	1450-85-7	NI
5	2-methylpentane	107-83-5	NI
6	3,3-Dimethylpentane	562-49-2	NI
7	3-Methoxy-1,2-propanediol	623-39-2	NI
8	3-methylhexane	589-34-4	NI
9	Aluminum Hydroxide	21645-51-2	NI
10	Diisobutyl Ketone	108-83-8	NI
11	Ethyl acetate	141-78-6	NI
12	Ethyl trimethyl acetate	3938-95-2	NI
13	Ethylenediaminetetraacetic acid dipotassium salt dehydrate	25102-12-9	NI
14	Gluconolactone	90-80-2	NI
15	Glycerol	56-81-5	NI
16	Iminodibenzyl	494-19-9	NI
17	Iso-octyl acrylate	29590-42-9	NI
18	Methyl amyl ketone	110-43-0	NI
19	Methyl cyclopentane	96-37-7	NI
20	Methyl isobutyl ketone	108-10-1	NI
21	n,n-Dimethylguanidine sulfate	598-65-2	NI
22	n-Butyl acetate	123-86-4	NI
23	Phenothiazine	92-84-2	NI
24	Polyethylene glycol 400	25322-68-3	NI
25	Potassium tetrafluoroborate	14075-53-7	NI
26	Toluene	108-88-3	NI
27	Tween 20	9005-64-5	NI
28	Xylene	1330-20-7	NI
29	1-Octanol	111-87-5	2B
30	2, 6-dichlorobenzoyl chloride	4659-45-4	2A
31	2-Ethyl-1-hexanol	104-76-7	2A
32	2-Methyl-1-pentanol	105-30-6	2
33	Acetone	67-64-1	2A
34	Benzalkonium chloride (10%)	71-36-3	1
35	Butanol	79-92-5	2A
36	Camphen	111-87-5	2
37	Cetylpyridinium bromide (6%)	140-72-7	1
38	Chlorhexidine	55-56-1	1
39	Cyclohexanol	108-93-0	1
40	Dibenzoyl-L-tartaric acid (100%)	2743-38-6	1
41	Dibenzyl phosphate	1623-08-1	2A
42	Diethylethanolamine	100-37-8	1
43	Ethanol	64-17-5	2A
44	Ethyl-2-methylacetoacetate	609-14-3	2B
45	Isopropanol	67-63-0	2A
46	Lactic Acid 100% (liquid)	50-21-5	1
47	Maneb	12427-38-2	2
48	m-Dinitrobenzene	99-65-0	2
49	Methoxyethyl acrylate	3121-61-7	1
50	Methyl acetate	79-20-9	2A
51	Methyl cyanoacetate	105-34-0	2A
52	Methyl ethyl ketone (MEK)	78-93-3	2A
53	n-Hexanol	111-27-3	2
54	Promethazine Hydrochloride	58-33-3	1
55	Quinacrine	69-05-6	1
56	Sodium hydroxide (1%)	1310-73-2	2B
57	Sodium monochloroacetate	3926-62-3	2
58	Tetrahydrofuran	109-99-9	1
59	Tetraoctylammonium bromide	14866-33-2	1
60	Triton X-100 (5%)	9002-93-1	2A

Table 66 Prediction of eye irritancy at various concentrations in the LDM-MTT assay

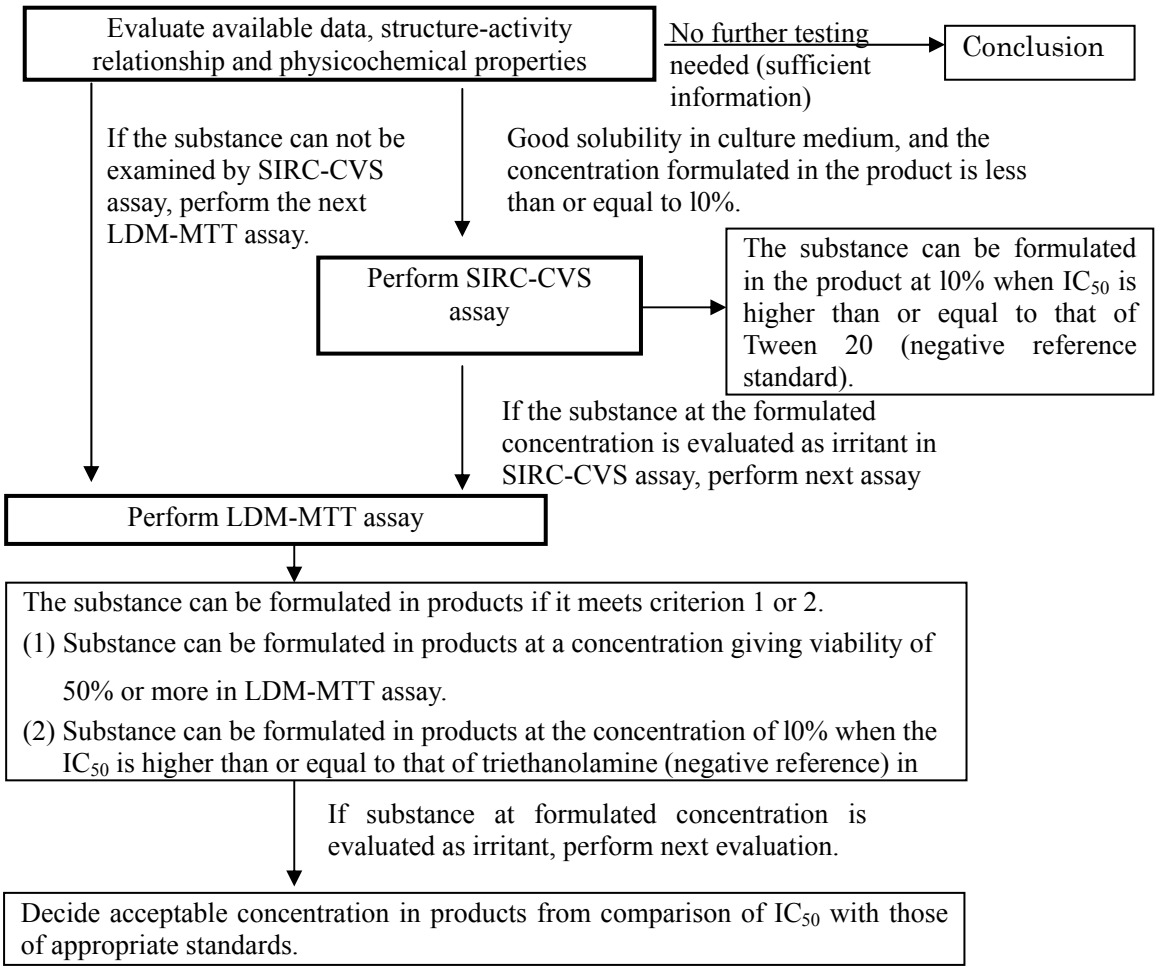
		In vitro (Classification by LDM-MTT assay using a viability of 50% as cut-off point)	
		Positive	Negative
In vivo (Estimated classification by GHS)	1, 2A or 2B	1-Octanol 2, 6-dichlorobenzoyl chloride 2-Ethyl-1-hexanol 2-Methyl-1-pentanol Acetone Benzalkonium chloride（10%) Butanol Camphen Cetylpyridinium bromide(6%) Chlorhexidine Cyclohexanol Dibenzoyl-L-tartaric acid Dibenzyl phosphate Diethylethanolamine Ethanol Ethyl-2-methylacetoacetate Isopropanol Lactic Acid Maneb m-Dinitrobenzene Methoxyethyl acrylate Methyl acetate Methyl cyanoacetate Methyl ethyl ketone n-Hexanol Promethazine Hydrochloride Quinacrine Sodium hydroxide(1%) Sodium monochloroacetate Tetrahydrofuran Tetraoctylammonium bromide Triton X-100(5%) 32	0
	NI	1-Decanol 2,4-Dichloro-5-sulfamoylbenzoic acid 2-Aminophenol 3,3-Dimethylpentane Diisobutyl Ketone Ethyl acetate Ethyl trimethyl acetate Ethylenediaminetetraacetic acid dipotassium salt dihydrate Gluconolactone Iminodibenzyl Iso-octyl acrylate Methyl amyl ketone Methyl cyclopentane Methyl isobutyl ketone n,n-Dimethylguanidine sulfate n-Butyl acetate Polyethylene glycol 400 Potassium tetrafluoroborate Toluene Tween 20 Xylene 21	2-Mercaptopyrimidine 2-methylpentane 3-Methoxy-1,2-propanediol 3-methylhexane Aluminum Hydroxide Glycerol Phenothiazine 7

Table 67 Prediction of eye irritancy at various concentrations in the LDM-MTT assay

		In vitro (Classification by LDM-MTT assay using a viability of 50% as cut-off point)	
		Positive	Negative
In vivo (Estimated classification by GHS)	1, 2A or 2B	Calcium thioglycolate (100, 10%) Lactic acid (100, 10%) Sodium lauryl sulfate (10%) Benzyl alcohol (100, 10%) Diisopropanolamine (10%) Monoethanolamine (10%) Acid red 92 (100, 10%) Glycolic acid (10%) Sodium hydrogenated tallow L-glutamate (10%) Chlorhexidine gluconate (20% solution) (10%) Butanol (10%) Potassium laurate (10%) Polyoxyethylene octylphenylether (10 E.O.) (10%) Di (2-ethylhexyl) sodium sulfosuccinate (10%) Acetic acid (10%) Cetyltrimethylammonium bromide (10%) Benzalkonium chloride (10%) Stearyltrimethylammonium chloride (10%) Cetylpyridinium chloride (10, 1%) Domiphen bromide (10%) Sucrose fatty acid ester (100%) Ethanol (100%) Sodium salicylate (100%) Distearyldimethylammonium chloride (100%) 29 2-Bromo-2-nitropropane-1,3-diol (100, 20, 10, 5%) Benzalkonium chloride (2, 1, 0.5%) Benzophenone-1 (100%) Benzophenone-2 (100%) Butoxyethanol (100, 15%) Cetrimonium chloride (2.5, 1.2, 0.5%) Chlorhexidine digluconate (20, 2%) Chlorophene (100, 3%) Chloroxylenol (100, 30%) Dioctyl sodium sulfosuccinate (10%) Hexylene glycol (100%) Lauramide DEA (20, 10%) Methoxyisopropyl acetate (100%) Phenethyl alcohol (100%) Phenethyl alcohol (15, 5%) Phenoxyethanol (100%) Phytantriol (100, 23%) Resorcinol (100%) Sodium naphthalenesulfonate (100%) Stearalkonium chloride (25, 4, 2.5%) TEA-Lauryl sulfate (20, 10, 5, 2.5, 1.25%) Triisopropanolamine (100%) 42 Cosmetic inredients 11 1-Octanol (100%) 2, 6-dichlorobenzoyl chloride (100%) 2-Ethyl-1-hexanol (100%) 2-Methyl-1-pentanol (100%) Acetone (100%) Benzalkonium chloride（10%) Butanol (100%) Camphen (100%) Cetylpyridinium bromide(6%) Chlorhexidine (100%) Cyclohexanol (100%) Dibenzoyl-L-tartaric acid (100%) Dibenzyl phosphate (100%) Diethylethanolamine (100%) Ethanol (100%) Ethyl-2-methylacetoacetate (100%) Isopropanol (100%) Lactic Acid (100%) Maneb (100%) m-Dinitrobenzene (100%) Methoxyethyl acrylate (100%) Methyl acetate (100%) Methyl cyanoacetate (100%) Methyl ethyl ketone (100%) n-Hexanol (100%) Promethazine Hydrochloride (100%) Quinacrine (100%) Sodium hydroxide(1%) Sodium monochloroacetate (100%) Tetrahydrofuran (100%) Tetraoctylammonium bromide (100%) Triton X-100(5%) 32 **114**	**0**
	NI	Polyoxyethylene sorbitan monooleate (20E.O.) (100, 10%) Sodium salicylate (10%) Triethanolamine (100, 10%) Polyoxyethylene sorbitan monolaurate (20 E.O.) (10%) Polyethyleneglycol monolaurate (10 E.O.) (10%) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) (10%) Sodium N-lauryl sarcosinate (30% solution) (10%) Sucrose fatty scid ester (10%) Methyl p-hydroxybenzoate (100%) Acid red 92 (1%) Cetylpyridinium chloride (0.1%) 13 2-Bromo-2-nitropropane-1,3-diol (2, 0.5%) Benzalkonium chloride (0.1, 0.01%) Benzethonium chloride (0.5%) Benzophenone-1 (16, 8, 4%) Benzophenone-2 (16, 8, 4%) Cetrimonium chloride (0.1%) Cetyl alcohol (100%) Cetyl palmitate (100%) Chlorhexidine digluconate (0.05%) Chlorophene (1, 0.3%) Diazolidinyl urea (30%) Diisopropyl adipate (100%) Dioctyl sodium sulfosuccinate (2, 0.5%) PEG-40 stearate (100%) Phytantriol (10, 3%) Propylene carbonate (100%) Sodium dehydroacetate (100%) Sodium lauroyl sarcosinate (5%) Sodium naphthalenesulfonate (2%) Sodium stearate (100%) Stearalkonium chloride (0.5%) Steareth-2 (60%) Steareth-20 (60%) Triacetin (100%) 33 Cosmetic ingredients 25 1-Decanol (100%) 2,4-Dichloro-5-sulfamoylbenzoic acid (100%) 2-Aminophenol (100%) 3,3-Dimethylpentane (100%) Diisobutyl Ketone (100%) Ethyl acetate (100%) Ethyl trimethyl acetate (100%) Ethylenediaminetetraacetic acid dipotassium salt dihydrate (100%) Gluconolactone (100%) Iminodibenzyl (100%) Iso-octyl acrylate (100%) Methyl amyl ketone (100%) Methyl cyclopentane (100%) Methyl isobutyl ketone (100%) n,n-Dimethylguanidine sulfate (100%) n-Butyl acetate (100%) Polyethylene glycol 400 (100%) Potassium tetrafluoroborate (100%) Toluene (100%) Tween 20 (100%) Xylene (100%) 21 **92**	Ethanol (10%) 2-Ethylhexyl p-dimethylamino benzonate (100, 10%) Glycerin (100, 10%) Polyethylene glycol 400 (10%) Polyoxyethylene hydrogenated caster oil (60 E.O.) (10%) Isopropyl myristate (100, 10%) Isotonic sodium chloride solution (100%) Silicic anhydride (100%) Benzyl alcohol (1%) 12 Acetyl tributyl citrate (100%) Buthoxyethanol (5%) Butylene glycol (100, 10%) Carnauba wax (50%) Decyl oleate (100%) Diethylhexyl adipate (100%) Ethylhexyl palmitate (100%) Ethylhexyl stearate (100%) Glyceryl stearate (100%) Hexylene glycol (25%) Isocetyl stearate (100%) Isopropyl myristate (100%) Isopropyl palmitate (100%) Oleyl alcohol (100%) PEG-2 stearate (100%) Phenethyl alcohol (0.3%) Phenoxyethanol (2.2%) Phenyl methyl pyrazolone (0.66%) Propylene carbonate (17.5, 10.5%) Castor seed oil (100%) Safflower oil (100%) Sesame oil (100%) Sodium hexametaphosphate (0.2%) Sorbitan oleate (100%) Sorbitan sesquioleate (100, 30%) Sorbitan stearate (30%) Squalane (100%) Stearyl alcohol (100%) Triethylene glycol (100%) Zinc stearate (100%) 33 Cosmetic ingredients 37 2-Mercaptopyrimidine 2-methylpentane 3-Methoxy-1,2-propanediol 3-methylhexane Aluminum Hydroxide Glycerol Phenothiazine 7 **89**

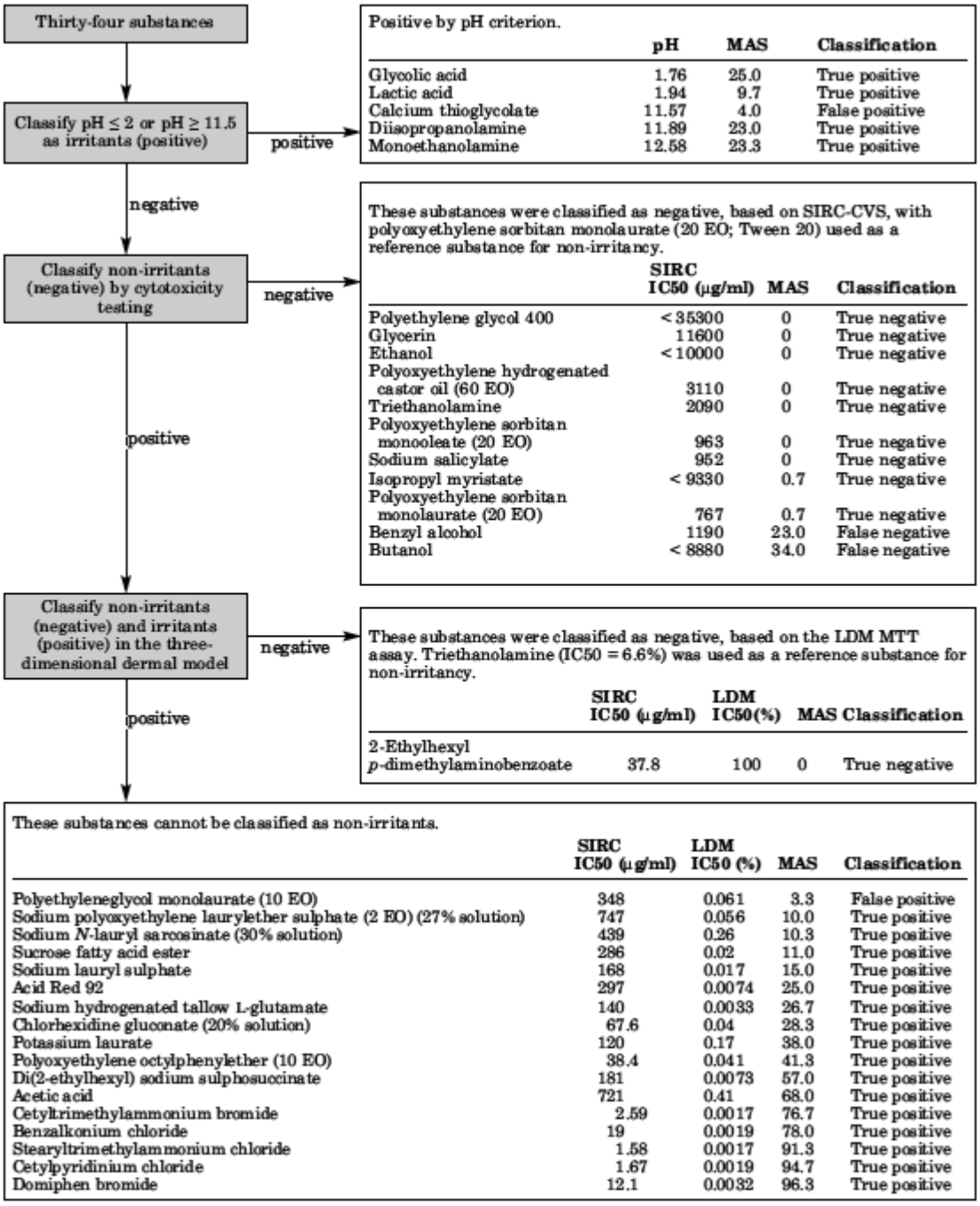
Thirty-two of 92 false positives were samples applied without dilution. Though these could not be negligible in the viewpoint of labelling of chemicals, the influence is relatively small for the evaluation of the cosmetic ingredients, that are virtually used with dilution.

Fig. 12 Schematic illustration of the tier evaluation using SIRC-CVS assay and LDM-MTT assay for the identification of non irritating ingredients.



The figure is the same as that reported by Hagino et al (2008).

Fig. 13 Verification of the tier evaluation method using monolayer cell culture and three-dimensional dermal model for the identification of non irritating ingredients.



Positive by pH criterion.

	pH	MAS	Classification
Glycolic acid	1.76	25.0	True positive
Lactic acid	1.94	9.7	True positive
Calcium thioglycolate	11.57	4.0	False positive
Diisopropanolamine	11.89	23.0	True positive
Monoethanolamine	12.58	23.3	True positive

These substances were classified as negative, based on SIRC-CVS, with polyoxyethylene sorbitan monolaurate (20 EO; Tween 20) used as a reference substance for non-irritancy.

	SIRC IC50 (μg/ml)	MAS	Classification
Polyethylene glycol 400	< 35300	0	True negative
Glycerin	11600	0	True negative
Ethanol	< 10000	0	True negative
Polyoxyethylene hydrogenated castor oil (60 EO)	3110	0	True negative
Triethanolamine	2090	0	True negative
Polyoxyethylene sorbitan monooleate (20 EO)	963	0	True negative
Sodium salicylate	952	0	True negative
Isopropyl myristate	< 9330	0.7	True negative
Polyoxyethylene sorbitan monolaurate (20 EO)	767	0.7	True negative
Benzyl alcohol	1190	23.0	False negative
Butanol	< 8880	34.0	False negative

These substances were classified as negative, based on the LDM MTT assay. Triethanolamine (IC50 = 6.6%) was used as a reference substance for non-irritancy.

	SIRC IC50 (μg/ml)	LDM IC50(%)	MAS	Classification
2-Ethylhexyl *p*-dimethylaminobenzoate	37.8	100	0	True negative

These substances cannot be classified as non-irritants.

	SIRC IC50 (μg/ml)	LDM IC50 (%)	MAS	Classification
Polyethyleneglycol monolaurate (10 EO)	348	0.061	3.3	False positive
Sodium polyoxyethylene laurylether sulphate (2 EO) (27% solution)	747	0.056	10.0	True positive
Sodium *N*-lauryl sarcosinate (30% solution)	439	0.26	10.3	True positive
Sucrose fatty acid ester	286	0.02	11.0	True positive
Sodium lauryl sulphate	168	0.017	15.0	True positive
Acid Red 92	297	0.0074	25.0	True positive
Sodium hydrogenated tallow L-glutamate	140	0.0033	26.7	True positive
Chlorhexidine gluconate (20% solution)	67.6	0.04	28.3	True positive
Potassium laurate	120	0.17	38.0	True positive
Polyoxyethylene octylphenylether (10 EO)	38.4	0.041	41.3	True positive
Di(2-ethylhexyl) sodium sulphosuccinate	181	0.0073	57.0	True positive
Acetic acid	721	0.41	68.0	True positive
Cetyltrimethylammonium bromide	2.59	0.0017	76.7	True positive
Benzalkonium chloride	19	0.0019	78.0	True positive
Stearyltrimethylammonium chloride	1.58	0.0017	91.3	True positive
Cetylpyridinium chloride	1.67	0.0019	94.7	True positive
Domiphen bromide	12.1	0.0032	96.3	True positive

The figure is the same as that reported by Hagino et al (2008). The data were taken from Ohno et al. (1999), Tani et al. (1999) and Ohuchi et al. (1999). Non irritants (= negative) was defined here as those having MAS of 5 or less in the Draize eye test. Eye irritancy (=MAS) of 10% solutions of the substances was predicted based on the IC_{50} in the two models after classification according to pH. The figure was the same as that reported by Hagino et al (2008).

Table 68 Predicted irritancy according to in vitro tier system consisting of SIRC-CVS assay and LDM-MTT assay
(Concentration: 10%, Negative reference: Tween 20 in the SIRC-CVS assay and Triethanolamine in the LDM-MTT assay)

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Sodium lauryl sulfate Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 14	Benzyl alcohol* Glycolic acid* Butanol* 3
	NI	Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester Diisopropanolamine 5	Ethanol* Glycerin* Polyethylene glycol 400* Polyoxyethylene hydrogenated caster oil (60 E.O.)* Polyoxyethylene sorbitan monooleate (20E.O.)* Sodium salicylate* Triethanolamine* Isopropyl myristate* Polyoxyethylene sorbitan monolaurate (20 E.O.)=Tween 20* Lactic acid* 2-Ethylhexyl p-dimethylamino benzonate Calcium thioglycolate 12

*: It was classified as negative by the SIRC-CVS assay.

Table 69 Predicted irritancy according to in vitro tier system consisting of SIRC-CVS assay and LDM-MTT assay
(Concentration: 10%, Negative reference: Tween 20 in the SIRC-CVS assay and Triethanolamine in the LDM-MTT assay)
-GHS classification by considering pH-

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Calcium thioglycolate Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 16	Lactic acid* Benzyl alcohol* Glycolic acid* Butanol* 4
	NI	Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester 4	Ethanol* Glycerin* Polyethylene glycol 400* Polyoxyethylene hydrogenated caster oil (60 E.O.)* Polyoxyethylene sorbitan monooleate (20E.O.)* Sodium salicylate* Triethanolamine* Isopropyl myristate* Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20* 2-Ethylhexyl p-dimethylamino benzonate 10

*: It was classified as negative by the SIRC-CVS assay.

Table 70 Predicted irritancy in LDM-MTT assay of 19 substances positive in SIRC-CVS assay and 11 with poor solubility in culture medium.
(Concentration: 10%, Negative reference: Tween 20 in the SIRC-CVS assay and Triethanolamine in the LDM-MTT assay)

		In vitro (Classification by LDM-MTT assay using triethanolamine as a reference substance for non-irritancy)		
		Positive	Negative	
In vivo (Classification by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive 1,2Aor2B in GHS	2-Bromo-2-Nitropropane-1,3-Diol Benzalkonium chloride Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulfate 9	0	
	Negative NI in GHS	Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20 7	Acetyl tributyl citrate Carnauba wax Cetyl alcohol Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate 14	Oleyl alcohol PEG-2 stearate Castor seed oil Sorbitan stearate Steareth-2 Stearyl alcohol Zinc stearate

*: It was classified as negative by the SIRC-CVS assay.

Table 71 Predicted irritancy according to in vitro tier system consisting of SIRC-CVS assay and LDM-MTT assay
(Concentration: 10%, Negative reference: Tween 20 in the SIRC-CVS assay and Triethanolamine in the LDM-MTT assay)

		In vitro (Classification by LDM-MTT assay using triethanolamine as a reference substance for non-irritancy)		
		Positive	Negative	
In vivo (Classification by Draize eye test at 10% concn) Corneal damage or MAS over 15 was classified as positive.	Positive 1,2Aor2B in GHS	2-Bromo-2-Nitropropane-1,3-Diol Benzalkonium chloride Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulfate 9	Phenethyl alcohol* 1	
	Negative NI in GHS	Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20 7	Butylene glycol* Diethylhexyl adipate* Diisopropyl adipate* Ethylhexyl palmitate* Hexylene glycol* Isocetyl stearate* Isopropyl myristate* Isopropyl palmitate* Propylene carbonate* Safflower oil* Sesame oil* Sodium dehydroacetate* Sorbitan oleate* Sorbitan sesquioleate* Squalane* Triacetin* Triethylene glycol* 31	Acetyl tributyl citrate Carnauba wax Cetyl alcohol Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate Oleyl alcohol PEG-2 stearate Castor seed oil Sorbitan stearate Steareth-2 Stearyl alcohol Zinc stearate

*: It was classified as negative by the SIRC-CVS assay.

Table 72 Predicted irritancy according to in vitro tier system consisting of SIRC-CVS assay and LDM-MTT assay
(Concentration: 10%, Negative reference: Tween 20 in the SIRC-CVS assay and Triethanolamine in the LDM-MTT assay)
-GHS classification by considering pH-

		In vitro (Classification by SIRC-CVS assay using Tween 20 as a reference substance for non-irritancy)	
		Positive	Negative
In vivo (Classification by GHS)	1, 2Aor 2B	Calcium thioglycolate Sodium lauryl sulfate Diisopropanolamine Monoethanolamine Acid red 92 Sodium hydrogenated tallow L-glutamate Chlorhexidine gluconate (20% solution) Potassium laurate Polyoxyethylene octylphenylether (10 E.O.) Di (2-ethylhexyl) sodium sulfosuccinate Acetic acid Cetyltrimethylammonium bromide Benzalkonium chloride Stearyltrimethylammonium chloride Cetylpyridinium chloride Domiphen bromide 2-Bromo-2-Nitropropane-1,3-Diol ~~Benzalkonium chloride~~ Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulfosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulfate 24	Lactic acid* Benzyl alcohol* Glycolic acid* Butanol* Phenethyl alcohol* 5
	NI	Polyethyleneglycol monolaurate (10 E.O.) Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27% solution) Sodium N-lauryl sarcosinate (30% solution) Sucrose fatty acid ester Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20 11	Ethanol* Glycerin* Polyethylene glycol 400* Polyoxyethylene hydrogenated caster oil (60 E.O.)* Polyoxyethylene sorbitan monooleate (20E.O.)* Sodium salicylate* Triethanolamine* Isopropyl myristate* Polyoxyethylene sorbitan monolaurate (20 E.O.) =Tween 20* 2-Ethylhexyl p-dimethylamino benzonate Butylene glycol* Diethylhexyl adipate* Diisopropyl adipate* Ethylhexyl palmitate* Hexylene glycol* Isocetyl stearate* ~~Isopropyl myristate*~~ Isopropyl palmitate* Propylene carbonate* Safflower oil* Sesame oil* Sodium dehydroacetate* Sorbitan oleate* Sorbitan sesquioleate* Squalane* Triacetin* Triethylene glycol* Acetyl tributyl citrate Carnauba wax Cetyl alcohol Cetyl palmitate Decyl oleate Ethylhexyl stearate Glyceryl stearate Oleyl alcohol PEG-2 stearate Castor seed oil Sorbitan stearate Steareth-2 Stearyl alcohol Zinc stearate 40

*: It was classified as negative by the SIRC-CVS assay.

Table 73 Abbriviations

CV	Coefficient of variation
CVS	Crystal violet staining
EC50	>> IC50 (EC50 is the same as IC50 here)
GHS	Globally harmonized system of classification and labelling of chemicals
IC50	Concentration that inhibits the viability of the cell to 50% of control
LDM	Living dermal model
MAS	Maximal average score
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NRU	Neutral red uptake
SD	Standard deviation
SIRC	Statens Seruminstitut Rubbit Cornea
SOP	Standard operating procedure

日本語の参考資料

表 1 Draize 眼粘膜刺激性試験から得られる情報と対応する代替法

ドレイズ試験からの情報	CAM	赤血球	皮膚モデル	SIRC	ヒト培養細胞	動物培養細胞	EYTEX
① 角膜混濁							
a 膜実質（コラーゲン）の変性	▲	×	○	×	×	×	○
b コラーゲンの膨潤（上皮・内皮の障害度に依存）	▲	×	○	×	×	×	○
c 上皮細胞の変性・剥離（細胞毒性による）	▲	▲	○	○	○	○	×
② 虹彩							
a 経角膜吸収と虹彩損傷性	×	×	×	×	×	×	×
b 対光反射	×	×	×	×	×	×	×
③ 結膜							
a 発赤（炎症性血管拡張）	○	×	▲	▲	▲	▲	×
b 浮腫（炎症性の浮腫）	▲	×	▲	▲	▲	▲	×
c 分泌物（涙液の過剰分泌・炎症性浸潤反応）	×	×	×	×	×	×	×
④ 経過観察からの情報							
a 修復性	▲	×	▲	▲	▲	▲	×
b 遅発性の有無	▲	▲	▲	▲	▲	▲	×
⑤ ドレイズの観察項目にない情報							
a 角膜潰瘍（角膜上皮の損傷・欠落）	×	×	×	×	×	×	×
b 角膜の凹凸（乾燥性・凹地形成）	×	×	×	×	×	×	×
c 洗浄による障害の軽減性	○	×	○	▲	▲	▲	×
d 痛みの評価（行動観察・瞬目回数・閉眼）	×	×	×	×	×	×	×
e 物理的刺激による障害の検出（不溶性物質）	×	×	×	×	×	×	×

注（バリデーション開始前の文献に基づく評価で，○：導入可能，▲：導入には検討の必要，×：導入不可能）

金子（1996）による表を引用

表 2 Draize 試験のスコアリング

Ⅰ 角膜	
A 不透明度:混濁の程度(もっとも混濁した領域を読み取る)	
不透明度なし	0
虹彩を明視できる程度の散在からび慢性の不透明化	1
虹彩の細部がわずかにぼやけて見える	2
虹彩の細部が観察できないが、瞳孔の大きさはかろうじて識別できる	3
虹彩が透視できない	4
B 角膜損傷域	
正常	0
0＜A＜1/4	1
1/4≦A＜1/2	2
1/2≦A＜3/4	3
3/4≦A	4
評点:A×B×5(最大値:80)	
Ⅱ 虹彩(A)	
正常	0
皺壁形成亢進、充血、腫脹、角膜周囲の充血(いずれか1つ、あるいは全て、若しくは組み合わせ)が見られるが、対光反射は認められる (緩除反応陽性)。	1
対光反射消失、出血、広範囲の破壊(いずれか1つ、あるいは全て)が見られる。	2
評点:A×5 (最大値:10)	
Ⅲ 結膜	
A 発赤(角膜及び虹彩を除く瞼、球結膜)	
正常	0
充血亢進	1
広範囲かつ深紅色となり、血管の識別困難	2
全域の深紅色化	3
B 結膜浮腫	
正常	0
腫脹亢進(瞬瞼を含む)	1
眼瞼の部分的外反を伴う腫脹	2
腫脹を伴う 1/2 程度の眼瞼閉鎖	3
腫脹を伴う 1/2 以上の眼瞼閉鎖	4
C 分泌物	
正常	0
常量以上の分泌物(正常な動物の内眥に見られる少量は含まない)	1
眼瞼及び眼瞼に接する被毛を湿潤	2
眼瞼及び眼の周囲を相当範囲湿潤	3
評点:(A＋B＋C)×2 (最大値:20)	

表 3 GHS（世界調和システム）による判定基準

GHS の区分	in vivo の試験（Draize 試験）結果による判定	既存の分類による判定
区分1	・少なくとも 1 匹の動物で角膜、虹彩、あるいは結膜に可逆的とは思われない障害を出現、あるいは処置後 21 日目でも障害が完全には回復しない場合。 ・3匹中2匹以上で処置後 24, 48, 72 時間目での評点の平均値が角膜混濁指標では3以上、虹彩指標では 1.5 より大きかった場合。	Severe あるいは Corrosive（非常に強い刺激性または腐食性 AOI 80 以上に相当）と分類された物質は区分1に分類（但し、非可逆的病変が観察されない場合は刺激性（区分2A）と判定）
区分 2A	・3 匹の動物を用いて実施した Draize 試験で2匹以上に処置後 24, 48, 72 時間目での評点の平均値が角膜混濁では1以上、虹彩炎では 1 以上、結膜発赤では2以上、結膜浮腫では2以上の場合。かつ、21日間の観察期間中に完全に回復する。	Moderate（強い刺激性　AOI 30−80 に相当）と分類された物質は区分2Aに分類
区分 2B	・3 匹の動物を用いて実施した Draize 試験で2匹以上に処置後 24, 48, 72 時間目での評点の平均値が角膜混濁では1以上、虹彩炎では 1 以上、結膜発赤では2以上、結膜浮腫では2以上の場合。かつ7日以内に回復する	Mild と分類された物質は区分2Bに分類

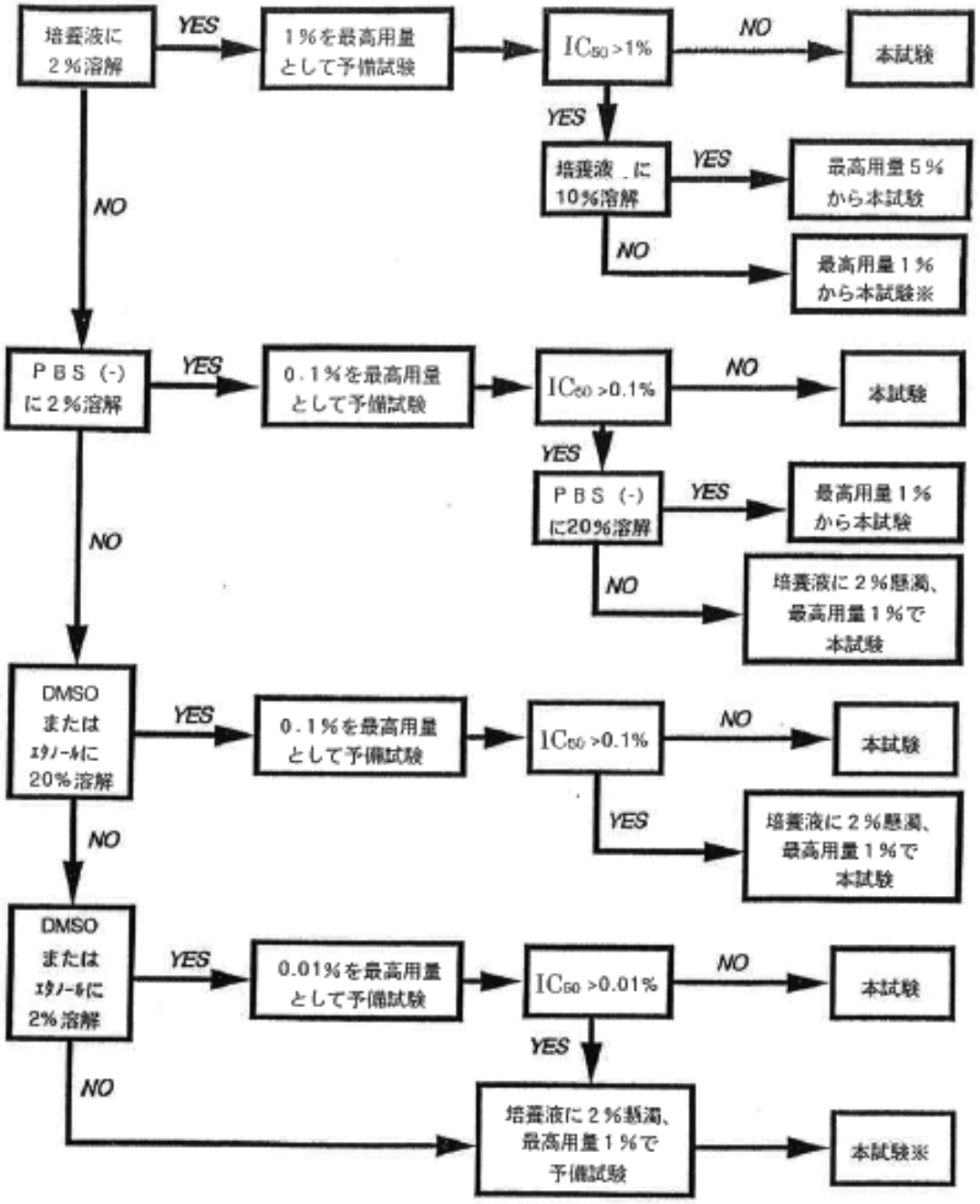
試験を行う前に、化学物質の眼に対する重篤な損傷性または眼刺激性を判定するのに、いくつかの要因を考慮するべきである。人および動物で蓄積された経験からは、眼に対する作用に直接関連する情報が得られるので、それが分析の第一段階に置かれるべきである。また、構造的に関連している化合物から有害性決定に十分な情報が得られる例もある。同様に、pH≦2 および≧11.5 など極端な pH は、特に有意な緩衝能力をともなっている場合は、眼に対する重篤な損傷作用があることを示唆している。そのような物質は眼に有意な作用を生じると予測される。皮膚腐食性物質について、局所的な作用である眼への試験を行うことを回避するために、眼に対する重篤な損傷性／刺激性を考えるに先立って、皮膚腐食性の可能性について評価しておかなければならない。有効性が確認され、承認されている in vitro 代替試験を用いて分類決定をおこなってもよい。

【出典：経済産業省 HP, http://www.meti.go.jp/policy/chemical_management/GHS/text/part3.3.htm , 2007 年 12 月 7 日アクセス. 】

表 4 化粧品・医薬部外品製造販売ガイドブック 2006 に掲載されている試験方法の例
－眼刺激性試験－

試験動物	原則として若齢成熟白色ウサギ
動物数	原則として1群3匹以上
用量	原則として 0.1mL（液体）又は 100mg（固体）
投与方法	片方の眼の下眼瞼を眼球より穏やかに引き離し，結膜嚢内に投与し，上下眼瞼を約 1 秒間穏やかに合わせる。他方の眼は未処置のまま残し，無処置対照眼とする。眼刺激性を示す物質は点眼後に洗眼を行う。
観察	原則として 1、 24、 48、72 及び 96 時間後に眼の観察を行う。持続性の角膜障害等が認められた場合には、その経過及び可逆性の有無について観察を続ける。

図 1 細胞毒性試験における被験物質の調製手順



小島(1999)による図を引用

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Reexamination of predictive capacity and applicability domain by using appropriate in vivo data

Evaluation of Draize eye test reference data was done by Barroso et al after the completion of this validation test. We examined predictive capacity and applicability domain except for chemical evaluated as "Should not be used" in single in vivo data. Table 1 shows one chemical excluded from analysis due to precipitation in in vitro test, one chemical excluded due to overlap and chemicals excluded due to inappropriate in vivo data. Twenty two chemicals were excluded from 120 chemicals and 98 chemicals were used for the analysis of predictive capacity.

Table 2 shows the predictive capacity of the SIRC-CVS: TEA test using in vitro and in vivo data of 98 chemicals. The SIRC-CVS: TEA test method demonstrated an accuracy of 50% (49/98), a sensitivity of 55% (27/49), and a specificity of 47% (22/47). There was little difference in predictive capacity before and after exclusion of chemicals with inappropriate in vivo data.

Further analysis was conducted to reduce false negatives by delimiting the applicability domain to certain chemical classes and properties of interest. Table 3 shows one chemical excluded from analysis due to precipitation in in vitro test, one chemical excluded due to overlap, chemicals excluded due to inappropriate in vivo data and chemicals excluded due to purity of less than 80%. Thirty three chemicals were excluded from 120 chemicals and 87 chemicals were used for analysis. Alcohols (The number of hydroxyl group≤2), esters, ethers, ketones, heterocyclic compounds, and carboxylic acid (containing salt) with a molecular weight of less than 180 as exclusion condition were used for the selection of the applicability domain in consideration of decreasing false negative, as shown in Table 4. Forty one out of 87 chemicals were excluded, and 46 chemicals were used for the analysis of predictive capacity. Table 5 shows the predictive capacity of the SIRC-CVS: TEA test using in vitro data and in vivo data of 46 chemicals. The SIRC-CVS:TEA test method demonstrated an accuracy of 57% (26/46), a sensitivity of 88% (14/16), and a specificity of 40% (12/30). False negative rate was improved to 12.5% (2/16). They suggest that the predictive capacity of the SIRC-CVS:TEA test can be improved by delimiting the applicability domain. Toluene was one of the two false negatives and was > Category 2B per TSCA in vivo data, but was classified no category, meaning “negative” per ECETOC in vivo data. Because 3,3-dithiodipropionic acid is a strong acid, it is evaluated as positive by prior information.

It was concluded that the SIRC-CVS:TEA test was useful alternative to the Draize eye test for distinguishing test chemicals that are ocular non irritants.

Table 1 Twenty two test chemicals excluded from the analysis of the predictive capacity

Code No	Chemical Name	Reason for exclusion from analysis
P2-002	2,5-Dimethylhexaediol	Inappropriate in vivo data
P2-016	1-Naphthaleneacetic acid	Inappropriate in vivo data
P3-026	Methylthioglycolate	Inappropriate in vivo data
P3-032	Disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	Inappropriate in vivo data
P3-039	1,2,4-Triazole,sodium salt	Inappropriate in vivo data
P3-041	Benzenamine,4,4'-(4-aimino-3-methylphenyl) (4-imino-3-methyl-2,5-cyclohexadien-1-ylidene) methyl-2-methy HCL	Inappropriate in vivo data
P3-047	2-Benzyloxyethanol	Inappropriate in vivo data
P3-051	Myristyl alcohol	Inappropriate in vivo data
P3-052	Hexyl cinnamic aldehyde	Inappropriate in vivo data
P3-054	Monoethanolamine	Inappropriate in vivo data
P3-058	Methoxyethyl acrylate	Inappropriate in vivo data
P3-065	2-Methylbutyric acid	Inappropriate in vivo data
P3-066	Calcium thioglycolate trihydrate	Inappropriate in vivo data (and in vitro data excluded by precipitation)
P3-067	Citric acid	Inappropriate in vivo data
P3-068	Potassium sorbate	Inappropriate in vivo data
P3-071	n-Lauroylsarcosine sodium salt	Inappropriate in vivo data
P3-072	Sodium lauryl sulfate	Inappropriate in vivo data
P3-090	Cetylpyridinium bromide	Inappropriate in vivo data
P3-093	Sodium hydroxide	Inappropriate in vivo data
P3-094	Glycolic acid	Inappropriate in vivo data
P3-095	3,3-Dithiodipropionic acid	Overlap (P3-095 was the same as P3-023)
P3-096	Sucrose fatty acid ester	Inappropriate in vivo data

Table 2 Predictive capacity of SIRC-CVS:TEA test

N=98	+ (SIRC-CVS)	− (SIRC-CVS)
+ (in vivo) GHS 1,2B 2A	27 P2-004 Ammonium nitrate P2-011 Sodium oxalate P2-015 Isobutyraldehyde P2-018 Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate P2-019 Camphene P3-016 3-Chloropropionitrile P3-019 Diethyl toluamide P3-021 Sodium chloroacetate P3-022 2,4,11,13-Tetraazatetra (Chlorohexidine glucocinate) P3-024 2-Amino-3-hydroxy pyridine P3-027 3-(2-Aminoethylamino)propyl]trimethoxysilane P3-028 Tetraethylene glycol P3-029 Dodecanoic acid P3-030 1,2-Benzisothiazol-3(2H)-one P3-031 2-Hydroxy-1,4-naphthoquinone P3-040 4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol] P3-045 (3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone P3-046 1-Octanol P3-049 Isobutyl alcohol P3-053 n-Butanal P3-055 m-Phenylenediamine P3-061 Imidazole P3-070 Distearyldimethylammonium chloride P3-073 Triton X-100 (5%) P3-075 Promethazine hydrochloride P3-076 2-Ethyl-1-hexanol P3-091 Triton X-100	24 P2-003 1-(2-Propoxy-1-methylethoxy)-2-propanol P2-009 Propylene glycol propyl ether P2-020 Cyclopentanol P3-017 2-Methyl-1-pentanol P3-018 Ethyl-2-methylacetoacetate P3-020 4-Nitrobenzoic acid P3-023 3,3-Dithiodipropionic acid P3-025 Sodium benzoate P3-033 Gamma-Butyrolactone P3-044 Isopropyl acetoacetate P3-048 Butanol P3-050 Isopropyl alcohol P3-059 Methyl acetate P3-060 Methyl cyanoacetate P3-062 Pyridine P3-069 Sodium salicylate P3-078 Cyclohexanol P3-079 Ethanol P3-080 n-Hexanol P3-083 Toluene P3-084 Acetone P3-087 Methyl ethyl ketone (2-butanone) P3-099 Benzyl alcohol P3-100 Lactic acid
− (in vivo) GHS NC	25 P2-001 Piperonylbutoxide P2-006 3,4,4'-Trichlorocarbanilide P2-007 1-Bromohexane P2-010 Ethyl thioglycolate P2-013 1-Bromo-4-chlorobutane P2-014 Sodium hydrogensulfite P2-017 Propyl 4-hydroxybenzoate P3-001 2-Ethoxyethyl methacrylate P3-003 Dipropyl disulfide P3-004 1-Bromo-octane P3-006 Dioctyl ether P3-007 3-Phenoxybenzyl alcohol P3-008 Glycidyl methacrylate P3-011 6-Hydroxy-2,4,5-triaminopyrimidine Sulfate P3-015 3,4-Dimethoxy benzaldehyde P3-035 4-(Methylmercapto)benzaldehyde P3-036 1,9-Decaine P3-042 1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol P3-043 3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien P3-074 2-Ethylhexyl p-dimethyl-amino benzoate P3-081 3,3-Dimethylpentane P3-082 Methyl cyclopentane P3-092 Tween20 P3-097 Methyl para-Hydroxybenzoate P3-098 Silic acid	22 P2-005 Potassium tetrafluoroborate P2-008 4,4'-Methylenebis(2,6-di-tert-butylphenol) P2-012 2-Phospho-L-ascorbic acid trisodium salt P3-002 Iso-octylthioglycolate P3-005 2-(2-Ethoxyethoxy)ethanol P3-009 2-Ethylhexylthioglycolate P3-010 n,n-Dimethylguanidine sulfate P3-012 Polyethylene hydrogenated caster oil (40E.O.) P3-013 2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl) -4-(1,1,3,3-tetramethylbutyl)phenol) P3-014 Cellulose 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride P3-034 1-Methylpropyl benzene P3-037 2,4-Dimethyl-3-pentanol P3-038 1-Ethyl-3-methylimidazolium ethylsulfate P3-056 Ethyl acetate P3-057 Isopropyl myristate P3-063 Isopropyl bromide P3-064 Cyclohexanone P3-077 3-Methoxy-1.2-propanediol P3-085 Gluconolactone P3-086 Methyl amyl ketone (2-heptanol) P3-088 Methyl isobutyl ketone(4-methyl 2-pentanol) P3-089 Glycerol

Table 3 Thirty three test chemicals excluded from the analysis of the predictive capacity and the applicability domain

Code	Chemical Name	Reason for exclusion from analysis
P2-002	2,5-Dimethylhexaediol	Inappropriate in vivo data
P2-014	Sodium hydrogensulfite	Purity<80%
P2-016	1-Naphthaleneacetic acid	Inappropriate in vivo data
P3-012	Polyethylene hydrogenated caster oil (40E.O.)	Purity<80%
P3-014	Cellulose 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride	Purity<80%
P3-022	2,4,11,13-Tetraazatetra (Chlorohexidine glucocinate)	Purity<80%
P3-026	Methylthioglycolate	Inappropriate in vivo data
P3-028	Tetraethylene glycol	Purity<80%
P3-032	Disodium 4,4'-bis(2-sulfonatostyryl)biphenyl	Inappropriate in vivo data
P3-039	1,2,4-Triazole,sodium salt	Inappropriate in vivo data
P3-041	Benzenamine,4,4'-(4-aimino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl-2-methy HCL	Inappropriate in vivo data
P3-047	2-Benzyloxyethanol	Inappropriate in vivo data
P3-051	Myristyl alcohol	Inappropriate in vivo data
P3-052	Hexyl cinnamic aldehyde	Inappropriate in vivo data
P3-054	Monoethanolamine	Inappropriate in vivo data
P3-058	Methoxyethyl acrylate	Inappropriate in vivo data
P3-063	Isopropyl bromide	Inappropriate in vivo data
P3-065	2-Methylbutyric acid	Inappropriate in vivo data
P3-066	Calcium thioglycolate trihydrate	Inappropriate in vivo data (and in vitro data excluded by precipitation)
P3-067	Citric acid	Inappropriate in vivo data
P3-068	Potassium sorbate	Inappropriate in vivo data
P3-070	Distearyldimethylammonium chloride	Inappropriate in vivo data
P3-071	n-Lauroylsarcosine sodium salt	Inappropriate in vivo data
P3-072	Sodium lauryl sulfate	Inappropriate in vivo data
P3-073	Triton X-100 (5%)	Purity<80%
P3-090	Cetylpyridinium bromide	Inappropriate in vivo data
P3-091	Triton X-100	Inappropriate in vivo data
P3-092	Tween20	Purity<80%
P3-093	Sodium hydroxide	Inappropriate in vivo data
P3-094	Glycolic acid	Inappropriate in vivo data
P3-095	3,3-Dithiodipropionic acid	Overlap (P3-095 was the same as P3-023)
P3-096	Sucrose fatty acid ester	Inappropriate in vivo data , Purity<80%
P3-098	Silic acid	Purity<80%

Table 4 Eighty seven test chemicals classified on the basis of applicability domain

Code	Chemical Name	Within(1)or outside(0) applicability domain
P2-001	Piperonylbutoxide	1
P2-004	Ammonium nitrate	1
P2-005	Potassium tetrafluoroborate	1
P2-006	3,4,4'-Trichlorocarbanilide	1
P2-007	1-Bromohexane	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	1
P2-013	1-Bromo-4-chlorobutane	1
P2-015	Isobutyraldehyde	1
P2-017	Propyl 4-hydroxybenzoate	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate	1
P2-019	Camphene	1
P3-002	Iso-octylthioglycolate	1
P3-003	Dipropyl disulfide	1
P3-004	1-Bromo-octane	1
P3-006	Dioctyl ether	1
P3-007	3-Phenoxybenzyl alcohol	1
P3-009	2-Ethylhexylthioglycolate	1
P3-010	n,n-Dimethylguanidine sulfate	1
P3-011	6-Hydroxy-2,4,5-triaminopyrimidine Sulfate	1
P3-013	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl) -4-（1,1,3,3-tetramethylbutyl)phenol)	1
P3-015	3,4-Dimethoxy benzaldehyde	1
P3-016	3-Chloropropionitrile	1
P3-019	Diethyl toluamide	1
P3-023	3,3-Dithiodipropionic acid	1
P3-027	3-(2-Aminoethylamino)propyl]trimethoxysilane	1
P3-029	Dodecanoic acid	1
P3-031	2-Hydroxy-1,4-naphthoquinone	1
P3-034	1-Methylpropyl benzene	1
P3-035	4-(Methylmercapto)benzaldehyde	1
P3-036	1,9-Decaine	1
P3-038	1-Ethyl-3-methylimidazolium ethylsulfate	1
P3-040	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1 -benzoxathiole-3,3-diyl)bis[2,6-dibromophenol]	1
P3-042	1-(9H-Carbozol-4-yloxy)-3 -[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	1
P3-043	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien	1
P3-045	(3R,4R)-4-Acetoxy-3-[(R) -(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	1
P3-053	n-Butanal	1
P3-055	m-Phenylenediamine	1
P3-057	Isopropyl myristate	1
P3-074	2-Ethylhexyl p-dimethyl-amino benzoate	1
P3-075	Promethazine hydrochloride	1
P3-081	3,3-Dimethylpentane	1
P3-082	Methyl cyclopentane	1
P3-083	Toluene	1
P3-085	Gluconolactone	1
P3-089	Glycerol	1
P2-003	1-(2-Propoxy-1-methylethoxy)-2-propanol	0
P2-009	Propylene glycol propyl ether	0
P2-010	Ethyl thioglycolate	0
P2-011	Sodium oxalate	0
P2-020	Cyclopentanol	0
P3-001	2-Ethoxyethyl methacrylate	0
P3-005	2-(2-Ethoxyethoxy)ethanol	0
P3-008	Glycidyl methacrylate	0

P3-017	2-Methyl-1-pentanol	0
P3-018	Ethyl-2-methylacetoacetate	0
P3-020	4-Nitrobenzoic acid	0
P3-021	Sodium chloroacetate	0
P3-024	2-Amino-3-hydroxy pyridine	0
P3-025	Sodium benzoate	0
P3-030	1,2-Benzisothiazol-3(2H)-one	0
P3-033	Gamma-Butyrolactone	0
P3-037	2,4-Dimethyl-3-pentanol	0
P3-044	Isopropyl acetoacetate	0
P3-046	1-Octanol	0
P3-048	Butanol	0
P3-049	Isobutyl alcohol	0
P3-050	Isopropyl alcohol	0
P3-056	Ethyl acetate	0
P3-059	Methyl acetate	0
P3-060	Methyl cyanoacetate	0
P3-061	Imidazole	0
P3-062	Pyridine	0
P3-064	Cyclohexanone	0
P3-069	Sodium salicylate	0
P3-076	2-Ethyl-1-hexanol	0
P3-077	3-Methoxy-1.2-propanediol	0
P3-078	Cyclohexanol	0
P3-079	Ethanol	0
P3-080	n-Hexanol	0
P3-084	Acetone	0
P3-086	Methyl amyl ketone (2-heptanol)	0
P3-087	Methyl ethyl ketone (2-butanone)	0
P3-088	Methyl isobutyl ketone(4-methyl 2-pentanol)	0
P3-097	Methyl para-Hydroxybenzoate	0
P3-099	Benzyl alcohol	0
P3-100	Lactic acid	0

Table 5 Predictive capacity of SIRC-CVS test except for test chemicals such as alcohols, esters, ethers, ketones, heterocyclic compounds, and carboxylic acids with a molecular weight of less than180

N=46	＋ (SIRC-CVS)	－ (SIRC-CVS)
＋ (in vivo) GHS 1,2B 2A	14 P2-004 Ammonium nitrate P2-015 Isobutyraldehyde P2-018 Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate P2-019 Camphene P3-016 3-Chloropropionitrile P3-019 Diethyl toluamide P3-027 3-(2-Aminoethylamino)propyl]trimethoxysilane P3-029 Dodecanoic acid P3-031 2-Hydroxy-1,4-naphthoquinone P3-040 4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol] P3-045 (3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone P3-053 n-Butanal P3-055 m-Phenylenediamine P3-075 Promethazine hydrochloride	2 P3-023 3,3-Dithiodipropionic acid P3-083 Toluene
－ (in vivo) GHS NC	18 P2-001 Piperonylbutoxide P2-006 3,4,4'-Trichlorocarbanilide P2-007 1-Bromohexane P2-013 1-Bromo-4-chlorobutane P2-017 Propyl 4-hydroxybenzoate P3-003 Dipropyl disulfide P3-004 1-Bromo-octane P3-006 Dioctyl ether P3-007 3-Phenoxybenzyl alcohol P3-011 6-Hydroxy-2,4,5-triaminopyrimidine Sulfate P3-015 3,4-Dimethoxy benzaldehyde P3-035 4-(Methylmercapto)benzaldehyde P3-036 1,9-Decaine P3-042 1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol P3-043 3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien P3-074 2-Ethylhexyl p-dimethyl-amino benzoate P3-081 3,3-Dimethylpentane P3-082 Methyl cyclopentane	12 P2-005 Potassium tetrafluoroborate P2-008 4,4'-Methylenebis(2,6-di-tert-butylphenol) P2-012 2-Phospho-L-ascorbic acid trisodium salt P3-002 Iso-octylthioglycolate P3-009 2-Ethylhexylthioglycolate P3-010 n,n-Dimethylguanidine sulfate P3-013 2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl) -4-(1,1,3,3-tetramethylbutyl)phenol) P3-034 1-Methylpropyl benzene P3-038 1-Ethyl-3-methylimidazolium ethylsulfate P3-057 Isopropyl myristate P3-085 Gluconolactone P3-089 Glycerol

References

Barroso, J. et al.(2016) Cosmetics Europe compilation of historical serious eye damage/eye irritation in vivo data analysed by drivers of classification to support the selection of chemicals for development and evaluation of alternative method

Physicochemical explanation of applicability domain

A study to establish a physiochemical explanation of the applicability domain resulted in the following criteria for exclusion that reduces false negatives to a similar level.

(1) pKa

・Chemicals with an acid dissociation constant (pKa) of 4 or less

・Organic salts consisting of weak acid and strong base (=Alkaline)

(The pKa of the weak acid is 3 or more, and the strong base is “sodium”, “potassium” and so on)

(2) Log P

・Chemicals with a distribution coefficient (log P) of greater than -1.5 and less than 2

#Basis of these criteria

Conditions of the SIRC-CVS test differ from in vivo. The test chemical is immersed in a buffer solution, which we think inhibits effects from hydrogen ions or hydroxide ions. The acid disassociation constant is a quantitative index of the strength of an acid in solution, and the smaller the pKa value, the stronger the acid. Chemicals with pKa of 4 or less should be excluded from applicability domain. Furthermore, organic salts consisting of weak acid and strong base (=Alkaline) may take false negative in the SIRC-CVS test on the basis of the above reason. Therefore, they should be excluded from applicability domain.

Examining the quantitative structure–activity relationship (QSAR) for the ocular irritation potential of 53 chemicals, Cronin et al focused on the partition coefficient (log P; equal to log Kow) and found that some amphiphilic chemicals are ocular irritants, as shown in Fig. 1. Conversely, this tendency was not found in non-irritants, as shown in Fig.2. A chemical with a low log P value will have excellent solubility in water but poor cellular membrane permeability. Conversely, a chemical with a high log P value will have both excellent lipid solubility and excellent membrane permeability. When conducting in vivo tests for ocular irritation, however, a layer of aqueous lacrimal fluid covering the cornea prevents the test chemical from coming in direct contact with the cornea. Chemicals with intermediate log P values are amphiphilic, capable of permeating both an aqueous layer of lacrimal fluid and lipid cellular membranes, and thereby affecting cells and cornea alike. But since amphiphilic ocular irritants (active ingredients) generally do not exhibit cytotoxicity at the level of concentration (0.5% or less) used in the SIRC-CVS test method, they yield false negative results.

#Reexamination of predictive capacity and applicability domain

In determining the applicability domain, we looked at 98 chemicals after the exclusion of 22 test chemicals (as shown in table 1 of appendix 8.14) from 120 chemicals tested in the validation study.

Ninty two chemicals were obtained after excluding 4 chemicals with an acid dissociation constant (pKa) of 4 or less (Table 1) and 2 organic salts consisting of weak acid and strong base (Table 2). Furthermore, 52 substances were obtained after excluding chemicals with a distribution coefficient (log P) of greater than -1.5 and less than 2 (Table 3). Table 4 shows the predictive capacity of SIRC-CVS: TEA test under this applicability domain. The SIRC-CVS:TEA test method demonstrated an accuracy of 58% (30/52), a sensitivity of 94% (15/16), and a specificity of 42% (15/36). False negative rate was improved to 6% (1/16). They suggest that the predictive capacity of the SIRC-CVS:TEA test can be improved by delimiting the applicability domain. Toluene was one of the two false negatives and was > Category 2B per TSCA in vivo data, but was classified No Category, meaning “negative,” per ECETOC in vivo data. Chemicals exhibiting false positive is considered that they has a possibility of having an effect on the eye. They are considered to be negative in vivo because they are discharged from the rabbit eye usually in about 5 minutes in vivo. Takahashi et al reported that rabbits excreted about 80% of applied materials from the conjunctival sac in 3-4 min. If they contact with the cornea sufficiently without being discharged in about 5 minutes from the eye, they may have an effect on the rabbit eye.

It was concluded that the SIRC-CVS:TEA test was useful alternative to the Draize eye test for distinguishing test chemicals that are ocular non irritants. A study to establish a physiochemical explanation of the applicability domain results in the better criteria of applicability domain.

References

1) Cronin, M.T.D, Basketter, D.A. and York, M.(1994) A quantitative structure-activity relationship (QSAR) investigation of a Draize eye irritation database, Toxicol. in Vitro, Vol.8, No.1, 21-28.

2) Takahashi, Y., Koike, M., Honda, H., Ito, Y., Sakaguchi, H. (2008) Development of the short time exposure (STE) test: An in vitro eye irritation test using SIRC cells, Toxicol. in Vitro, Vol.22, 760-770.

Fig. 1: Log P of some ocular irritants

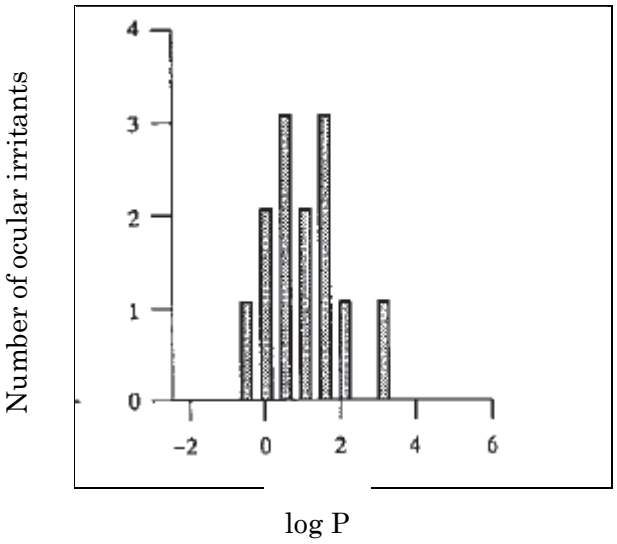

Fig 2: Log P of some ocular non-irritants

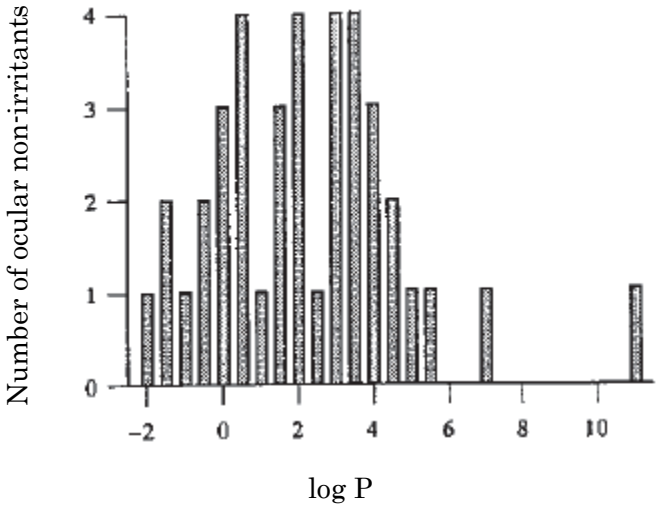

Table 1 Four test chemicals evaluated as eye irritants due to pKa value of 4 or less

Code No	Chemical Name	Substances with pKa value of 4 or less (Most Acidic Temp: 25°C by SciFinder)
P3-020	4-Nitrobenzoic acid	3.42 ±0.10
P3-023	3,3-Dithiodipropionic acid	3.94 ±0.10
P3-060	Methyl cyanoacetate	2.75 ±0.10
P3-100	Lactic acid	3.91 ±0.11

Excluding the above four chemicals, chemicals to be analyzed were decreased from 98 to 94.

Table 2 Two test chemicals evaluated as eye irritants due to organic salts containing strong base and weak acid with pKa of 3 or more

Code No	Chemical Name	pKa value (Most Acidic Temp: 25°C by SciFinder)
P3-025	Sodium benzoate	pKa of benzoic acid is 4.20±0.10
P3-069	Sodium salicylate	pKa of salicylic acid is 3.01±0.10

Excluding the above two chemicals, chemicals to be analyzed were decreased from 94 to 92.

Table 3 Ninety two test chemicals classified by log P

Code	Chemical Name	Log P (Log Kow KOWWIN v.1.68 estimate, EPI Suite)	Within(1)or outside(0) applicability domain
P2-001	Piperonylbutoxide	4.29	1
P2-004	Ammonium nitrate	-4.39	1
P2-005	Potassium tetrafluoroborate	-0.78	1
P2-006	3,4,4'-Trichlorocarbanilide	4.90	1
P2-007	1-Bromohexane	3.63	1
P2-008	4,4'-Methylenebis(2,6-di-tert-butylphenol)	8.99	1
P2-011	Sodium oxalate	-7.00	1
P2-012	2-Phospho-L-ascorbic acid trisodium salt	-9.96	1
P2-013	1-Bromo-4-chlorobutane	2.90	1
P2-014	Sodium hydrogensulfite	-7.51	1
P2-017	Propyl 4-hydroxybenzoate	2.98	1
P2-018	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridine propionate	2.01	1
P2-019	Camphene	4.35	1
P3-002	Iso-octylthioglycolate	3.68	1
P3-003	Dipropyl disulfide	3.84	1
P3-004	1-Bromo-octane	4.61	1
P3-005	2-(2-Ethoxyethoxy)ethanol	-0.69	1
P3-006	Dioctyl ether	6.94	1
P3-007	3-Phenoxybenzyl alcohol	3.13	1
P3-009	2-Ethylhexylthioglycolate	3.68	1
P3-011	6-Hydroxy-2,4,5-triaminopyrimidine Sulfate	-4.92	1
P3-012	Polyethylene hydrogenated caster oil (40E.O.)	17.71	1
P3-013	2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl) -4- (1,1,3,3-tetramethylbutyl)phenol)	12.46	1
P3-019	Diethyl toluamide	2.26	1
P3-021	Sodium chloroacetate	-3.47	1
P3-027	3-(2-Aminoethylamino)propyl]trimethoxy silane	-1.67	1
P3-029	Dodecanoic acid	5.00	1
P3-034	1-Methylpropyl benzene	3.94	1
P3-035	4-(Methylmercapto)benzaldehyde	2.31	1
P3-036	1,9-Decaine	4.98	1
P3-037	2,4-Dimethyl-3-pentanol	2.13	1
P3-040	4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromo phenol]	10.33	1
P3-042	1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol	3.05	1
P3-043	3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazape nta-1,4-dien	5.55	1
P3-046	1-Octanol	2.81	1
P3-057	Isopropyl myristate	7.17	1
P3-063	Isopropyl bromide	2.08	1
P3-070	Distearyldimethylammonium chloride	12.52	1
P3-073	Triton X-100 (5%)	4.86	1
P3-074	2-Ethylhexyl p-dimethyl-amino benzoate	5.77	1
P3-075	Promethazine hydrochloride	2.97	1

P3-076	2-Ethyl-1-hexanol	2.73	1
P3-077	3-Methoxy-1.2-propanediol	-1.15	1
P3-081	3,3-Dimethylpentane	3.67	1
P3-082	Methyl cyclopentane	3.10	1
P3-083	Toluene	2.54	1
P3-085	Gluconolactone	-1.98	1
P3-089	Glycerol	-1.65	1
P3-091	Triton X-100	4.86	1
P3-092	Tween20	-2.03	1
P3-097	Methyl para-Hydroxybenzoate	2.00	1
P3-098	Silic acid	-1.50	1
P2-003	1-(2-Propoxy-1-methylethoxy) -2-propanol	0.64	0
P2-009	Propylene glycol propyl ether	0.49	0
P2-010	Ethyl thioglycolate	0.81	0
P2-015	Isobutyraldehyde	0.74	0
P2-020	Cyclopentanol	1.15	0
P3-001	2-Ethoxyethyl methacrylate	1.49	0
P3-008	Glycidyl methacrylate	0.81	0
P3-010	n,n-Dimethylguanidine sulfate	No data	0
P3-014	Cellulose 2-(2-hydroxy-3-(trimethylammonio)propoxy) ethyl ether chloride	No data	0
P3-015	3,4-Dimethoxy benzaldehyde	1.36	0
P3-016	3-Chloropropionitrile	0.60	0
P3-017	2-Methyl-1-pentanol	1.75	0
P3-018	Ethyl-2-methylacetoacetate	0.21	0
P3-022	2,4,11,13-Tetraazatetra (Chlorohexidine glucocinate)	-0.33	0
P3-024	2-Amino-3-hydroxy pyridine	0.05	0
P3-028	Tetraethylene glycol	0.29	0
P3-030	1,2-Benzisothiazol-3(2H)-one	0.64	0
P3-031	2-Hydroxy-1,4-naphthoquinone	0.78	0
P3-033	Gamma-Butyrolactone	-0.31	0
P3-038	1-Ethyl-3-methylimidazolium ethylsulfate	No data	0
P3-044	Isopropyl acetoacetate	0.21	0
P3-045	(3R,4R)-4-Acetoxy-3-[(R)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone	No data	0
P3-048	Butanol	0.84	0
P3-049	Isobutyl alcohol	0.77	0
P3-050	Isopropyl alcohol	0.28	0
P3-053	n-Butanal	0.82	0
P3-055	m-Phenylenediamine	-0.39	0
P3-056	Ethyl acetate	0.86	0
P3-059	Methyl acetate	0.37	0
P3-061	Imidazole	0.06	0
P3-062	Pyridine	0.80	0
P3-064	Cyclohexanone	1.13	0
P3-078	Cyclohexanol	1.64	0
P3-079	Ethanol	-0.14	0
P3-080	n-Hexanol	1.82	0
P3-084	Acetone	-0.24	0
P3-086	Methyl amyl ketone (2-heptanol)	1.73	0

P3-087	Methyl ethyl ketone (2-butanone)	0.26	0
P3-088	Methyl isobutyl ketone (4-methyl 2-pentanol)	1.16	0
P3-099	Benzyl alcohol	1.08	0

Table 4 Predictive capacity of SIRC-CVS test

N=52	＋ (SIRC-CVS)	－ (SIRC-CVS)
＋ (in vivo) GHS 1,2B 2A	15 P2-004 Ammonium nitrate P2-011 Sodium oxalate P2-018 Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropionate P2-019 Camphene P3-019 Diethyl toluamide P3-021 Sodium chloroacetate P3-027 3-(2-Aminoethylamino)propyl]trimethoxysilane P3-029 Dodecanoic acid P3-040 4,4'-(4,5,6,7-Tetrabromo-1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis[2,6-dibromophenol] P3-046 1-Octanol P3-070 Distearyldimethylammonium chloride P3-073 Triton X-100 (5%) P3-075 Promethazine hydrochloride P3-076 2-Ethyl-1-hexanol P3-091 Triton X-100	1 P3-083 Toluene
－ (in vivo) GHS NC	21 P2-001 Piperonylbutoxide P2-006 3,4,4'-Trichlorocarbanilide P2-007 1-Bromohexane P2-013 1-Bromo-4-chlorobutane P2-014 Sodium hydrogensulfite P2-017 Propyl 4-hydroxybenzoate P3-003 Dipropyl disulfide P3-004 1-Bromo-octane P3-006 Dioctyl ether P3-007 3-Phenoxybenzyl alcohol P3-011 6-Hydroxy-2,4,5-triaminopyrimidine Sulfate P3-035 4-(Methylmercapto)benzaldehyde P3-036 1,9-Decaine P3-042 1-(9H-Carbozol-4-yloxy)-3-[[2-(2-methoxy phenoxy)ethyl] amino]-2-propanol P3-043 3-Methyl-1,5-di(2,4-xylyl)-1,3,5-Triazapenta-1,4-dien P3-074 2-Ethylhexyl p-dimethyl-amino benzoate P3-081 3,3-Dimethylpentane P3-082 Methyl cyclopentane P3-092 Tween20 P3-097 Methyl para-Hydroxybenzoate P3-098 Silic acid	15 P2-005 Potassium tetrafluoroborate P2-008 4,4'-Methylenebis(2,6-di-tert-butylphenol) P2-012 2-Phospho-L-ascorbic acid trisodium salt P3-002 Iso-octylthioglycolate P3-005 2-(2-Ethoxyethoxy)ethanol P3-009 2-Ethylhexylthioglycolate P3-012 Polyethylene hydrogenated caster oil (40E.O.) P3-013 2,2'-Methylene-bis-(6-(2Hbenzotriazol-2-yl) -4-(1,1,3,3-tetramethylbutyl)phenol) P3-034 1-Methylpropyl benzene P3-037 2,4-Dimethyl-3-pentanol P3-057 Isopropyl myristate P3-063 Isopropyl bromide P3-077 3-Methoxy-1.2-propanediol P3-085 Gluconolactone P3-089 Glycerol

Physicochemical explanation of applicability domain by using the additional data from Shiseido

A study to establish a physiochemical explanation of the applicability domain resulted in the following criteria for exclusion that reduces false negatives to a similar level. They were also obtained by using the additional data from Shiseido.

(1) pKa
・Chemicals with an acid dissociation constant (pKa) of 4 or less
・Organic salts consisting of weak acid and strong base (=Alkaline)
(The pKa of the weak acid is 3 or more, and the strong base is “sodium”, “potassium” and so on)
(2) Log P
・Chemicals with a distribution coefficient (log P) of greater than -1.5 and less than 2

The predictive capacity of SIRC-CVS:TEA test was analyzed by the additional data from Shiseido. Shiseido’s data were taken from the report used in the peer review by JaCVAM eye irritation test evaluating committee in 2009-2011, and their data sheets was checked during the peer review.
Table 1 shows whether or not 46 chemicals falls within the scope of applicability domain when classified by Log P.
Table 2 shows the predicative capacity when examined with 46 chemical substances before being classified by Log P. The SIRC-CVS:TEA test method demonstrated an accuracy of 63% (29/46), a sensitivity of 81% (17/21), and a specificity of 48% (12/25). False negative rate was 19% (4/21).
Table 3 shows the predictive capacity of SIRC-CVS: TEA test under this applicability domain classified by Log P. The SIRC-CVS:TEA test method demonstrated an accuracy of 65% (20/31), a sensitivity of 100% (11/11), and a specificity of 45% (9/20). False negative rate was 0% (0/11). They suggest that the predictive capacity of the SIRC-CVS:TEA test can be improved by delimiting the applicability domain.
It was concluded that the SIRC-CVS:TEA test was useful alternative to the Draize eye test for distinguishing test chemicals that are ocular non irritants. A study to establish a physiochemical explanation of the applicability domain results in the better criteria of applicability domain.

Table 1

Code	Chemical name	CAS	Log P (Log Kow KOWWIN v.1.68 estimate, EPI Suite)	Applicable (1) or unapplicable (0)	in vitro	in vivo
1	Butylene glycol	107-88-0	-0.29	0	N	N
2	Propylene carbonate	108-32-7	0.08	0	N	N
3	2,4-Pentanediol	625-69-4	0.13	0	N	N
4	Resorcinol	108-46-3	1.03	0	P	P
5	Butoxyethanol	111-76-2	0.57	0	N	P
6	Hexylene glycol	107-41-5	0.58	0	N	P
7	Phenethyl alcohol	1960/12/8	1.57	0	P	P
8	Methoxyisopropyl acetate	108-65-6	0.52	0	N	P
9	6-Methyl purine	2004/3/7	-0.27	0	P	P
10	Phenoxyethanol	122-99-6	1.10	0	N	P
11	Di-iso-butyl ketone	108-83-8	2.56	1	N	N
12	Triethylene glycol	112-27-6	-1.75	1	N	N
13	Chloroxylenol	88-04-0	3.25	1	P	P
14	2,4-Difluoronitrobenzene	446-35-5	2.21	1	P	N
15	iso-Octyl acrylate	29590-42-9	4.09	1	P	N
16	Sodium dehydroacetate	4418-26-2	-0.32	0	P	N
17	Triisopropanolamine	122-20-3	-1.22	0	P	P
18	2-Bromo-2-Nitropropane-1,3-Diol	52-51-7	-0.64	0	P	P
19	2-(n-Dodecylthio)ethanol	1462-55-1	5.35	1	P	N
20	Benzophenone-1	131-56-6	2.96	1	P	P
21	Triacetin	102-76-1	0.36	0	P	N
22	Chlorophene	120-32-1	4.18	1	P	P
23	Sodium naphthalenesulfonate	532-02-5	-1.78	1	P	P
24	Diisopropyl adipate	6938-94-9	3.20	1	P	N
25	tetra-Aminopyrimidine sulfate	5392-28-9	-5.37	1	P	N
26	Cetyl alcohol	36653-82-4	6.73	1	P	N
27	Benzophenone-2	131-55-5	2.78	1	P	P
28	Oleyl alcohol	143-28-2	7.50	1	P	N
29	Benzalkonium chloride	8001-54-5	2.93	1	P	P
30	Lauramide DEA	120-40-1	2.89	1	P	P
31	Isopropyl Palmitate	142-91-6	8.16	1	N	N
32	Sodium stearate	822-16-2	4.13	1	P	N
33	Cetrimonium chloride	112-02-7	3.18	1	P	P
34	Phytantriol	74563-64-7	6.36	1	P	P
35	Ethylhexyl palmitate	29806-73-3	10.61	1	N	N
36	Diethylhexyl adipate	103-23-1	8.12	1	N	N
37	TEA-Lauryl sulfate 40% solution	139-96-8	0.55	0	P	P
38	Squalane	111-01-3	14.63	1	N	N
39	Stearalkonium chloride	122-19-0	5.87	1	P	P
40	Sorbitan oleate	1338-43-8	5.89	1	P	N
41	Dioctyl sodium sulfosuccinate	577-11-7	3.95	1	P	P
42	Isocetyl stearate	25339-09-7	15.52	1	N	N
43	PEG-40 stearate	9004-99-3	6.16	1	P	N
44	Safflower (Carthamus tinctorius) oil	8001-23-8	22.65	1	N	N
45	Sesame (Sesamum indicum) oil	8008-74-0	22.80	1	N	N
46	Sorbitan sesquioleate	8007-43-0	13.83	1	P	N

P: 1,2B or 2A in GHS

N: NC in GHS

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Table 2 Predictive capacity of SIRC-CVS:TEA test evaluated in Shiseido's additional data

N=46	＋ (SIRC-CVS)	－ (SIRC-CVS)
＋ (in vivo) GHS 1,2B 2A	17 Resorcinol Phenethyl alcohol 6-Methyl purine Chloroxylenol Triisopropanolamine 2-Bromo-2-Nitropropane-1,3-Diol Benzophenone-1 Chlorophene Sodium naphthalenesulfonate Benzophenone-2 Benzalkonium chloride Lauramide DEA Cetrimonium chloride Phytantriol TEA-Lauryl sulfate 40% solution Stearalkonium chloride Dioctyl sodium sulfosuccinate	4 Butoxyethanol Hexylene glycol Methoxyisopropyl acetate Phenoxyethanol
－ (in vivo) GHS NC	13 2,4-Difluoronitrobenzene iso-Octyl acrylate Sodium dehydroacetate 2-(n-Dodecylthio)ethanol Triacetin Diisopropyl adipate tetra-Aminopyrimidine sulfate Cetyl alcohol Oleyl alcohol Sodium stearate Sorbitan oleate PEG-40 stearate Sorbitan sesquioleate	12 Butylene glycol Propylene carbonate 2,4-Pentanediol Di-iso-butyl ketone Triethylene glycol Isopropyl Palmitate Ethylhexyl palmitate Diethylhexyl adipate Squalane Isocetyl stearate Safflower (Carthamus tinctorius) oil Sesame (Sesamum indicum) oil

Table 3 Predictive capacity of SIRC-CVS:TEA test in the applicability domain classified by log P using Shiseido's additional data

N=31	＋ (SIRC-CVS)	－ (SIRC-CVS)
＋ (in vivo) GHS 1,2B 2A	11 Chloroxylenol Benzophenone-1 Chlorophene Sodium naphthalenesulfonate Benzophenone-2 Benzalkonium chloride Lauramide DEA Cetrimonium chloride Phytantriol Stearalkonium chloride Dioctyl sodium sulfosuccinate	0
－ (in vivo) GHS NC	11 2,4-Difluoronitrobenzene iso-Octyl acrylate 2-(n-Dodecylthio)ethanol Diisopropyl adipate tetra-Aminopyrimidine sulfate Cetyl alcohol Oleyl alcohol Sodium stearate Sorbitan oleate PEG-40 stearate Sorbitan sesquioleateChloroxylenol	9 Di-iso-butyl ketone Triethylene glycol Isopropyl Palmitate Ethylhexyl palmitate Diethylhexyl adipate Squalane Isocetyl stearate Safflower (Carthamus tinctorius) oil Sesame (Sesamum indicum) oil