新規試験法提案書

# 眼刺激性試験代替法
# *In vitro* 短時間曝露法（STE 法）

平成28年 3 月

国立医薬品食品衛生研究所

# 新規試験法提案書

平成 28 年 3 月 10 日
No.　2015-03

**眼刺激性試験代替法 *In vitro* 短時間曝露法 (STE 法)**
**に関する提案**

平成 28 年 3 月 3 日に東京、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）において以下の提案がなされた。

**提案内容：** *In vitro* 短時間曝露法（The Short Time Exposure *In Vitro* Test Method：STE 法）は、化学物質による眼刺激性を評価でき、トップダウン方式において重篤な眼の傷害を起こす物質 (United Nations Globally Harmonized System of Classification and Labeling of Chemicals(UN GHS) 区分 1）物質を検出する方法、ならびにボトムアップ方式において眼刺激性物質とは分類されない物質（UN GHS 区分外）を検出する方法として、行政的利用が可能である。

この提案書は、日本動物実験代替法学会 (JSAAE)とJaCVAMのバリデーション報告書、Interagency Coordinating Committee on the Validation of Alternative Methods(ICCVAM)/ National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) による第三者評価報告書および Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG)491をもとに、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、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として眼刺激性試験代替法 STE 法の使用を提案するものである。

大野泰雄

JaCVAM 評価会議　議長

西川秋佳

JaCVAM 運営委員会　委員長

JaCVAM 評価会議

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| 吉田武美 | （日本毒性学会） |
| 吉村　功 | （座長推薦） |

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日

JaCVAM 運営委員会

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| 中村高敏 | （独立行政法人　医薬品医療機器総合機構） |
| 日田　充 | （厚生労働省　医薬・生活衛生局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） |
| 本間正充 | （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　変異遺伝部） |
| 美上憲一 | （厚生労働省　医薬・生活衛生局化学物質安全対策室） |
| 三澤　馨 | （厚生労働省　医薬・生活衛生局審査管理課） |
| 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 Short Time Exposure *In Vitro* Test Method for assessing ocular irritation

At a meeting held on 3 March 2016 at the National Institute of Health Sciences (NIHS) in Tokyo, Japan, the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

**Proposal:** The Short Time Exposure (STE) *In Vitro* Test Method is a suitable means for assessing ocular irritation potency in a regulatory context as part of either a top-down approach to screening test chemicals that potentially induce serious eye damage (Category 1) or a bottom-up approach to screening test chemicals that potentially induce neither eye irritation nor serious eye damage and therefore do not require classification (No Category) under the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS).

This statement was prepared following a review of a validation report prepared by the Japanese Society for Alternatives to Animal Experiments (JSAAE) and JaCVAM, a peer review report prepared by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 491 "Short Time Exposure *In Vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage," as well as other materials prepared by the Ocular Irritation Testing JaCVAM Editorial Committee to acknowledge that the results of a review and study by the JaCVAM Regulatory Acceptance Board have confirmed the usefulness of this assay.
Based on the above, we propose the Short Time Exposure *In Vitro* Test Method as a useful means for assessing ocular irritation potency during safety assessments by regulatory agencies.

Yasuo Ohno
Chairperson
JaCVAM Regulatory Acceptance Board

Akiyoshi Nishikawa
Chairperson
JaCVAM Steering Committee

10 March 2016

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. Yasuo Ohno (nominee by JaCVAM Steering Committee) : Chairperson
Mr. Naofumi Iizuka (Pharmaceuticals and Medical Devices Agency)
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)
Mr. Yuji Ishii (Biological Safety Research Center: BSRC, NIHS)
Ms. Yumiko Iwase (Japan Pharmaceutical Manufacturers Association)
Mr. Kazuhiro Kaneko (Japan Chemical Industry Association)
Mr. Eiji Maki (Japanese Society of Immunotoxicology)
Mr. Takeshi Morita (Japanese Environmental Mutagen Society)
Mr. Akiyoshi Nishikawa (BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)
Ms. Koko Tanigawa (Japanese Society for Alternatives to Animal Experiments)
Mr. Takashi Yamada (National Institute of Technology and Evaluation)
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)
Mr. Takemi Yoshida (Japanese Society of Toxicology)
Mr. Isao Yoshimura (nominee by Chairperson)

Term: From 1st April 2014 to 31st March 2016

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

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson
Mr. Toru Kawanishi (NIHS)
Mr. Mitsuru Hida (Ministry of Health, Labour and Welfare)
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Atsushi Kato (National Institute of Infectious Diseases)
Mr. Kenichi Mikami (Ministry of Health, Labour and Welfare)
Mr. Kaoru Misawa (Ministry of Health, Labour and Welfare)
Mr. Takatoshi Nakamura (Pharmaceutical & Medical Devices Agency)
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)
Mr. Kazutoshi Shinoda (Pharmaceuticals and Medical Devices Agency)
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)
Mr. Masaaki Tsukano (Ministry of Health, Labour and Welfare)
Mr. Hajime Kojima (Division of Risk Assessment, BSRC, NIHS): Secretary

# 眼刺激性試験代替法 *In vitro* 短時間曝露法（STE 法）

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# 眼刺激性試験代替法の評価会議報告書

*In vitro* 短時間曝露法
(The Short Time Exposure *In Vitro* Test Method: STE 法)

JaCVAM 評価会議

平成 27 年（2015 年）12 月　18 日



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JaCVAM 評価会議

大野泰雄　（運営委員会推薦）：座長

飯塚尚文　（独立行政法人　医薬品医療機器総合機構）

五十嵐良明　（国立医薬品食品衛生研究所　生活衛生化学部）

石井雄二　（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　病理部）

岩瀬裕美子　（日本製薬工業協会）

金子和弘　（日本化学工業協会）

篠田和俊　（独立行政法人　医薬品医療機器総合機構）

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横関博雄　（日本皮膚アレルギー・接触皮膚炎学会）

吉田武美　（日本毒性学会）

吉村　功　（座長推薦）

任期：平成 26 年 4 月 1 日～平成 28 年 3 月 31 日



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*In vitro* 短時間曝露法（The Short Time Exposure *In Vitro* Test Method：STE 法）は、ウサギを用いた Draize 眼刺激性試験法の代替試験法であり、ウサギ角膜由来の上皮細胞株（SIRC 細胞）に対する化学物質の細胞毒性を指標に用いて眼刺激性を評価する試験法である。トップダウン方式において重篤な眼の損傷を起こす物質（United Nations Globally Harmonized System of Classification and Labeling of Chemicals: UN GHS 区分 1）を検出する方法、およびボトムアップ方式において眼刺激性物質とは分類されない物質（UN GHS 区分外）を検出する方法[1)] としてバリデーションが行われ、2015 年に OECD TG491 として採択された[2)]。JaCVAM 評価会議は、この TG491 の妥当性について検討した。

１．試験法の定義

名称： *In vitro* 短時間曝露法（The Short Time Exposure *In Vitro* Test Method：STE 法）

代替する対象毒性試験： Draize 眼刺激性試験

試験法の概略：

コンフルエントになるように単層培養した SIRC 細胞に被験物質希釈液を 5 分間曝露した後、細胞を洗浄し、その後、MTT（3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide）の取り込み量で測定した細胞生存率を指標に用いる。これは MTT がミトコンドリア内の脱水素酵素の基質となる性質を利用し、細胞内に取り込まれた MTT が還元され、生成するホルマザン量が生存細胞数に比例することを利用している（MTT 還元法）。

２．評価に用いた資料および評価内容の科学的妥当性

眼に異物が入った場合、眼の刺激性は角膜や結膜の細胞傷害から始まる。本試験法はこれに注目して、角膜上皮細胞に対する細胞毒性を指標として眼刺激性を評価する試験法である。眼に溶液が入った場合、ヒトではその大部分が 1～2 分で眼内から排出され、ウサギでは 3～4 分で 80%が排出されると報告されている[3)]。このような実際の曝露状況を考慮して、本試験法は通常の細胞毒性試験と比べて短時間曝露の試験として設計されている。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。

本試験法は日本動物実験代替法学会[4)] と JaCVAM[5)] のバリデーション試験を経て、トップダウン方式において UN GHS 区分 1 物質を検出する方法およびボトムアップ方式において UN GHS 区分外物質を検出する方法として ICCVAM(Interagency Coordinating Committee on the Validation of Alternative Methods)/NICEATM(National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods)により第三者評価を受け、その報告書が 2013 年 6 月に公表された[6)]。その後、2015 年 7 月に OECD によりトップダウン・ボトムアップ両方式で利用できる眼刺激性試験代替法として TG491 が採択された[2)]。



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ICCVAM/NICEATM によるトップダウン方式の正確性の検討には 125 物質が、ボトムアップ方式の正確性の検討には 130 物質が用いられ、十分な数が ICCVAM/NICEATM により第三者評価委員会により評価されている。JaCVAM 眼刺激性試験資料編纂委員会は、それらの資料を用いて本試験法を評価しており、妥当である。

３．本試験法の有用性と適用限界

本試験法のバリデーションの結果、トップダウン方式の偽陽性率は 1.2%、ボトムアップ方式の偽陰性率は、適用限界を考慮しない場合 12.3% 考慮する場合 1.9%という優れた値である。

本試験法は、短時間で安価に実施でき、使用細胞は公的な細胞バンクから入手可能であり、特殊な機材や試薬を必要としないことから、基本的な細胞培養の技術と設備を持っている施設であれば実施可能であり、技術移転性は高い。2つのプレバリデーション試験[7,8] においても技術移転性についての問題は指摘されていないが、OECDのガイドラインに示された熟達度確認物質[2, 9]を用いて、実施する試験施設へ適切な技術移転が達成できたことを確かめる必要がある。

本試験法には試験の性質と正確性の観点から以下の制限が設けられている。

1) 生理食塩水、5% (w/w) DMSOを含む生理食塩水、およびミネラルオイルのいずれにも溶解しない、あるいはそれら溶媒中で均一分散している状態が5分以上保たれない物質は適用不可能である。
2) ボトムアップ方式で単一物質に適用する場合、蒸気圧が6 kPaを超える物質および界面活性剤ではない固体は、高い偽陰性率を示すことから、適用可能物質から除外される。固体混合物に適用する場合は、界面活性剤のみから構成される物質は対象にするが、それ以外の物質は除外する。また、蒸気圧が6 kPaを超える物質を含む混合物に適用する場合は、眼刺激性が過小評価される可能性があるので注意する必要がある。
3) トップダウン方式で単一物質に適用することには制限がなく、バリデーションデータは限られてはいるものの、混合物質への適用にも制限がない。

以上の点から、TG491 に準拠して実施した場合、トップダウン方式において重篤な眼の損傷を起こす（すなわち、UN GHS 区分 1）物質を検出する方法、およびボトムアップ方式において眼刺激性物質とは分類されない（すなわち、UN GHS 区分外）物質を検出する方法として有用である。

４．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：

本試験法はウサギ角膜由来の上皮細胞株（SIRC 細胞）に対する化学物質の細胞毒性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、短時間で安価に実施でき、使用細胞の入手は容易であり、特殊な機材や試薬を必要としないことか

ら、基本的な細胞培養の技術と設備を持っている施設であれば実施可能であり、技術移転性は高い。以上より、本試験法は社会的受け入れ性が高い。

行政上の利用性：

本試験法は、化学物質による眼刺激性を評価でき、トップダウン方式において重篤な眼の傷害を起こす物質（UN GHS 区分 1）物質を検出する方法、ならびにボトムアップ方式において眼刺激性物質とは分類されない物質（UN GHS 区分外）を検出する方法として、行政的利用が可能である。

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# 眼刺激性試験代替法の評価報告書

## *In vitro* 短時間曝露法
## （Short Time Exposure *In Vitro* Test Method: STE 法）

眼刺激性代替法資料編纂委員会

2015 年 9 月 29 日

眼刺激性代替法資料編纂委員会

竹内　小苗　　（P&G イノベーション合同会社）
小坂　忠司　　（一般財団法人　残留農薬研究所）
佐々木　正治　（アッヴィ合同会社）
森村　智美　　（一般財団法人　食品薬品安全センター）
吉村　功　　（東京理科大学名誉教授）

略語

AD: Applicable Domain

BCOP: Bovine Corneal Opacity and Permeability

CAS: Chemical Abstracts Services

CM: Cytosensor Microphysiometer

DMSO: Dimethyl Sulfoxide

ETDA: Ethylenediaminetetraacetic Acid

FL: Fluorescein Leakage

GHS: Globally Harmonized System of Classification and Labeling of Chemicals

ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods

ICE: Isolated Chicken Eye

JaCVAM: Japanese Center for the Validation of Alternative Methods

kPa: kiloPascals

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NICEATM: National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods

OD: Optical Density

OECD: Organization for Economic Co-operation and Development

SD: Standard Deviation

SRD: Summary Review Document

SIRC: Statens Seruminstitut Rabbit Cornea

SLS: Sodium Lauryl Sulfate

STE: Short Time Exposure *In Vitro* Test

TG: Test Guideline

UN: United Nations

要旨

*in vitro* 短時間曝露法（Short Time Exposure *In Vitro* Test Method：STE 法）は、ウサギ由来の角膜上皮細胞に対する化学物質の細胞毒性を指標に用いて眼刺激性を評価する試験法である。トップダウン方式において UN GHS 区分 1 物質を検出する方法、およびボトムアップ方式において UN GHS 区分外物質を検出する方法としてバリデーションが行われ、2015 年に OECD のテストガイドライン TG 491 として採択された。本報告書は、ICCVAM/NICEATM の SRD や発表論文などをもとに、採択された STE 法の概要を説明し、JaCVAM 眼刺激性代替法資料編纂委員会の意見を述べたものである。

ウサギを用いた Draize 眼刺激性試験と比較したとき、STE 法のトップダウン方式およびボトムアップ方式における一致度は 83.2%および 84.6%、偽陽性率は 1.2%および 19.3%、偽陰性率は 51.3%および 12.3%であった。ボトムアップ方式において適用除外物質を除いた場合は、一致度、偽陽性率、偽陰性率が 90.2%、18.8%、1.9%であった。施設内・施設間再現性、技術移転性に関しては、懸念される結果がなかった。

TG 491 に準拠して実施すれば、STE 法はトップダウン方式で UN GHS 区分 1 物質を検出する方法、およびボトムアップ方式で UN GHS 区分外物質を検出する方法として用いることができると本委員会は考える。

1. まえおき

化学物質の眼刺激性を評価する方法としては、ウサギの眼を用いた Draize 眼刺激性試験が従来使用されてきた。しかし、近年の動物福祉に対する関心の高まりや欧州における法規制改正により、その代替法の開発が進められ・バリデーションが行われてきた。現在 OECD によりテストガイドラインとして採択された代替法は、ウシ摘出角膜の混濁および透過性試験（BCOP 法、TG 437）、ニワトリ摘出眼球を用いた眼刺激性試験法（ICE 法、TG 438）、およびフルオレセイン漏出試験法（FL 法、TG 460）の 3 試験法である。BCOP 法と ICE 法は食用などの目的で処分された動物より摘出した器官・組織を用いて化学物質の曝露により生じる角膜の物理的特性の変化を指標に眼刺激性を評価する試験法で、トップダウン方式において重篤な眼の傷害を起こす（すなわち、UN GHS 区分 1）物質を検出する方法として、またボトムアップ方式において眼に対する重篤な損傷性を有する物質および眼刺激性物質とは分類されない（すなわち、UN GHS 区分外）物質を検出する方法として用いられる。FL 法は単層培養した尿細管上皮細胞を用いて化学物質の曝露により生じる細胞間結合の傷害を指標に眼刺激性を評価する試験法で、トップダウン方式でのみ用いられる。

*in vitro* 短時間曝露法（Short Time Exposure *In Vitro* Test Method、以下 STE 法）は、角膜上皮の損傷の作用機序である角膜上皮細胞に対する細胞毒性を指標に用いて、眼刺激性を評価する試験法である。すなわち STE 法では、ウサギ角膜由来株化細胞である SIRC 細胞に被験物質の希釈液を 5 分間曝露した後、MTT の取り込み量を基に細胞生存率を測定し（MTT 還元法）、被験物質（未希釈）の眼刺激性を評価している（Takahashi et al. 2008）。

STE 法は日本動物実験代替法学会（Sakaguchi et al. 2011）と JaCVAM（Kojima et al. 2013）のバリデーションを経て、トップダウン方式において UN GHS 区分 1 物質を検出する方法およびボトムアップ方式において UN GHS 区分外物質を検出する方法として ICCVAM/NICEATM により第三者評価を受け、その報告書（Summary Review Document: SRD）が 2013 年 6 月に公表された。その後、2015 年 7 月に OECD によりトップダウン・ボトムアップ両方式の眼刺激性試験代替法としてテストガイドラインに採択された（TG 491）。

本報告書は ICCVAM/NICEATM の SRD や発表論文などをもとに OECD TG 491 に採択された STE 法の概要を説明し、本委員会の意見および評価を述べたものである。

2．試験法の位置づけ

STE 法は、UN GHS 区分 1 物質である単一物質および混合物をトップダウン方式で検出する際に最初に用いる試験法である。また、STE 法は、UN GHS 区分外物質である単一物質および混合物をボトムアップ方式で検出する際に最初に用いる試験法でもある。

3．試験法の原理

眼に異物が入った場合、眼の刺激性は最表面の細胞傷害から始まる。STE 法はこれに

注目して、角膜上皮細胞に対する細胞毒性を指標として眼刺激性を評価する試験法である。コンフルエントに単層培養した SIRC 細胞に被験物質希釈液を 5 分間曝露した後、MTT の取り込み量で測定した細胞生存率をエンドポイントに用いる。これは、MTT が脱水素酵素の基質となる性質を利用し、細胞内に取り込まれた MTT がミトコンドリア内脱水素酵素により還元され、生成されたホルマザン量が生存細胞数に比例することを利用している（MTT 還元法）。

眼に溶液が入った場合、ヒトではその大部分が 1～2 分で眼内から排出され、ウサギでは3～4分で80%が排出されると報告されている。このような実際の曝露状況を考慮して、STE 法は通常の細胞毒性試験と比べて短時間曝露の試験として設計されている。

4. 試験手順

STE 法の手順の詳細は TG 491 に書かれているが、概要は以下の通りである。

4-1. 細胞の準備

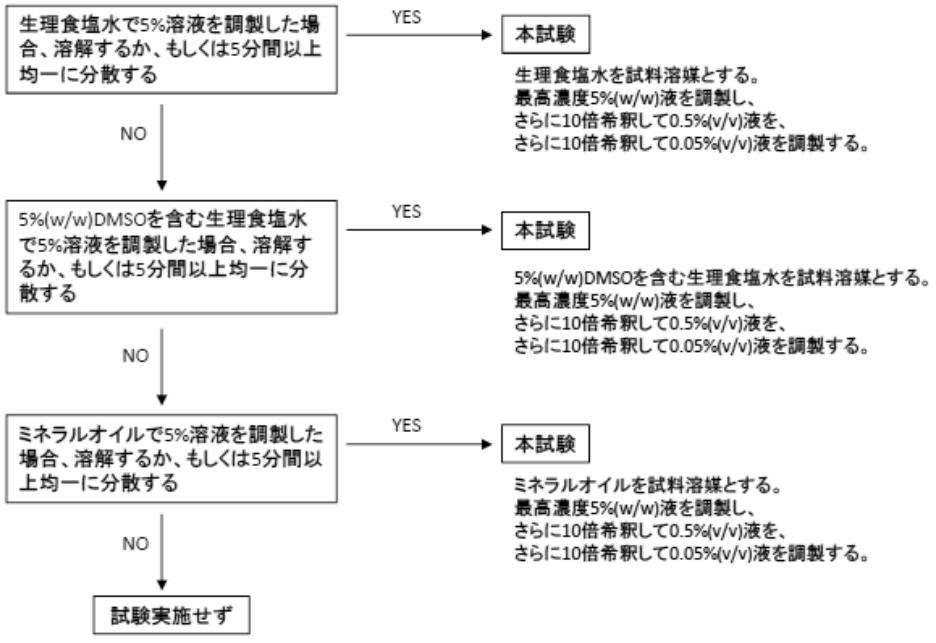
SIRC 細胞は 10%（v/v）ウシ胎児血清、2 mM L-グルタミン、50～100 unit/mL ペニシリン、50～100 µg/mL ストレプトマイシンを添加した Eagle 最小必須培地にて 37˚C、5% $CO_2$ 存在下で培養する。コンフルエントになるまで培養した細胞をトリプシン EDTA 溶液により剥がし、継代用培養フラスコに播種する。試験には継代数 25 回までの細胞を使用する。

試験に用いる際は、試験実施時にコンフルエント（>80%）になるように、前培養期間が 4 日間ならばウェルあたり $6.0 \times 10^3$ cells、5 日間ならばウェルあたり $3.0 \times 10^3$ cells に調製し 96 ウェル平板プレートに 200 µL ずつ播種する。

4-2. 試験サンプルの調製

生理食塩水、5%（w/w）DMSO（CAS# 67-68-5）を含む生理食塩水、ミネラルオイル

図１．試験溶媒の選択方法



（CAS# 8042-47-5）の中から適切な溶媒を図 1 に従って選択する。

試験には被験物質の 5% および 0.05% 希釈液（溶液あるいは懸濁液）を供する。陽性対照には SLS の 0.01% 生理食塩水溶液を用いる。これに加えて、溶媒対照、操作対照（培地対照）およびブランク（細胞なし培地のみ）も設ける。

4-3. 曝露・細胞生存率の算出

SIRC 細胞を前培養してコンフルエントとなった 96 ウェル平板プレートを以下のように処理する。各ウェルから培地を除去し、被験物質希釈液（5%、0.05%）および対照物質をそれぞれ 200 µL ずつ添加し 5 分間室温で曝露する。曝露終了後、試験サンプルを除去し、リン酸緩衝液 200 µL で 2 回洗浄する。MTT 溶液を 200 µL 添加し、37˚C、5% $CO_2$ 存在下で 2 時間反応させる。反応後 MTT 溶液を除去し、ホルマザン抽出液を 200 µL 添加し、1 時間室温暗所でホルマザンを抽出し、その吸光度をプレートリーダーにより 570 nm（$OD_{570}$）で測定する。

試験サンプル毎に 3 ウェルを用い、3 ウェルの平均 $OD_{570}$ を細胞生存率算出に用いる。

$$\text{細胞生存率} = \frac{OD_{570\ \text{試験サンプル}} - OD_{570\ \text{ブランク}}}{OD_{570\ \text{溶媒対照}} - OD_{570\ \text{ブランク}}} \times 100$$

なお、試験サンプル処理群の細胞生存率がマイナスになった場合は、生存率をゼロとする。

4-4. 眼刺激性判定

以上の手順で実験を 3 回実施し、それぞれで得られた細胞生存率の平均を最終判定に用いる。眼刺激性 GHS 分類の判定は表 1 に示した通りである。

表 1. 眼刺激性 GHS 分類の判定

| 被験物質希釈濃度 | 平均生存率 | 被験物質の GHS 分類 |
|---|---|---|
| 0.05% | > 70% | 区分外 |
| 5% | > 70% | |
| 0.05% | > 70% | 分類できない |
| 5% | ≤ 70% | |
| 0.05% | ≤ 70% | 区分 1 |
| 5% | ≤ 70% | |

4-5. 結果の承認基準

以下の 4 条件を満たした場合、試験の結果を承認する。

1) 操作対照の吸光度がブランク減算後 0.3 以上であること。
2) 実験に用いた溶媒対照すべてにおいて、細胞生存率が操作対照の 80%以上であること。

3) 陽性対照の細胞生存率が妥当な範囲[注)] に収まること。
4) 3 回の実験の細胞生存率の SD が被験物質 5%、0.05% 希釈液どちらでも 15%未満であること。

1)～4) の条件のいずれかが満たされない場合にはデータをすべて破棄し、新たに独立した 3 回の実験を実施する。

5. 試験法の正確性

以下のバリデーション試験等の結果が STE 法の正確性の検討に用いられた。

- Takahashi et al.（2009）– プレバリデーション試験 I　44 物質
- Takahashi et al.（2010）– プレバリデーション試験 II　70 物質
- Sakaguchi et al.（2011）– バリデーション試験 I　25 物質
- Kojima et al.（2013）–　バリデーション試験 II　40 物質
- 花王社内データ –Takahashi et al.（2011）および未発表社内データ　23 物質
- 花王社内データ（追加分）– 界面活性剤および界面活性剤含有の処方　52 物質
- Saito et al.（2015）–　混合物　40 物質

Saito et al.のデータを除く 169 物質のデータを用いて ICCVAM/NICEATM による正確性の検討が行われた。そのうち、GHS の眼刺激性分類を行うのに適切な動物試験のデータがない物質は正確性の検討対象から除かれた。1 つの物質に対して複数の試験施設での STE 法の判定がある場合は、試験施設数が最も多い判定を採用した。その試験施設数が同数になった場合は、刺激性の高い判定の方を採用した。なお、ICCVAM/NICEATM の検討では MTT を直接還元する物質（ボトムアップ方式では区分外と判定された物質のみ）は検討対象から外されていたが、2 次元細胞培養系を用いる STE 法においては洗浄により被験物質は十分取り除かれるので、以下の正確性の検討では採用した。

Saito et al. のデータは 5%希釈の結果のみを含み、ボトムアップ方式での混合物の評価における正確性を検討している。

5-1. トップダウン方式－GHS 区分 1 の物質の検出

トップダウン方式の正確性の検討には 125 物質のデータが用いられた（Appendix 1）。その結果は、正確度 83.2%（104/125）、偽陽性率 1.2%（1/86）、偽陰性率 51.3%（20/39）であった。偽陰性を示した 20 物質のうち、アルコール類が 8 物質、カルボン酸類が 5 物質、また塩類が 3 物質あった。そのほか、エステル類やヘテロサイクリック化合物などで偽陰性を示した物質があった。偽陽性となった 1 物質は非イオン性界面活性剤であった。アルコール類やエステル類など動物試験で得られる眼刺激性分類と一致しない物質の割合が高かった化学物質群を除いても、正確性の数値に顕著な改善は見られなかった。

注) 実施施設の過去のデータから求められる平均細胞生存率±2SD に収まること。上限・下限値は 3 ヶ月ごとに更新すべきである。実施実績が 1 ヶ月に 1 回未満の施設においては上限・下限値の更新は試験実施ごとに行うこと。過去のデータが不十分な施設においては、STE 法開発施設が設定した上限・下限値（21.1%～62.3%）を用いて構わない。

すでにトップダウン方式でのバリデーションが終了している他の代替法と STE 法の正確性の比較を表 2 にまとめた。

5-2. ボトムアップ方式－GHS 区分外の物質の検出

ボトムアップ方式の正確性の検討には 130 物質のデータが用いられた（Appendix 1）。その結果は、正確度 84.6%（110/130）、偽陽性率 19.3%（11/57）、偽陰性率 12.3%（9/73）であった。高い偽陰性率を示した物質は、塩類、炭化水素、アルコール類などがあった。また、固体（13.5%）の方が液体（4.3%）よりも偽陰性率が高かった。これらの化学物質群のデータをそれぞれ除いても、正確性の数値に顕著な改善は見られなかった。適用範囲として、蒸気圧が高い物質（> 6 kPa）と界面活性剤ではない固体を除いた場合は、正確度 90.2%（92/102）、偽陽性率 18.8%（9/48）、偽陰性率 1.9%（1/54）であった。偽陰性を示した物質は動物試験での GHS 分類が区分 2B である Toluene であった。すでにボトムアップ方式でのバリデーションが終了している他の代替法と STE 法の正確性の比較を表 3 にまとめた。

表 2. トップダウン方式での STE 法とバリデーションが終了している代替法との正確性の比較

| 試験法 | 物質数 | 正確度 | | 偽陽性率 | | 偽陰性率 | |
|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. |
| STE | 125 | 83.2 | 104/125 | 1.2 | 1/86 | 51.3 | 20/39 |
| BCOP | 188 | 79.3 | 149/188 | 23.6 | 29/123 | 15.4 | 10/65 |
| ICE | 144 | 83.3 | 120/144 | 7.9 | 9/114 | 50.0 | 15/30 |
| CM | 82 | 90.2 | 74/82 | 2.1 | 1/48 | 20.6 | 7/34 |
| FL | 151 | 77.5 | 117/151 | 6.8 | 7/103 | 56.3 | 27/48 |

表 3. ボトムアップ方式での STE 法とバリデーションが終了している代替法との正確性の比較

| 試験法 | 物質数 | 正確度 | | 偽陽性率 | | 偽陰性率 | |
|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. |
| STE | 130 | 84.6 | 110/130 | 19.3 | 11/57 | 12.3 | 9/73 |
| BCOP | 188 | 66.5 | 125/188 | 69.2 | 63/91 | 0.0 | 0/97 |
| ICE | 152 | 82.2 | 125/152 | 32.9 | 26/79 | 1.4 | 1/73 |
| CM | 53 | 67.9 | 36/53 | 68.0 | 17/25 | 0.0 | 0/28 |
| STE* | 102 | 90.2 | 92/102 | 18.8 | 9/48 | 1.9 | 1/54 |

* 蒸気圧 > 6 kPa の物質、および、界面活性剤以外の固体を除いた場合

5-3. 混合物

前述の ICCVAM/NICEATM による正確性の検討には混合物が 10 物質含まれている。そのうち、区分 1 の 1 物質は分類不可と判定された。区分外の 4 物質中 3 物質は区分外と判定された。

ICCVAM/NICEATM による正確性の検討とは別に、Saito et al. の 40 混合物を用いたボトムアップ方式における正確性の検討によると、正確度 87.5% （35/40）、偽陽性率 50.0%（5/10）、偽陰性率 0.0%（0/30）であった。

6. 試験法の信頼性

以下のバリデーション試験の結果が STE 法の信頼性の検討に用いられた。

- Takahashi et al.（2009）– プレバリデーション試験 I
- Takahashi et al.（2010）– プレバリデーション試験 II
- Sakaguchi et al.（2011）– バリデーション試験 I
- Kojima et al.（2013）– バリデーション試験 II

6-1. 技術移転性

STE 法を実施するために必要な装置・器具は、一般的な無菌化の細胞培養に用いるもので、入手は容易である。手技も技術的に複雑なものではない。2 つのプレバリデーション試験においても技術移転性についての問題は指摘されていない。

6-2. 施設内再現性

STE 法は 1 つの試験で 3 つの独立した実験を行うため、個々の実験から得られた細胞生存率が施設内再現性の検討に用いられた。区分外と判定された物質の 5%希釈液での細胞生存率の変動係数は 0.3～23.5%であった。区分外と判定されなかった物質では生存率が極めて低い値になるため、その変動係数は高い値を示した。陽性対照として用いられる 0.01% SLS の平均細胞生存率は 41.7%（n = 71）であり、変動係数は 24.7%であった。

6-3. 施設間再現性

5 施設で行われた Sakaguchi et al.のバリデーション試験 I において、それぞれの施設で得られた希釈濃度ごとの 25 物質の平均細胞生存率の SD は 0.3～20.1%であった。SD は基本的に小さく、良好な施設間再現性が確認された。25 物質の GHS 分類の判定について 5 施設すべてで判定が一致したのは 21 物質、1 施設で判定が異なったのは 3 物質で、2 施設で判定が異なったのは 1 物質であった。

STE 法を実施する試験施設へ適切な技術移転が達成できたことを確かめるには、Appendix 2 の熟達度確認物質を用いる。

7. 試験法の適用範囲

STE 法の適用には試験法の性質上、また、正確性の観点から以下の制限が設けられる。

1) 生理食塩水、5%（w/w）DMSO を含む生理食塩水、およびミネラルオイルのいずれにも溶解しない、あるいはそれら溶媒中で均一分散している状態が 5 分以上保たれない物質は適用不可能である。
2) ボトムアップ方式を単一物質に適用する場合、蒸気圧が 6 kPa を超える物質および界面活性剤ではない固体は、これら物質群が高い偽陰性率を示すことから、適用か

ら除外される。固体混合物に適用する場合は、界面活性剤のみから構成される物質は対象にするが、それ以外の物質は除外する。また、蒸気圧の高い（> 6 kPa）物質を含む混合物に適用する場合は眼刺激性が過小評価される可能性があるので、STE 法を用いる正当性を個別に検討すべきである。

3) トップダウン方式では、単一物質を用いる場合は制限がなく、また、混合物についてもバリデーションデータは限られてはいるものの、適用から除外する必要はない。

8. 本委員会の結論

STE 法は、すでにバリデーションが終了している他の代替法と比較した場合、正確性、再現性は同程度であった。

短時間で安価に実施でき、使用細胞の供給源に制限がなく、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ないから、技術移転性は高いと判断できる。ガイドラインに示された熟達度確認物質を用いて、実施する試験施設へ適切な技術移転が達成できたことを確かめる必要がある。

使用溶媒に不溶な物質、あるいは均一に分散しない物質は試験に適さない。ボトムアップ方式では、界面活性剤以外の固体や蒸気圧の高い（> 6 kPa）物質を除外することになっているが、その妥当性の確認は十分でない。

TG 491 に準拠して実施した場合、トップダウン方式で UN GHS 区分 1 物質を検出する方法、およびボトムアップ方式で UN GHS 区分外物質を検出する方法として用いることができると本委員会は考える。

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Appendix 1

STE 法の評価に用いられた物質（その１）

| | トップダウン方式 | ボトムアップ方式 | ボトムアップ方式<br>適用制限物質を除外 |
|---|---|---|---|
| 全体 | 125 | 130 | 102 |
| 化学物質区分 | | | |
| アルコール類 | 39 | 40 | 36 |
| アルデヒド類 | 2 | 2 | 0 |
| アルカリ | 1 | 1 | 0 |
| アミン類 | 9 | 10 | 8 |
| ホウ素化合物 | 4 | 4 | 2 |
| カルボン酸類 | 23 | 24 | 19 |
| 塩素化合物 | 1 | 1 | 0 |
| エステル類 | 17 | 18 | 17 |
| エーテル類 | 16 | 16 | 16 |
| 製剤（混合物） | 10 | 10 | 10 |
| グリコール類 | 1 | 1 | 1 |
| ヘテロサイクリック化合物 | 9 | 10 | 7 |
| 炭化水素 | 23 | 24 | 18 |
| 無機化合物 | 1 | 1 | 0 |
| ケトン類 | 8 | 8 | 5 |
| ラクトン | 1 | 1 | 1 |
| 油類 | 5 | 7 | 6 |
| ニトリル化合物 | 1 | 1 | 1 |
| 窒素化合物 | 1 | 1 | 1 |
| オニウム化合物 | 11 | 12 | 11 |
| フェノール類 | 1 | 1 | 0 |
| 多環式化合物 | 1 | 1 | 1 |
| 塩類 | 17 | 18 | 14 |
| 硫黄化合物 | 15 | 16 | 12 |

注）物質によっては複数の化学物質区分にまたがっている場合もあるため、合計は物質総数とは一致しない。

Appendix 1

STE 法の評価に用いられた物質（その２）

| | トップダウン方式 | ボトムアップ方式 | ボトムアップ方式<br>適用制限物質を除外 |
|---|---|---|---|
| 全体 | 125 | 130 | 102 |
| 物質の特性 | | | |
| 液体 | 92 | 93 | 79 |
| 蒸気圧 >6kPa | - | 14 | 0 |
| 蒸気圧 ≤6kPa | - | 61 | 61 |
| 蒸気圧　不明 | - | 18 | 18 |
| 固体 | 33 | 37 | 23 |
| 界面活性剤 | - | 23 | 23 |
| 非界面活性剤 | - | 14 | 0 |
| 蒸気圧 >6kPa | - | 0 | 0 |
| 蒸気圧 ≤6kPa | - | 23 | 10 |
| 蒸気圧　不明 | - | 14 | 13 |
| 製品区分 | | | |
| 界面活性剤 | 45 | 49 | 49 |
| 溶剤 | 34 | 35 | 26 |
| 化学中間産物 | 24 | 24 | 20 |
| 医薬品 | 20 | 20 | 13 |
| 香料 | 20 | 20 | 13 |
| 殺虫剤・抗菌剤・防腐剤 | 10 | 10 | 7 |
| 塗料 | 11 | 10 | 7 |

注）製品区分は物質数の多いものを抽出した。物質によっては複数の製品区分にまたがっている場合もある。

Appendix 2

STE 法の熟達度確認物質

| 物質名 | CAS 番号 | 化学分類 | 性状 | GHS 分類 | STE 法での使用溶媒 | STE 法判定 |
|---|---|---|---|---|---|---|
| Benzalkonium chloride (10% aqueous) | 8001-54-5 | オニウム化合物 | 液体 | 区分 1 | 生理食塩水 | 区分 1 |
| Triton X-100 (100%) | 9002-93-1 | エーテル類 | 液体 | 区分 1 | 生理食塩水 | 区分 1 |
| Acid Red 92 | 18472-87-2 | ﾍﾃﾛｻｲｸﾘｯｸ化合物；臭化化合物；塩素化合物 | 固体 | 区分 1 | 生理食塩水 | 区分 1 |
| Sodium hydroxide | 1310-73-2 | アルカリ；無機化合物 | 固体 | 区分 1 | 生理食塩水 | 区分 1 |
| Butyrolactone | 96-48-0 | ラクトン類；ﾍﾃﾛｻｲｸﾘｯｸ化合物 | 液体 | 区分 2A | 生理食塩水 | 分類不可 |
| 1-Octanol | 111-87-5 | アルコール類 | 液体 | 区分 2A/B | ﾐﾈﾗﾙｵｲﾙ | 分類不可 |
| Cyclopentanol | 96-48-0 | アルコール類；炭化水素（環状） | 液体 | 区分 2A/B | 生理食塩水 | 分類不可 |
| 2-Ethoxyethyl acetate | 111-15-9 | アルコール類；エーテル類 | 液体 | 区分外 | 生理食塩水 | 区分外 |
| Dodecane | 112-40-3 | 炭化水素（非環状） | 液体 | 区分外 | ﾐﾈﾗﾙｵｲﾙ | 区分外 |
| Methyl isobutyl ketone | 108-10-1 | ケトン類 | 液体 | 区分外 | ﾐﾈﾗﾙｵｲﾙ | 区分外 |
| n,n-Dimethylguanidine sulfate | 598-65-2 | アミジン類；硫黄化合物 | 固体 | 区分外 | 生理食塩水 | 区分外 |

Adopted:
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# OECD GUIDELINE FOR THE TESTING OF CHEMICALS

## Short Time Exposure *In Vitro* Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

### INTRODUCTION

1. The Short Time Exposure (STE) test method is an *in vitro* method that can be used under certain circumstances and with specific limitations for hazard classification and labeling of chemicals (substances and mixtures) that induce serious eye damage as well as those that do not require classification for either serious eye damage or eye irritation, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (1).

2. For many years, the eye hazard potential of chemicals has been evaluated primarily using an *in vivo* rabbit eye test (TG 405). It is generally accepted that, in the foreseeable future, no single *in vitro* alternative test will be able to fully replace the *in vivo* rabbit eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of alternative test methods used in a (tiered) testing strategy may well be able to fully replace the rabbit eye test (2). The top-down approach is designed for the testing of chemicals that can be expected, based on existing information, to have a high irritancy potential or induce serious eye damage. Conversely, the bottom-up approach is designed for the testing of chemicals that can be expected, based on existing information, not to cause sufficient eye irritation to require a classification. While the STE test method is not considered to be a complete replacement for the *in vivo* rabbit eye test, it is suitable for use as part of a tiered testing strategy for regulatory classification and labeling, such as the top-down/bottom-up approach, to identify without further testing (i) chemicals inducing serious eye damage (UN GHS Category 1) and (ii) chemicals (excluding highly volatile substances and all solid chemicals other than surfactants) that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (1) (2). However, a chemical that is neither predicted to cause serious eye damage (UN GHS Category 1) nor UN GHS No Category (does not induce either serious eye damage or eye irritation) by the STE test method would require additional testing to establish a definitive classification. Furthermore, the appropriate regulatory authorities should be consulted before using the STE in a bottom-up approach under classification schemes other than the UN GHS.

3. The purpose of this test guideline (TG) is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in the Short Time Exposure Test method. The cytotoxic effect of chemicals on corneal epithelial cells is an important mode of action (MOA) leading to corneal epithelium damage and eye irritation. Cell viability in the STE test method is

assessed by the quantitative measurement, after extraction from cells, of blue formazan salt produced by the living cells by enzymatic conversion of the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), also known as Thiazolyl Blue Tetrazolium Bromide (3). The obtained cell viability is compared to the solvent control (relative viability) and used to estimate the potential eye hazard of the test chemical. A test chemical is classified as UN GHS Category 1 when both the 5% and 0.05% concentrations result in a cell viability smaller than or equal to (≤) 70%. Conversely, a chemical is predicted as UN GHS No Category when both 5% and 0.05% concentrations result in a cell viability higher than (>) 70%.

4. The term "test chemical" is used in this Test Guideline to refer to what is tested and is not related to the applicability of the STE test method to the testing of substances and/or mixtures. Definitions are provided in Annex I.

**INITIAL CONSIDERATIONS AND LIMITATIONS**

5. This Test Guideline is based on a protocol developed by Kao Corporation (4), which was the subject of two different validation studies: one by the Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE) (5) and another by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6). A peer review was conducted by NICEATM/ICCVAM based on the validation study reports and background review documents on the test method (7).

6. When used to identify chemicals (substances and mixtures) inducing serious eye damage (UN GHS Category 1 (1), data obtained with the STE test method on 125 chemicals (including both substances and mixtures), showed an overall accuracy of 83% (104/125), a false positive rate of 1% (1/86), and a false negative rate of 51% (20/39) as compared to the *in vivo* rabbit eye test (7). The false negative rate obtained is not critical in the present context, since all test chemicals that induce a cell viability of ≤ 70% at a 5% concentration and > 70% at 0.05% concentration would be subsequently tested with other adequately validated *in vitro* test methods or, as a last option, in the *in vivo* rabbit eye test, depending on regulatory requirements and in accordance with the sequential testing strategy and weight-of-evidence approaches currently recommended (1) (8). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The STE test method showed no other specific shortcomings when used to identify test chemicals as UN GHS Category 1. Investigators could consider using this test method on test chemicals, whereby cell viability ≤ 70% at both 5% and 0.05% concentration should be accepted as indicative of a response inducing serious eye damage that should be classified as UN GHS Category 1 without further testing.

7. When used to identify chemicals (substances and mixtures) not requiring classification for eye irritation and serious eye damage (i.e. UN GHS No Category), data obtained with the STE test method on 130 chemicals (including both substances and mixtures), showed an overall accuracy of 85% (110/130), a false negative rate of 12% (9/73), and a false positive rate of 19% (11/57) as compared to the *in vivo* rabbit eye test (7). If highly volatile substances and solid substances other than surfactants are excluded from the dataset, the overall accuracy improves to 90% (92/102), the false negative rate to 2% (1/54), and the false positive to 19% (9/48) (7). As a consequence, the potential shortcomings of the STE test method when used to identify test chemicals not requiring classification for eye irritation and serious eye damage (UN GHS No Category) are a high false negative rate for i) highly volatile substances with a vapor pressure over 6 kPa and ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants. Such chemicals are excluded from the applicability domain of the STE test method (7).

8. In addition to the chemicals mentioned in paragraphs 6 and 7, the STE test method generated dataset also contains in-house data on 40 mixtures, which when compared to the *in vivo* Draize eye test, showed an accuracy of 88% (35/40), a false positive rate of 50% (5/10), and a false negative rate of 0% (0/30) for predicting mixtures that do not require classification under the UN GHS classification system (9). The STE test method can therefore be applied to identify mixtures as UN GHS No Category in a bottom-up approach with the exception of solid mixtures other than those composed only of surfactants as an extension of its limitation to solid substances. Furthermore, mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

9. The STE test method cannot be used for the identification of test chemicals as UN GHS Category 2, Category 2A (eye irritation) or UN GHS Category 2B (mild eye irritation), due to the considerable number of UN GHS Category 1 chemicals under-predicted as UN GHS Category 2, 2A, or 2B and UN GHS No Category chemicals over-predicted as UN GHS Category 2, 2A, or 2B (7). For this purpose, further testing with another suitable method may be required.

10. The STE test method is suitable for test chemicals that are dissolved or uniformly suspended for at least 5 minutes in physiological saline, 5% dimethyl sulfoxide (DMSO) in saline, or mineral oil. The STE test method is not suitable for test chemicals that are insoluble or cannot be uniformly suspended for at least 5 minutes in physiological saline, 5% DMSO in saline, or mineral oil. The use of mineral oil in the STE test method is possible because of the short-time exposure. Therefore, the STE test method is suitable for predicting the eye hazard potential of water-insoluble test chemicals (*e.g.*, long-chain fatty alcohols or ketones) provided that they are miscible in at least one of the three above proposed solvents (4).

11. The term "test chemical" is used in this Test Guideline to refer to what is being tested[11] and is not related to the applicability of the STE test method to the testing of substances and/or mixtures.

**PRINCIPLE OF THE TEST**

11. The STE test method is a cytotoxicity-based *in vitro* assay that is performed on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells, cultured on a 96-well polycarbonate microplate (4). After five-minute exposure to a test chemical, the cytotoxicity is quantitatively measured as the relative viability of SIRC cells using the MTT assay (4). Decreased cell viability is used to predict potential adverse effects leading to ocular damage.

12. It has been reported that 80% of a solution dropped into the eye of a rabbit is excreted through the conjunctival sac within three to four minutes, while greater than 80% of a solution dropped into the human eye is excreted within one to two minutes (10). The STE test method attempts to approximate these exposure times and makes use of cytotoxicity as an endpoint to assess the extent of damage to SIRC cells following a five-minute exposure to the test chemical.

**DEMONSTRATION OF PROFICIENCY**

13. Prior to routine use of the STE test method described in this test guideline, laboratories should demonstrate technical proficiency by correctly classifying the eleven substances recommended in Table 1. These substances were selected to represent the full range of responses for serious eye damage or eye irritation based on results of *in vivo* rabbit eye tests (TG 405) and the UN GHS classification system (1). Other selection criteria included that the substances should be commercially available, that high-quality *in vivo* reference data should be available, and that high-quality *in vitro* data from the STE test method should

[1] In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term “test chemical” describing what is being tested should now be applied in new and updated Test Guidelines.

be available (3). In situations where a listed substance is unavailable or where justifiable, another substance for which adequate *in vivo* and *in vitro* reference data are available could be used provided that the same criteria as described here are used.

**Table 1: List of Proficiency Substances**

| Substance | CASRN | Chemical class[1] | Physical state | *In Vivo UN GHS Cat.*[2] | Solvent in STE test | STE UN GHS Cat. |
|---|---|---|---|---|---|---|
| Benzalkonium chloride (10%, aqueous) | 8001-54-5 | Onium compound | Liquid | Category 1 | Saline | Category 1 |
| Triton X-100 (100%) | 9002-93-1 | Ether | Liquid | Category 1 | Saline | Category 1 |
| Acid Red 92 | 18472-87-2 | Heterocyclic compound; Bromine compound; Chlorine compound | Solid | Category 1 | Saline | Category 1 |
| Sodium hydroxide | 1310-73-2 | Alkali; Inorganic chemical | Solid | Category 1[3] | Saline | Category 1 |
| Butyrolactone | 96-48-0 | Lactone; Heterocyclic compound | Liquid | Category 2A | Saline | No prediction can be made |
| 1-Octanol | 111-87-5 | Alcohol | Liquid | Category 2A/B[4] | Mineral Oil | No prediction can be made |
| Cyclopentanol | 96-41-3 | Alcohol; Hydrocarbon, cyclic | Liquid | Category 2A/B[5] | Saline | No prediction can be made |
| 2-Ethoxyethyl acetate | 111-15-9 | Alcohol; Ether | Liquid | No Category | Saline | No Category |
| Dodecane | 112-40-3 | Hydrocarbon, acyclic | Liquid | No Category | Mineral Oil | No Category |
| Methyl isobutyl ketone | 108-10-1 | Ketone | Liquid | No Category | Mineral Oil | No Category |
| n,n-Dimethylguanidine sulfate | 598-65-2 | Amidine; Sulfur compound | Solid | No Category | Saline | No Category |

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1]Chemical classes were assigned using information obtained from previous NICEATM publications and if not available, using the National Library of Medicine's Medical Subject Headings (MeSH®) (via ChemIDplus® [National Library of Medicine], available at http://chem.sis.nlm.nih.gov/chemidplus/) and structure determinations made by NICEATM.

[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS (1).

[3]Classification as Cat.1 is based on skin corrosive potential of 100% sodium hydroxide (listed as a proficiency chemical with skin corrosive potential in OECD TG 435) and the criterion for UN GHS category 1 (1).

[4]Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 2 out of 6 vs 4 out of 6 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* dataset included 2 studies with 3 animals each. In one study two out of three animals showed effects at day 7 warranting a Cat. 2A classification (11), whereas in the second study all endpoints in all three animals recovered to a score of zero by day 7 warranting a Cat. 2B classification (12).

[5]Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these

two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from corneal opacity and conjunctivae redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 and a conjunctivae redness of 1 (at day 7) that fully recovered at day 14 (11).

**PROCEDURE**

***Preparation of the Cellular Monolayer***

14. The rabbit cornea cell line, SIRC should be used for performing the STE test method. It is recommended that SIRC cells are obtained from a well-qualified cell bank, such as American Type Culture Collection CCL60.

15. SIRC cells are cultured at 37°C under 5% $CO_2$ and humidified atmosphere in a culture flask containing a culture medium comprising Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50–100 units/mL penicillin and 50–100 µg/mL streptomycin. Cells that have become confluent in the culture flask should be separated using trypsin-ethylenediaminetetraacetic acid solution, with or without the use of a cell scraper. Cells are propagated (e.g. 2 to 3 passages) in a culture flask before being employed for routine testing, and should undergo no more than 25 passages from thawing.

16. Cells ready to be used for the STE test are then prepared at the appropriate density and seeded into 96-well plates. The recommended cell seeding density is $6.0 \times 10^3$ cells per well when cells are used four days after seeding, or $3.0 \times 10^3$ cells per well when cells are used five days after seeding, at a culture volume of 200 µL. Cells used for the STE test that are seeded in a culture medium at the appropriate density will reach a confluence of more than 80% at the time of testing, *i.e.*, four or five days after seeding.

***Application of the Test Chemicals and Control Substances***

17. The first choice of solvent for dissolving or suspending test chemicals is physiological saline. If the test chemical demonstrates low solubility or cannot be dissolved or suspended uniformly for at least five minutes in saline, 5% DMSO (CAS#67-68-5) in saline is used as a second choice solvent. For test chemicals that cannot be dissolved or suspended uniformly for at least five minutes in either saline or 5% DMSO in saline, mineral oil (CAS#8042-47-5) is used as a third choice solvent.

18. Test chemicals are dissolved or suspended uniformly in the selected solvent at 5% (w/w) concentration and further diluted by serial 10-fold dilution to 0.5% and 0.05% concentration. Each test chemical is to be tested at both 5% and 0.05% concentrations. Cells cultured in the 96-well plate are exposed to 200 µL/well of either a 5% or a 0.05% concentration of the test chemical solution (or suspension), for five minutes at room temperature. Test chemicals (mono-constituent substances or multi-constituent substances or mixtures) are considered as neat substances and diluted or suspended according to the method, regardless of their purity.

19. The culture medium described in paragraph 15 is used as a medium control in each plate of each repetition. Furthermore, cells are to be exposed also to solvent control samples in each plate of each repetition. The solvents listed in paragraph 17 have been confirmed to have no adverse effects on the viability of SIRC cells.

20. In the STE test method, 0.01% Sodium lauryl sulfate (SLS) in saline is to be used as a positive control in each plate of each repetition. In order to calculate cell viability of the positive control, each plate of each repetition has to also include a saline solvent control.

21. A blank is necessary to determine compensation for optical density and should be performed on wells containing only phosphate buffered saline, but no calcium and magnesium (PBS-) or cells.

22. Each sample (test chemical at 5% and 0.05%, medium control, solvent control, and positive control) should be tested in triplicate in each repetition by exposing the cells to 200 µL of the appropriate test or control chemical for five minutes at room temperature.

23. Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses.

***Cell Viability Measurement***

24. After exposure, cells are washed twice with 200 µL of PBS and 200 µL of MTT solution (0.5 mg MTT/mL of culture medium) is added. After a two-hour reaction time in an incubator (37˚C, 5% $CO_2$), the MTT solution is decanted, MTT formazan is extracted by adding 200 µL of 0.04 N hydrochloric acid-isopropanol for 60 minutes in the dark at room temperature, and the absorbance of the MTT formazan solution is measured at 570 nm with a plate reader. Interference of test chemicals with the MTT assay (by colorants or direct MTT reducers) only occurs if significant amount of test chemical is retained in the test system following rinsing after exposure which is the case for 3D Reconstructed human cornea or Reconstructed human epidermis tissues but is not relevant for the 2D cell cultures used for the STE test method.

***Interpretation of Results and Prediction Model***

25. The optical density (OD) values obtained for each test chemical are then used to calculate cell viability relative to the solvent control, which is set at 100%. The relative cell viability is expressed as a percentage and obtained by dividing the OD of test chemical by the OD of the solvent control after subtracting the OD of blank from both values.

$$\text{Cell viability (\%)} = \frac{(OD_{570} \text{ of test chemical}) - (OD_{570} \text{ of blank})}{(OD_{570} \text{ of solvent control}) - (OD_{570} \text{ of blank})} \times 100$$

Similarly, the relative cell viability of each solvent control is expressed as a percentage and obtained by dividing the OD of each solvent control by the OD of the medium control after subtracting the OD of blank from both values.

26. Three independent repetitions, each containing three replicate wells (*i.e.*, n=9), should be performed. The arithmetic mean of the three wells for each test chemical and solvent control in each independent repetition is used to calculate the arithmetic mean of relative cell viability. The final arithmetic mean of the cell viability is calculated from the three independent repetitions.

27. The cell viability cut-off values for identifying test chemicals inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter.

**Table 2: Prediction model of the STE test method**

| Cell viability | | UN GHS Classification | Applicability |
|---|---|---|---|
| At 5% | At 0.05% | | |
| > 70% | > 70% | No Category | Substances and mixtures, with the exception of:<br>i) highly volatile substances with a vapor pressure over 6 kPa[1] and<br>ii) Solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants |
| ≤ 70% | > 70% | No prediction can be made | Not applicable |
| ≤ 70% | ≤ 70% | Category 1 | Substances and mixtures [2] |

[1] Mixtures containing substances with vapour pressure higher than 6kPa should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

[2] Based on results obtained mainly with mono-constituent substances, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. Before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

*Acceptance Criteria*

28. Test results are judged to be acceptable when the following criteria are all satisfied:

a) Optical density of the medium control (exposed to culture medium) should be 0.3 or higher after subtraction of blank optical density.

b) Viability of the solvent control should be 80% or higher relative to the medium control. If multiple solvent controls are used in each repetition, each of those controls should show cell viability greater than 80% to qualify the test chemicals tested with those solvents.

c) The cell viability obtained with the positive control (0.01% SLS) should be within two standard deviations of the historical mean. The upper and lower acceptance boundaries for the positive control should be frequently updated i.e., every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e., less than once a month). Where a laboratory does not complete a sufficient number of experiments to establish a statistically robust positive control distribution, it is acceptable that the upper and lower acceptance boundaries established by the method developer are used, i.e., between 21.1% and 62.3% according to its laboratory historical data, while an internal distribution is built during the first routine tests.

d) Standard deviation of the final cell viability derived from three independent repetitions should be less than 15% for both 5% and 0.05% concentrations of the test chemical.

If one or several of these criteria is not met, the results should be discarded and another three independent repetitions should be conducted.

**DATA AND REPORTING**

***Data***

29. Data for each individual well (e.g., cell viability values) of each repetition as well as overall mean, SD, and classification are to be reported.

***Test Report***

30. The test report should include the following information:

***Test Chemical and Control Substances***

- Mono-constituent substance : chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;

- Multi-constituent substance, UVCB and mixture: Characterization as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

- Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;

- Purity, chemical identity of impurities as appropriate and practically feasible, etc;

- Treatment prior to testing, if applicable (e.g., warming, grinding);

- Storage conditions and stability to the extent available.

***Test Method Conditions and Procedures***

- Name and address of the sponsor, test facility and study director;

- Description of the test method used;

- Cell line used, its source, passage number and confluence of cells used for testing;

- Details of test procedure used;

- Number of repetitions and replicates used;

- Test chemical concentrations used (if different than the ones recommended);

- Justification for choice of solvent for each test chemical;

- Duration of exposure to the test chemical (if different than the one recommended);

- Description of any modifications of the test procedure;

- Description of evaluation and decision criteria used;

- Reference to historical positive control mean and Standard Deviation (SD):

- Demonstration of proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or demonstration of reproducible performance of the test method over time.

***Results***

- For each test chemical and control substance, and each tested concentration, tabulation should be given for the individual OD values per replicate well, the arithmetic mean OD values for each independent repetition, the % cell viability for each independent repetition, and the final arithmetic mean % cell viability and SD over the three repetitions;

- Results for the medium, solvent and positive control demonstrating suitable study acceptance criteria;

- Description of other effects observed;

- The overall derived classification with reference to the prediction model/decision criteria used.

***Discussion of the Results***

***Conclusions***

## LITERATURE


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## ANNEX I

## DEFINITIONS

**Accuracy:** The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (13).

**Benchmark substance:** A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

**Bottom-Up Approach**: A step-wise approach used for a test chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome)

**Chemical:** means a substance or mixture.

**Eye irritation:** Production of change in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with "reversible effects on the eye" and with UN GHS Category 2 (1)

**False negative rate:** The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance.

**False positive rate:** The proportion of all negative chemicals that are falsely identified by a test method as positive. It is one indicator of test method performance.

**Hazard:** Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

**Medium control:** An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

**Mixture:** A mixture or a solution composed of two or more substances in which they do not react (1).

**Mono-constituent substance:** A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

**MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

**Multi-constituent substance:** A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

**OD:** Optical Density.

**Positive control:** A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

**Relevance:** Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (10).

**Reliability:** Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (13).

**Sensitivity:** The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (10).

**Serious eye damage:** Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with “irreversible effects on the eye” and with UN GHS Category 1 (1).

**Solvent/vehicle control:** An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical-treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

**Specificity:** The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (13).

**Substance**: Chemical elements and their compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing it composition (1).

**Surfactant:** Also called surface-active agent, this is a chemical such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

**Test chemical**: The term "test chemical" is used to refer to what is being tested.

**Tiered testing strategy:** A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight of evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made.

**Top-Down Approach:** step-wise approach used for a test chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

**United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS):** A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

**UN GHS Category 1:** See "Serious eye damage".

**UN GHS Category 2:** See "Eye irritation".

**UN GHS No Category**: Chemicals that are not classified as UN GHS Category 1 or 2 (2A or 2B).

**UVCB:** substances of unknown or variable composition, complex reaction products or biological materials.

# Short Time Exposure (STE) Test Method

# Summary Review Document

**Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)**

**National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)**

**National Institute of Environmental Health Sciences**
**National Institutes of Health**
**U.S. Public Health Service**
**Department of Health and Human Services**

**2013**

**National Toxicology Program**
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# Table of Contents



**Supplements**

Supplements containing files all of the available documentation related to the short time exposure (STE) test method review have been provided on the enclosed CD-ROM. These documents are also available on the NICEATM website at http://iccvam.niehs.nih.gov/STEReview.htm. Supplement A contains the Kao Corporation STE background review document (A1) and appendices (A2 to A10) as submitted. Supplement B (B1 to B3) contains the data and information used by NICEATM to conduct a technical review of the STE test method.

**Supplement A**
**Kao Corporation Background Review Document and Appendices**

| | |
|---|---|
| Supplement A1 | Kao Background Review Document: Current Status of *In Vitro* Test Methods for Identifying Ocular Irritants: Short Time Exposure (STE) Test |
| Supplement A2 | Short Time Exposure (STE) Test Protocol |

# List of Tables

# List of Abbreviations and Acronyms

| | |
|---|---|
| AD | Applicability domain |
| BCOP | Bovine corneal opacity and permeability |
| BRD | Background review document |
| CASRN | CAS Registry Number® (American Chemical Society) |
| CV | Coefficient of variation |
| EPA | U.S. Environmental Protection Agency |
| GHS | Globally Harmonized System of Classification and Labelling of Chemicals |
| ICCVAM | Interagency Coordinating Committee on the Validation of Alternative Methods |
| ICE | Isolated chicken eye |
| ILS | Integrated Laboratory Systems, Inc. |
| JaCVAM | Japanese Center for the Validation of Alternative Methods |
| kPa | Kilopascals |
| MeSH® | Medical Subject Headings (National Library of Medicine) |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NICEATM | National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods |
| NIEHS | National Institute of Environmental Health Sciences |
| NLM | National Library of Medicine |
| NTP | National Toxicology Program |
| OECD | Organisation for Economic Co-operation and Development |
| SIRC | Statens Seruminstitut rabbit cornea |
| STE | Short time exposure |
| TG | Test Guideline |
| UN | United Nations |

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# Executive Summary

The short time exposure (STE) test method is an *in vitro* method for identifying ocular irritants. Developed by Takahashi et al. (2008), the STE test method assesses cytotoxicity in a rabbit corneal epithelial cell line (SIRC cells) through a 5-minute exposure to the test substance. In March 2011, Kao Corporation (Tochigi, Japan) submitted a background review document (BRD) titled "Current Status of *In Vitro* Test Methods for Identifying Ocular Irritants: Short Time Exposure (STE) Test" to the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NICEATM conducted a preliminary evaluation of the BRD and requested additional information, which resulted in several revisions to the BRD. Kao Corporation drafted a final BRD in May 2012 (**Supplement A**). The BRD contains all data and information that were available in the peer-reviewed literature and Kao Corporation in–house data to describe the current validation status of the STE test method, including what was known about its accuracy and reliability.

This summary review document presents an evaluation of STE test method accuracy, sensitivity, specificity, false positive rate, and false negative rate based on test substances with corresponding *in vivo* data. The analysis in a top-down and a bottom-up approach was based on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2011) and U.S. Environmental Protection Agency (EPA 2012) classification systems for eye hazard classification and labeling.

In a top-down approach, the STE test method is used to distinguish and label severe eye irritants/corrosives from all other hazard categories. Any substance not identified as a severe eye irritant/corrosive by the STE test method requires additional testing with other methods. A top-down approach requires a low false positive rate to avoid overclassification of substances. The false negative rate is not as critical because substances that test negative in the STE test method would be tested with another method.

In contrast, a bottom-up approach is used to distinguish substances not labeled as eye irritants from all other hazard categories. Any substance that tests positive in a bottom-up approach requires additional testing with other methods to determine the appropriate hazard classification and labeling. A bottom-up approach requires a low false negative rate to avoid irritants being classified and mislabeled as irritants when the correct eye hazard classification is GHS Not classified or EPA Category IV (minimal effects clearing in less than 24 hours). The false positive rate is not as critical because substances that test positive in the STE test method would be tested with another method.

The Kao Corporation BRD describes their analyses of 119 tests substances in four studies (Kojima et al. [Kao BRD]; Sakaguchi et al. 2011; Takahashi et al. 2009, 2010), with additional in-house data provided by Kao Corporation. In September 2012, Kao Corporation provided data on 52 additional surfactant or surfactant-containing substances, for a total of 169 substances with *in vitro* STE and *in vivo* rabbit eye test data (**Supplement B**).

The analyses in this report used consensus calls for both STE and rabbit eye test data when results were available from more than one laboratory or study. When equivocal results were obtained in two or more laboratories or in different studies, the more severe hazard classification was used.

**Table 1** summarizes overall performance of the STE test method in a top-down approach for all substances in the database. The overall false positive rate in a top-down approach ranged from 1.2% (1/84) for GHS classification to 2.3% (2/87) for EPA classification. Exclusion of alcohols reduced the rate to 0% (data not shown). The performance of the STE test method is compared to other validated *in vitro* test methods in **Table 2**.

**Table 1 Overall STE Performance in a Top-Down Approach**

| Regulatory System | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. | % | No. | % | No. |
| GHS | 120 | 85 | 102/120 | 53 | 19/36 | 99 | 83/84 | 1.2 | 1/84 | 47 | 17/36 |
| EPA | 120 | 87 | 104/120 | 58 | 19/33 | 98 | 85/87 | 2.3 | 2/87 | 42 | 14/33 |

Abbreviations: EPA = U.S. Environmental Protection Agency; GHS = Globally Harmonized System of Classification and Labelling of Chemicals; N = number of substances; STE = short time exposure.

**Table 2 Top-Down Performance of Validated *In Vitro* Test Methods Compared to the STE Test Method**

| GHS | N | Accuracy | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. |
| BCOP | 188 | 79 | 149/188 | 24 | 29/123 | 15 | 10/65 |
| ICE | 144 | 83 | 120/144 | 8.0 | 9/114 | 50 | 15/30 |
| CM | 82 | 90 | 74/82 | 2.0 | 1/48 | 21 | 7/34 |
| STE | 120 | 85 | 102/120 | 1.2 | 1/84 | 47 | 17/36 |

Abbreviations: BCOP = bovine corneal opacity and permeability; CM = Cytosensor microphysiometer; GHS = Globally Harmonized System of Classification and Labelling of Chemicals; ICE = isolated chicken eye; N = number of substances.

The STE overall performance in a bottom-up approach is shown in **Table 3.** The overall false negative rate in a bottom-up approach ranged from 12.3% (9/73) for GHS classification to 24.7% (24/97) for EPA classification.

**Table 3 Overall STE Performance in a Bottom-Up Approach**

| Regulatory System | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. | % | No. | % | No. |
| GHS | 129 | 85 | 109/129 | 88 | 64/73 | 80 | 45/56 | 20 | 11/56 | 12 | 9/73 |
| EPA | 129 | 80 | 103/129 | 75 | 73/97 | 94 | 30/32 | 6.3 | 2/32 | 25 | 24/97 |

Abbreviations: EPA = U.S. Environmental Protection Agency; GHS = Globally Harmonized System of Classification and Labelling of Chemicals; N = number of substances; STE = short time exposure.

The applicability domain was evaluated to reduce the false positive rate and increase performance for both GHS and EPA classifications in a bottom-up approach. Improvements in the applicability domain were determined by analyzing assay performance by chemical class and physical properties. As a result, two applicability domains were evaluated based on excluding certain chemical and product classes, or physical characteristics. Applicability domain one excludes liquids with vapor pressures ≥6 kilopascals (kPa) solid alcohols, hydrocarbons, and salts while applicability domain two excludes liquids with vapor pressures ≥6 kilopascals (kPa) and nonsurfactant solids (**Table 4)**. The performance of the STE test method is compared to other validated *in vitro* test methods in **Table 5**.

**Table 4 Overall STE Performance in a Bottom-Up Approach After Excluding Discordant Categories**

| Regulatory System | N | Accuracy | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. |
| Applicability Domain 1 - Exclusion of liquids with vapor pressures ≥6 kilopascals (kPa) solid alcohols, hydrocarbons, and salts | | | | | | | |
| GHS | 94 | 90 | 85/94 | 18 | 8/45 | 2 | 1/49 |
| EPA | 94 | 83 | 78/94 | 7.7 | 2/26 | 21 | 14/68 |
| Applicability Domain 2 - Exclusion of liquids with vapor pressures ≥6 kilopascals (kPa) and nonsurfactant solids | | | | | | | |
| GHS | 101 | 90 | 91/101 | 19 | 9/47 | 1.9 | 1/54 |
| EPA | 101 | 85 | 86/101 | 7.1 | 2/28 | 18 | 13/73 |

Abbreviations: EPA = U.S. Environmental Protection Agency; GHS = Globally Harmonized System of Classification and Labelling of Chemicals; No. = number; STE = short time exposure.

**Table 5 Bottom-Up Performance of Validated *In Vitro* Test Methods Compared to the STE Test Method**

| GHS | N | Accuracy | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|
| | | % | No. | % | No. | % | No. |
| BCOP | 188 | 66 | 125/188 | 69 | 63/91 | 0 | 0/97 |
| CM | 53 | 68 | 36/53 | 68 | 17/25 | 0 | 0/28 |
| STE – AD1 | 94 | 91 | 85/94 | 18 | 8/45 | 2 | 1/49 |
| STE – AD2 | 101 | 90 | 91/101 | 19 | 9/47 | 1.9 | 1/54 |

Abbreviations: AD = applicability domain; BCOP = bovine corneal opacity and permeability; CM = Cytosensor microphysiometer; N = number of substances; STE = short time exposure.

This evaluation of the STE performance shows that this method is able to distinguish substances as severe irritants or corrosives (i.e., GHS Category 1 or EPA Category I) from all other hazard categories (GHS Category 2A, 2B, Not Classified or EPA Category II, III, IV) in a top-down approach, with false positive rates ranging from 1.2% (1/84) to 2.3% (2/87) for the GHS and EPA classification systems, respectively. Exclusion of discordant chemical classes (e.g., alcohols, ethers, hydrocarbons, or nonionic surfactants) reduced the false positive rate to 0%. In a bottom-up approach to distinguish substances that were either not classified or minimal irritants (i.e., GHS Not Classified or EPA Category IV) from all other hazard categories (i.e., GHS Category 1, 2A, 2B or EPA Category I, II, III), the STE false negative rates ranged from 12.3% (9/73) to 24.7% (24/97). The range of false negative rates in a bottom-up approach was decreased to 2% (1/49) and 21% (14/68) for the GHS and EPA classification systems, respectively, when liquids with vapor pressures >6kPa solid alcohols, hydrocarbons, and salts were excluded. The range of false negative rates in a bottom-up approach was decreased to 1.9% (1/54) and 18% (13/73) for the GHS and EPA classification systems, respectively, when liquids with vapor pressures >6kPa and nonsurfactant solids were excluded.

This SRD along with the original Kao BRD and other supporting documentation were forward by NTP to four external scientific reviewers. The reviewers were provided a list of questions that included a request to comment on the adequacy of the database used for evaluating STE, the adequacy of the performance evaluation, and to provide comments for regulators using the test

method. In response, the reviewers indicated that the database of compounds was generally sufficient and the review thorough. A summary of reviewer comments is provided in **Section 5.0**.

## 1.0 Introduction and Background

The Draize rabbit eye test has been the primary method used to determine the ocular irritation potential of chemicals (Draize et al. 1944). However, public interest in animal welfare has increased the pressure to develop non-animal alternatives. The development of alternative methods is also accelerating due to regulations banning animal ocular irritation tests for cosmetics in the European Union (Directive 2003/15/EC; European Union 2003). As a result, numerous alternative ocular irritation methods that use cell lines and tissues are being developed around the world (Balls et al. 1999; Eskes et al. 2005; Ohno et al. 1999). The test guidelines for the bovine corneal opacity and permeability (BCOP) test method and isolated chicken eye (ICE) test method were accepted by the Organisation for Economic Co-operation and Development (OECD) for predicting severe ocular irritation (OECD 2012a, 2012b). However, no other test guidelines have been accepted for *in vitro* ocular irritation tests.

The short time exposure (STE) test method is an alternative ocular irritation method developed by Kao Corporation (Takahashi et al. 2008). The STE test method uses a cultured cell line (SIRC cells) derived from rabbit cornea and uses shorter exposure times than many other cytotoxicity-based methods. Generally, cytotoxicity tests using cultured cells have the advantage of being simple, quick procedures with a low evaluation cost. The facility requirements necessary to conduct the STE test include a standard laboratory setup for cell culture. The cornea is one of the main targets during accidental eye exposures, and damage to the cornea can result in visual impairment. A final advantage of the STE test method is that it can be used to evaluate poorly water-soluble chemicals like toluene, octanol, and hexanol by using mineral oil as the vehicle (Takahashi et al. 2008).

The STE test method involves exposing SIRC cells to 5% and 0.05% concentrations of test substance for 5 minutes. Following exposure to 5% test substance concentration, substances that reduce cell viability below 70% are classified as irritants. Using this classification scheme, Kao Corporation assessed the performance of the STE test method in a bottom-up approach to distinguish substances not labeled as irritants from all other categories. Kao Corporation also proposed a second approach to establish an ocular irritation potency ranking that differentiates severely irritating substances from mild and moderate irritants. This approach uses a point system based on the test concentration and relative viability resulting from an exposure to 5% or 0.05% of test material (Takahashi et al. 2008). This second approach was used to review the STE test method in a top-down approach to distinguish corrosives/severe irritants from all other categories.

In March 2011, the Japanese Center for the Validation of Alternative Methods (JaCVAM), as part of the International Cooperation on Alternative Test Methods agreement, requested that the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) conduct a technical review of the STE test method. In support of the review, Kao Corporation submitted their STE test method BRD and subsequently provided NICEATM with a revised May 2012 BRD (**Supplement A**). The BRD contains STE and rabbit eye test data for 119 substances from four *in vitro–in vivo* comparative studies, with additional in-house data on 23 substances provided by Kao Corporation. After the preliminary analysis, additional data were requested and provided for 52 surfactants or surfactant-containing formulations that increased the STE database to 169 substances.

To assess the ability of the STE test method to predict the regulatory hazard classification identified in the rabbit eye test, the STE rank results were converted to Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and U.S. Environmental Protection Agency (EPA) classifications (UN 2011, EPA 2012). An STE rank of 1 (nonirritant), 2 (mild or moderate irritant), or 3 (severe irritant) was converted to GHS Not Classified, Category 2A/2B, or Category 1, respectively,

or to the U.S. EPA Label Review Manual classification of eye irritation as Category IV, Category III/II, or Category I, respectively.

STE test method performance was also evaluated in a top-down approach (i.e., distinguishing GHS Category 1 or EPA Category I substances from those in all other categories) or in a bottom-up approach (i.e., distinguishing GHS Not Classified or EPA Category IV substances from all other categories) for substances with corresponding *in vitro–in vivo* data. For a top-down approach, 120 substances had corresponding *in vitro* and *in vivo* classification data using the GHS or EPA classification systems, respectively, that were suitable for accuracy analysis. For a bottom-up approach, 129 substances had suitable in *vitro–in vivo* data for GHS and EPA classifications.

A variety of chemical categories were tested in the STE test method, and the chemical categories with the greatest amount of test data are alcohols, carboxylic acids, esters, ethers/polyethers, heterocyclic compounds, ketones/lactones, onium compounds, and salts. Physical properties of these substances have also been evaluated (pH, solids, liquids, and surfactants [nonionic, anionic, cationic]). This summary review document describes evaluations of the STE test method performance in a top-down or bottom-up approach.

## 2.0 STE Test Method Database

The May 2012 BRD submitted by Kao Corporation (**Supplement A**) includes information on the STE test method, the test method protocol, and data and performance analyses based on:

- Takahashi et al. (2009) – Prevalidation study 1 on 44 substances
- Takahashi et al. (2010) – Prevalidation study 2 on 70 substances
- Sakaguchi et al. (2011) – Phase I validation study on 25 substances
- Kojima et al. (Kao BRD) – Phase II validation study on 40 substances, then combined with Phase I substances for a total of 63 substances
- Kao in-house data on 22 of 23 substances in the original BRD

Additional information on the test substances are found in **Supplement B**. **Supplement B1** contains the *in vivo* data used to develop consensus *in vivo* classifications for substances evaluated in the STE test method. **Supplement B2** shows the test substances along with CAS Registry Number® (American Chemical Society), concentration tested, STE test data (mean viability value, standard deviation, number of replicates), category classification, and the reference. **Supplement B3** provides the same information but indicates the consensus STE classification.

The STE database includes test substances in the Kao Corporation BRD, with additional data on 52 surfactants and surfactant-containing formulations provided by Kao Corporation. However, the database used to assess performance consists of consensus classifications when a single substance was tested in multiple laboratories or in different studies. *In vivo* data are typically generated by testing neat chemicals. Twenty-three substances that were tested in the STE test method at a concentration less than 100% and that did not produce a severe irritant effect were excluded from these analyses because a mild/moderate irritant or nonirritant classification of a diluted chemical may be classified as a severe irritant when tested neat *in vitro*.

Chemicals that directly reduce MTT in the absence of cells have been shown to artificially inflate viability measures and underpredict cytotoxicity (Huang 2004; Sims and Plattner 2009). Kao Corporation assessed chemicals for their ability to directly reduce MTT by incubating the test substances with MTT and visually inspecting for color development. Test substances that were identified as direct MTT reducers were removed from top-down analysis and those classified as STE nonirritants were removed from bottom-up analysis, as these could be false negative.

Finally, *in vivo* data were analyzed to calculate the appropriate ocular irritation hazard classification. These data include cornea, iris, and conjunctiva scores for each animal at 24, 48, and 72 hours following test substance administration and/or assessment of lesions at 7, 14, and 21 days. Some test substances had insufficient *in vivo* data to assign a hazard classification. Thus, these substances were not used to evaluate STE accuracy and reliability.

The STE database contains 169 test substances representing a variety of chemical classes and physicochemical properties. **Table 2-1** provides information on the test substances evaluated in a top-down approach to identify severe eye irritants or corrosives. **Table 2-2** provides information evaluated in a bottom-up approach to identify GHS Not Classified or EPA Category IV substances. These substances had corresponding *in vivo* data, were assigned a GHS (UN 2011) or EPA (EPA 2012) eye hazard classification, and met other assay criteria as discussed in **Section 3.2**. **Table 2-3** shows the substances used to assess the STE test method in a bottom-up approach applicability domain one excluding liquids with vapor pressure >6 kilopascals alcohols, hydrocarbons, and salts and applicability domain two excluding liquids with vapor pressure >6 kilopascals and nonsurfactant solids.

**Table 2-1 Test Substances Used to Evaluate STE Performance in a Top-Down Approach[a]**

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| 1-Bromo-4-chlorobutane | 6940-78-9 | Liquid | 0.164 | 100% | Not Classified | Category IV | 1 |
| 1-Dodecanaminium, N-(2-hydroxy-3-sulfopropyl)-N,N-dimethyl-, inner salt | 13197-76-7 | Liquid | 1.16E-21 | 100% | Category 1 | Category I | 2 |
| 1-Methylpropyl benzene | 135-98-8 | Liquid | 0.176 | 100% | Not Classified | Category IV | 1 |
| 1-Octanol | 111-87-5 | Liquid | 0.013 | 100% | Category 2A | Category II | 2 |
| 1,3-Di-isopropylbenzene | 99-62-7 | Liquid | 0.041 | 100% | Not Classified | Category IV | 1 |
| 1,5-Hexadiene | 592-42-7 | Liquid | 28.6 | 100% | Not Classified | Category III | 1 |
| 1,9-Decadiene | 1647-16-1 | Liquid | 0.320 | 100% | Not Classified | Category IV | 1 |
| 2-Benzyloxyethanol | 622-08-2 | Liquid | 2.9E-4 | 100% | Category 2A | Category II | 2 |
| 2-Ethoxyethyl acetate (Cellosolve acetate) | 111-15-9 | Liquid | 0.397 | 100% | Not Classified | Category III | 1 |
| 2-Ethyl-1-hexanol | 104-76-7 | Liquid | 0.025 | 100% | Category 2A | Category II | 2 |
| 2-Ethylhexyl p-dimethylamino benzoate | 21245-02-3 | Liquid | 4.72E-06 | 100% | Not Classified | Category IV | 1 |
| 2-Methyl-1-pentanol | 105-30-6 | Liquid | 0.191 | 100% | Category 2B | Category III | 2 |
| 2-Methylbutyric acid | 116-53-0 | Liquid | 0.149 | 100% | Category 1 | Category I | 2 |
| 2-Methylpentane | 107-83-5 | Liquid | 27.8 | 100% | Not Classified | Category IV | 1 |
| 2-Naphthalenesulfonic acid,6-hydroxy-,monosodium salt, polymer with formaldehyde and hydroxymethylbenzenesulfonic aid monosodium salt | 85255-76-1 | Liquid | NA | 100% | Category 1 | Category II | 2 |
| 2,2-Dimethyl-3-pentanol | 3970-62-5 | Liquid | 0.413 | 100% | Not Classified | Category III | 1 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| 2,4-Pentanediol | 625-69-4 | Liquid | 7.3E-03 | 100% | Not Classified | Category IV | 1 |
| 2,5-Dimethyl-2,5-hexanediol | 110-03-2 | Solid | 5.78E-04 | 100% | Category 1 | Category I | 1 |
| 3-Methoxy-1,2-propanediol | 623-39-2 | Liquid | 1.92E-03 | 100% | Not Classified | Category IV | 1 |
| 3-Methylhexane | 589-34-4 | Liquid | 8.29 | 100% | Not Classified | Category IV | 1 |
| 3,3-Dimethylpentane | 562-49-2 | Liquid | 10.1 | 100% | Not Classified | Category IV | 1 |
| Acetone | 67-64-1 | Liquid | 33.2 | 100% | Category 2A | Category II | 2 |
| Acid red 92 | 18472-87-2 | Solid | 5.71E-24 | 100% | Category 1 | Category I | 3 |
| Acrylic acid homopolymer sodium salt | 9003-04-7 | Solid | 4.56E-04 | 100% | Not Classified | Category IV | 1 |
| Ammonium nitrate | 6484-52-2 | Solid | 4.48E-16 | 100% | Category 2B | Category III | 1 |
| Benzalkonium chloride | 8001-54-5 | Liquid | NA | 100% | Category 1 | Category I | 3 |
| Benzalkonium chloride (10%) | 63449-41-2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Benzene, 1,1'-oxybis-, tetrapropylene derivatives, sulfonated, sodium salts | 119345-04-9 | Solid | NA | 100% | Category 1 | Category I | 3 |
| Benzyl alcohol | 100-51-6 | Liquid | 7.14E-03 | 100% | Category 1 | Category I | 2 |
| Body shampoo A | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Butanol | 71-36-3 | Liquid | 1.04 | 100% | Category 1 | Category I | 2 |
| Butyl acetate | 123-86-4 | Liquid | 1.59 | 100% | Not Classified | Category III | 1 |
| Butyl cellosolve | 111-76-2 | Liquid | 0.0633 | 100% | Category 1 | Category II | 2 |
| Butylnaphthalenesulfonic acid sodium salt | 25638-17-9 | Solid | NA | 100% | Category 1 | Category I | 2 |
| Butyrolactone | 96-48-0 | Liquid | 0.0394 | 100% | Category 2A | Category II | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Camphene | 79-92-5 | Solid | 0.237 | 100% | Category 2B | Category III | 1 |
| Cetylpyridinium bromide (10%) | 140-72-7 | Solid | 3.47E-07 | 10% | Category 1 | Category I | 3 |
| Cetylpyridinium chloride | 6004-24-6 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cetyltrimethylammonium bromide (10%) | 57-09-0 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cyclohexanol | 108-93-0 | Liquid | 0.087 | 100% | Category 1 | Category I | 2 |
| Cyclohexanone | 108-94-1 | Liquid | 0.539 | 100% | Not Classified | Category III | 2 |
| Cyclopentanol | 96-41-3 | Liquid | 0.307 | 100% | Category 2B | Category II | 2 |
| Di-n-propyl disulphide | 629-19-6 | Liquid | 0.0664 | 100% | Not Classified | Category IV | 1 |
| Di(2-Ethylhexyl) sodium sulfosuccinate | 577-11-7 | Solid | 1.63E-15 | 10% | Category 1 | Category I | 3 |
| Di(propylene glycol) propyl ether | 29911-27-1 | Liquid | 2.38E-04 | 100% | Category 2B | Category III | 2 |
| Diisobutyl ketone | 108-83-8 | Liquid | 0.287 | 100% | Not Classified | Category IV | 1 |
| Dimethyl sulfoxide | 67-68-5 | Liquid | 0.0829 | 100% | Not Classified | Category III | 1 |
| Dodecane | 112-40-3 | Liquid | 0.0315 | 100% | Not Classified | Category III | 1 |
| Domiphen bromide | 538-71-6 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Ethanol | 64-17-5 | Liquid | 812 | 100% | Category 2A | Category I | 1 |
| Ethyl 2-methylacetoacetate | 609-14-3 | Liquid | 0.0915 | 100% | Category 2B | Category III | 2 |
| Ethyl acetate | 141-78-6 | Liquid | 13.1 | 100% | Not Classified | Category III | 2 |
| Ethyl trimethyl acetate | 3938-95-2 | Liquid | 2.24 | 100% | Not Classified | Category III | 1 |
| Ethylhexyl salicylate | 118-60-5 | Liquid | 9.51E-07 | 100% | Not Classified | Category IV | 1 |
| Glycerol | 56-81-5 | Liquid | 1.06E-05 | 100% | Not Classified | Category IV | 1 |
| Glycidyl methacrylate | 106-91-2 | Liquid | 0.0829 | 100% | Not Classified | Category III | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Imidazole | 288-32-4 | Solid | 5.78E-04 | 100% | Category 1 | Category I | 2 |
| Iso-octyl acrylate | 29590-42-9 | Liquid | 0.0204 | 100% | Not Classified | Category IV | 1 |
| Isobutanal | 78-84-2 | Liquid | 21.9 | 100% | Category 2B | Category III | 2 |
| Isobutyl alcohol | 78-83-1 | Liquid | 1.78 | 100% | Category 1 | Category I | 2 |
| Isopropyl alcohol | 67-63-0 | Liquid | 6.61 | 100% | Category 2A | Category III | 1 |
| Isopropyl bromide | 75-26-3 | Liquid | 28.5 | 100% | Not Classified | Category IV | 1 |
| Isopropyl myristate | 110-27-0 | Liquid | 1.08E-04 | 100% | Not Classified | Category IV | 1 |
| Lactic acid | 50-21-5 | Liquid | 3.81E-03 | 100% | Category 1 | Category I | 2 |
| Lauryldimethylamine oxide | 1643-20-5 | Solid | 1.68E-15 | 100% | Category 1 | Category I | 3 |
| Lotion A | NA | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| m-Phenylene diamine | 108-45-2 | Solid | 251E-04 | 100% | Category 1 | Category I | 2 |
| Methoxyethyl acrylate | 3121-61-7 | Liquid | 0.598 | 100% | Category 1 | ≥Category III | 2 |
| Methyl acetate | 79-20-9 | Liquid | 7.03 | 100% | Category 2A | Category II | 1 |
| Methyl amyl ketone | 110-43-0 | Liquid | 0.655 | 100% | Not Classified | Category III | 1 |
| Methyl cyanoacetate | 105-34-0 | Liquid | 0.047 | 100% | Category 2A | Category II | 2 |
| Methyl cyclopentane | 96-37-7 | Liquid | 17.8 | 100% | Not classified | Category III | 1 |
| Methyl ethyl ketone (2-Butanone) | 78-93-3 | Liquid | 13.1 | 100% | Category 2A | Category III | 2 |
| Methyl isobutyl ketone | 108-10-1 | Liquid | 2.90 | 100% | Not Classified | Category III | 1 |
| Methyl trimethyl acetate | 598-98-1 | Liquid | 4.76 | 100% | Not Classified | Category IV | 1 |
| Myristyl alcohol | 112-72-1 | Solid | 269E-05 | 100% | Category 2A | Category III | 1 |
| n-Hexanol | 111-27-3 | Liquid | 0.117 | 100% | Category 2A | Category II | 2 |
| n-Hexyl bromide | 111-25-1 | Liquid | 0.541 | 100% | Not Classified | Category IV | 1 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| n-Octyl bromide | 111-83-1 | Liquid | 0.0691 | 100% | Not Classified | Category IV | 1 |
| n,n-Dimethylguanidine sulfate | 598-65-2 | Solid | 4.04 | 100% | Not Classified | Category III | 1 |
| Naphthalenesulfonic acid, butyl-, polymer with formaldehyde and 2-naphthalenesulfonic acid, sodium salt | 188070-49-7 | Solid | NA | 100% | Category 2A | Category II | 2 |
| Polyethylene glycol 400 | 25322-68-3 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Polyethyleneglycol monolaurate (10 E.O.) | 9004-81-3 | Liquid | 0 | 100% | Not Classified | Category IV | 2 |
| Polyoxyethylene hydrogenated castor oil (60E.O.) | 61788-85-0 | Solid | NA | 100% | Not Classified | Category IV | 1 |
| Polyoxyethylene(10) poly oxypropylene(1.5) lauryl-myristyl ether | 68439-51-0 | Liquid | NA | 100% | Category 1 | Category I | 3 |
| Polyoxyethylene(13) (mo no-, di-, tri-) styrenated phenyl ether | 104376-75-2 | Liquid | NA | 100% | Not Classified | Category III | 3 |
| Polyoxyethylene(14) tribe nzylated phenyl ether | 116998-28-8 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Polyoxyethylene(160) sor bitan triisostearate | 54392-28-8 | Solid | NA | 100% | Not Classified | Category IV | 1 |
| Polyoxyethylene(19) (mo no-, di-, tri-) styrenated phenyl ether | 104376-75-2 | Liquid | NA | 100% | Not Classified | Category II | 2 |
| Polyoxyethylene(23) laury l ether | 9002-92-0 | Solid | 2.03E-13 | 100% | Category 2A | Category III | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Polyoxyethylene(40) hydrogenated castor oil | 61788-85-0 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Potassium laurate | 10124-65-9 | Solid | 0 | 10% | Category 1 | Category I | 3 |
| Potassium oleate | 143-18-0 | Solid | 4.93E-10 | 100% | Not Classified | Category III | 2 |
| Promethazine hydrochloride | 58-33-3 | Solid | 0 | 100% | Category 1 | Category I | 3 |
| Propasol solvent P | 1569-01-3 | Liquid | 0.180 | 100% | Category 2B | Category II | 2 |
| Propylene glycol | 57-55-6 | Liquid | 0.0148 | 100% | Not Classified | Category IV | 1 |
| Pyridine | 110-86-1 | Liquid | 2.58 | 100% | Category 1 | Category I | 2 |
| Rinse A | NA | Liquid | NA | 100% | Not Classified | Category III | 2 |
| Rinse B | NA | Liquid | NA | 100% | Category 2B | Category III | 2 |
| Rinse C | NA | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Rinse D | NA | Liquid | NA | 100% | Not Classified | Category III | 1 |
| Shampoo A | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Shampoo B | NA | Liquid | NA | 100% | Category 1 | Category I | 2 |
| Shampoo C | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Shampoo D | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Sodium 2-naphthalenesulfonate | 532-02-5 | Solid | NA | 100% | Not Classified | Category III | 2 |
| Sodium hydroxide | 1310-73-2 | Solid | 6.53E-21 | 10% | Category 1 | Category I | 3 |
| Sodium lauryl sulfate | 151-21-3 | Solid | 2.40E-13 | 100% | ≥Category 2A | Category III | 3 |
| Sodium lauryl sulfate (15%) | 151-21-3 | Solid | NA | 15% | Category 1 | Category I | 3 |
| Sodium monochloroacetate | 3926-62-3 | Solid | 4.23E-09 | 100% | Category 2B | Category III | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Sodium polyoxyethylene(3) lauryl ether sulfate | 9004-82-4 | Liquid | 2.27E-13 | 100% | Category 1 | Category I | 3 |
| Sodium salicylate | 54-21-7 | Solid | 4.84E-12 | 100% | Category 1 | Category I | 1 |
| Sorbitan monolaurate | 1338-39-2 | Liquid | 1.25E-15 | 100% | Not Classified | Category IV | 2 |
| Stearyltrimethylammonium chloride | 112-03-8 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Styrene | 100-42-5 | Liquid | 0.673 | 100% | Not Classified | Category III | 1 |
| Toluene | 108-88-3 | Liquid | 3.16 | 100% | ≥Category 2B | Category III | 1 |
| Triethanolamine | 102-71-6 | Liquid | 4.51E-07 | 100% | Not Classified | Category III | 1 |
| Triethanolamine polyoxyethylene(3.0) lauryl ether sulfate | 27028-82-6 | Liquid | 2.50E-10 | 100% | Category 1 | Category I | 3 |
| Triton X-100 | 9002-93-1 | Liquid | 0 | 100% | Category 1 | Category I | 3 |
| Triton X-100 (10%) | 9002-93-1 | Liquid | 9.32E-04 | 10% | Category 1 | Category I | 2 |
| Tween 20 | 9005-64-5 | Liquid | 0 | 100% | Not Classified | Category III | 2 |
| Tween 80 | 9005-65-6 | Liquid | 0 | 100% | Not Classified | Category IV | 1 |

Abbreviations: CASRN = CAS Registry Number® (American Chemical Society); EPA = U.S. Environmental Protection Agency; GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; JaCVAM = Japanese Center for the Validation of Alternative Methods; kPa = kilopascals; NA = not available; STE = short time exposure.

[a] A top-down approach is used to distinguish severe eye irritants or corrosives (i.e., GHS Category 1, EPA Category I, or STE Rank 3) from all other hazard or no hazard categories (i.e., GHS Category 2A, 2B, Not Classified; EPA Category II, III, IV; or STE Rank 1 or 2).

[b] Vapor pressure is expressed in kilopascals at 25°C. Vapor pressures were found using the Hazardous Substances Data Bank (HSDB® [U.S. National Library of Medicine]), available at http://toxnet.nlm.nih.gov (accessed 2/25/2013) or from ChemSpider (available at www.chemspider.com [accessed 2/25/2013]). If actual values were not available, predicted values were obtained from the U.S. EPA EPI (Estimation Programs Interface) Suite™ for Microsoft® Windows, v. 4.11) or ACD/Labs' ACD/PhysChem Suite available at http://www.acdlabs.com/products/pc_admet/physchem/physchemsuite/ (accessed 2/25/2013). Data from the EPI Suite and ACD/PhysChem Suite programs were also available in ChemSpider.

[c] The concentration as tested in the rabbit eye test, based on NICEATM data. For substances tested at 100%, the starting material was tested neat/undiluted.

[d] The consensus classification of two or more studies. When there was no consensus using either the GHS (UN 2011) or EPA (EPA 2012) eye hazard classification system (e.g., one GHS Category 2A and one GHS Category 2B), the more hazardous classification (i.e., GHS Category 2A) was used as the consensus classification.

[e] STE rank scores from Kao Corporation were equated to the GHS or EPA classification of eye hazard (i.e., UN 2011 and EPA 2012) such that an STE rank of 3 was considered a severe eye irritant or corrosive (i.e., GHS Category 1 or EPA Category I); an STE rank of 2 was considered a moderate to mild eye irritant (i.e., GHS Category 2A or 2B or EPA Category II or III); and an STE rank of 1 was considered to be equivalent to GHS Not Classified or EPA Category IV (minimal effects clearing in less than 24 hours).

**Table 2-2 Test Substances Used to Evaluate STE Performance in a Bottom-Up Approach[a]**

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| 1-Bromo-4-chlorobutane | 6940-78-9 | Liquid | 0.164 | 100% | Not Classified | Category IV | 1 |
| 1-Dodecanaminium, N-(2-hydroxy-3-sulfopropyl)-N,N-dimethyl-, inner salt | 13197-76-7 | Liquid | 1.16E-21 | 100% | Category 1 | Category I | 2 |
| 1-Methylpropyl benzene | 135-98-8 | Liquid | 0.176 | 100% | Not Classified | Category IV | 1 |
| 1-Octanol | 111-87-5 | Liquid | 0.0132 | 100% | Category 2A | Category II | 2 |
| 1,3-Di-isopropylbenzene | 99-62-7 | Liquid | 0.041 | 100% | Not Classified | Category IV | 1 |
| 1,5-Hexadiene | 592-42-7 | Liquid | 28.6 | 100% | Not Classified | Category III | 1 |
| 1,9-Decadiene | 1647-16-1 | Liquid | 0.320 | 100% | Not Classified | Category IV | 1 |
| 2-Benzyloxyethanol | 622-08-2 | Liquid | 294E-04 | 100% | Category 2A | Category II | 2 |
| 2-Ethoxyethyl acetate (Cellosolve acetate) | 111-15-9 | Liquid | 0.397 | 100% | Not Classified | Category III | 1 |
| 2-Ethyl-1-hexanol | 104-76-7 | Liquid | 0.245 | 100% | Category 2A | Category II | 2 |
| 2-Ethylhexyl p-dimethylamino benzoate | 21245-02-3 | Liquid | 4.72E-06 | 100% | Not Classified | Category IV | 1 |
| 2-Methyl-1-pentanol | 105-30-6 | Liquid | 0.191 | 100% | Category 2B | Category III | 2 |
| 2-Methylbutyric acid | 116-53-0 | Liquid | 0.149 | 100% | Category 1 | Category I | 2 |
| 2-Methylpentane | 107-83-5 | Liquid | 27.8 | 100% | Not Classified | Category IV | 1 |
| 2-Naphthalenesulfonic acid,6-hydroxy-,monosodium salt, polymer with formaldehyde and hydroxymethylbenzenesulfonic aid monosodium salt | 85255-76-1 | Liquid | NA | 100% | Category 1 | Category II | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| 2,2-Dimethyl-3-pentanol | 3970-62-5 | Liquid | 0.413 | 100% | Not Classified | Category III | 1 |
| 2,4-Pentanediol | 625-69-4 | Liquid | 7.3E-03 | 100% | Not Classified | Category IV | 1 |
| 2,5-Dimethyl-2,5-hexanediol | 110-03-2 | Solid | 578E-04 | 100% | Category 1 | Category I | 1 |
| 3-Methoxy-1,2-propanediol | 623-39-2 | Liquid | 1.92E-03 | 100% | Not Classified | Category IV | 1 |
| 3-Methylhexane | 589-34-4 | Liquid | 8.29 | 100% | Not Classified | Category IV | 1 |
| 3,3-Dimethylpentane | 562-49-2 | Liquid | 10.1 | 100% | Not Classified | Category IV | 1 |
| Acetic acid | 64-19-7 | Liquid | 2.29 | 10% | Category 1 | Category I | 2 |
| Acetone | 67-64-1 | Liquid | 33.2 | 100% | Category 2A | Category II | 2 |
| Acid red 92 | 18472-87-2 | Solid | 5.71E-24 | 100% | Category 1 | Category I | 3 |
| Acrylic acid homopolymer sodium salt | 9003-04-7 | Solid | 4.56E-04 | 100% | Not Classified | Category IV | 1 |
| Ammonium nitrate | 6484-52-2 | Solid | 4.48E-16 | 100% | Category 2B | Category III | 1 |
| Benzalkonium chloride | 8001-54-5 | Liquid | NA | 100% | Category 1 | Category I | 3 |
| Benzalkonium chloride (10%) | 63449-41-2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Benzene, 1,1'-oxybis-, tetrapropylene derivatives, sulfonated, sodium salts | 119345-04-9 | Solid | NA | 100% | Category 1 | Category I | 3 |
| Benzyl alcohol | 100-51-6 | Liquid | 7.14E-03 | 100% | Category 1 | Category I | 2 |
| Body shampoo A | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Butanol | 71-36-3 | Liquid | 1.04 | 100% | Category 1 | Category I | 2 |
| Butyl acetate | 123-86-4 | Liquid | 1.595 | 100% | Not Classified | Category III | 1 |
| Butyl cellosolve | 111-76-2 | Liquid | 0.0633 | 100% | Category 1 | Category II | 2 |
| Butylnaphthalenesulfonic acid sodium salt | 25638-17-9 | Solid | NA | 100% | Category 1 | Category I | 2 |
| Butyrolactone | 96-48-0 | Liquid | 0.0394 | 100% | Category 2A | Category II | 2 |
| Calcium thioglycolate | 5793-98-6 | Solid | 4.20E-03 | 100% | Category 1 | Category I | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Camphene | 79-92-5 | Solid | 0.237 | 100% | Category 2B | Category III | 1 |
| Cetyl trimethyl ammonium chloride | 112-02-7 | Liquid | NA | 5% | Category 1 | Category I | 2 |
| Cetylpyridinium bromide | 140-72-7 | Solid | 3.47E-07 | 10% | Category 1 | Category I | 3 |
| Cetylpyridinium chloride | 6004-24-6 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cetyltrimethylammonium bromide | 57-09-0 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cetyltrimethylammonium bromide (10%) | 57-09-0 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cyclohexanol | 108-93-0 | Liquid | 0.0866 | 100% | Category 1 | Category I | 2 |
| Cyclohexanone | 108-94-1 | Liquid | 539 | 100% | Not Classified | Category III | 2 |
| Cyclopentanol | 96-41-3 | Liquid | 307 | 100% | Category 2B | Category II | 2 |
| Di-n-propyl disulphide | 629-19-6 | Liquid | 0.0664 | 100% | Not Classified | Category IV | 1 |
| Di(2-Ethylhexyl) sodium sulfosuccinate | 577-11-7 | Solid | 1.63E-15 | 10% | Category 1 | Category I | 3 |
| Di(propylene glycol) propyl ether | 29911-27-1 | Liquid | 2.38E-04 | 100% | Category 2B | Category III | 2 |
| Diethylethanolamine | 100-37-8 | Liquid | 0.0863 | 100% | Category 1 | Category I | 2 |
| Diisobutyl ketone | 108-83-8 | Liquid | 0.287 | 100% | Not Classified | Category IV | 1 |
| Dimethyl sulfoxide | 67-68-5 | Liquid | 0.0829 | 100% | Not Classified | Category III | 1 |
| Distearyldimethylammonium chloride | 107-64-2 | Solid | 2.55E-15 | 100% | Category 1 | Category I | 2 |
| Dodecane | 112-40-3 | Liquid | 0.0315 | 100% | Not Classified | Category III | 1 |
| Domiphen bromide | 538-71-6 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Ethanol | 64-17-5 | Liquid | 8.12 | 100% | Category 2A | Category I | 1 |
| Ethyl 2-methylacetoacetate | 609-14-3 | Liquid | 0.0915 | 100% | Category 2B | Category III | 2 |
| Ethyl acetate | 141-78-6 | Liquid | 13.1 | 100% | Not Classified | Category III | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Ethyl trimethyl acetate | 3938-95-2 | Liquid | 2.240 | 100% | Not Classified | Category III | 1 |
| Ethylhexyl salicylate | 118-60-5 | Liquid | 9.51E-07 | 100% | Not Classified | Category IV | 1 |
| Glycerol | 56-81-5 | Liquid | 1.06E-05 | 100% | Not Classified | Category IV | 1 |
| Glycidyl methacrylate | 106-91-2 | Liquid | 0.0829 | 100% | Not Classified | Category III | 2 |
| Imidazole | 288-32-4 | Solid | 5.78E-04 | 100% | Category 1 | Category I | 2 |
| Iso-octyl acrylate | 29590-42-9 | Liquid | 0.0204 | 100% | Not Classified | Category IV | 1 |
| Isobutanal | 78-84-2 | Liquid | 21.9 | 100% | Category 2B | Category III | 2 |
| Isobutyl alcohol | 78-83-1 | Liquid | 1.78 | 100% | Category 1 | Category I | 2 |
| Isopropyl alcohol | 67-63-0 | Liquid | 6.61 | 100% | Category 2A | Category III | 1 |
| Isopropyl bromide | 75-26-3 | Liquid | 28.5 | 100% | Not Classified | Category IV | 1 |
| Isopropyl myristate | 110-27-0 | Liquid | .108E-04 | 100% | Not Classified | Category IV | 1 |
| Lactic acid | 50-21-5 | Liquid | 3.81E-03 | 100% | Category 1 | Category I | 2 |
| Lauric acid | 143-07-7 | Solid | 2.13E-09 | 100% | ≥Category 2A | ≥Category II | 2 |
| Lauryldimethylamine oxide | 1643-20-5 | Solid | 1.68E-15 | 100% | Category 1 | Category I | 3 |
| Lotion A | NA | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| m-Phenylene diamine | 108-45-2 | Solid | 2.51E-04 | 100% | Category 1 | Category I | 2 |
| Methoxyethyl acrylate | 3121-61-7 | Liquid | 0.598 | 100% | Category 1 | ≥Category III | 2 |
| Methyl acetate | 79-20-9 | Liquid | 7.03 | 100% | Category 2A | Category II | 1 |
| Methyl amyl ketone | 110-43-0 | Liquid | 0.655 | 100% | Not Classified | Category III | 1 |
| Methyl cyanoacetate | 105-34-0 | Liquid | 0.0469 | 100% | Category 2A | Category II | 2 |
| Methyl cyclopentane | 96-37-7 | Liquid | 17.8 | 100% | Not Classified | Category III | 1 |
| Methyl ethyl ketone (2-Butanone) | 78-93-3 | Liquid | 13.1 | 100% | Category 2A | Category III | 2 |
| Methyl isobutyl ketone | 108-10-1 | Liquid | 2.90 | 100% | Not Classified | Category III | 1 |
| Methyl trimethyl acetate | 598-98-1 | Liquid | 4.76 | 100% | Not Classified | Category IV | 1 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Myristyl alcohol | 112-72-1 | Solid | 2.69E-05 | 100% | Category 2A | Category III | 1 |
| n-Butanal | 123-72-8 | Liquid | 14.4 | 100% | Category 2B | Category III | 2 |
| n-Hexanol | 111-27-3 | Liquid | 0.117 | 100% | Category 2A | Category II | 2 |
| n-Hexyl bromide | 111-25-1 | Liquid | 0.541 | 100% | Not Classified | Category IV | 1 |
| n-Octyl bromide | 111-83-1 | Liquid | 0.0691 | 100% | Not Classified | Category IV | 1 |
| n,n-Dimethylguanidine sulfate | 598-65-2 | Solid | 4.04 | 100% | Not Classified | Category III | 1 |
| Naphthalenesulfonic acid, butyl-, polymer with formaldehyde and 2-naphthalenesulfonic acid, sodium salt | 188070-49-7 | Solid | NA | 100% | Category 2A | Category II | 2 |
| Polyethylene glycol 400 | 25322-68-3 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Polyethyleneglycol monolaurate (10 E.O.) | 9004-81-3 | Liquid | 0 | 100% | Not Classified | Category IV | 2 |
| Polyoxyethylene hydrogenated castor oil (60E.O.) | 61788-85-0 | Solid | NA | 100% | Not Classified | Category IV | 1 |
| Polyoxyethylene(10) polyoxy propylene(1.5) lauryl-myristyl ether | 68439-51-0 | Liquid | NA | 100% | Category 1 | Category I | 3 |
| Polyoxyethylene(13) (mono-, di-, tri-)styrenated phenyl ether | 104376-75-2 | Liquid | NA | 100% | Not Classified | Category III | 3 |
| Polyoxyethylene(14) tribenzy lated phenyl ether | 116998-28-8 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Polyoxyethylene(160) sorbita n triisostearate | 54392-28-8 | Solid | NA | 100% | Not Classified | Category IV | 1 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Polyoxyethylene(19) (mono-, di-, tri-) styrenated phenyl ether | 104376-75-2 | Liquid | NA | 100% | Not Classified | Category II | 2 |
| Polyoxyethylene(20) hydrogenated tallow amine | 61790-82-7 | Solid | NA | 100% | ≥Category 2A | ≥Category II | 3 |
| Polyoxyethylene(23) lauryl ether | 9002-92-0 | Solid | 2.03E-13 | 100% | Category 2A | Category III | 2 |
| Polyoxyethylene(40) hydrogenated castor oil | 61788-85-0 | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Potassium laurate | 10124-65-9 | Solid | 0 | 10% | Category 1 | Category I | 3 |
| Potassium oleate | 143-18-0 | Solid | 4.93E-10 | 100% | Not Classified | Category III | 2 |
| Promethazine hydrochloride | 58-33-3 | Solid | 0 | 100% | Category 1 | Category I | 3 |
| Propasol solvent P | 1569-01-3 | Liquid | 0.180 | 100% | Category 2B | Category II | 2 |
| Propylene glycol | 57-55-6 | Liquid | 0.0148 | 100% | Not Classified | Category IV | 1 |
| Pyridine | 110-86-1 | Liquid | 2.58 | 100% | Category 1 | Category I | 2 |
| Quaternary ammonium compounds, benzyl-C12-16-alkyldimethyl, chlorides | 68424-85-1 | Solid | NA | 1% | Category 1 | Category I | 2 |
| Quaternary ammonium compounds, di-C12-15-alkyldimethyl, chlorides | 68910-56-5 | Solid | NA | 10% | Category 1 | Category I | 2 |
| Rinse A | NA | Liquid | NA | 100% | Not Classified | Category III | 2 |
| Rinse B | NA | Liquid | NA | 100% | Category 2B | Category III | 2 |
| Rinse C | NA | Liquid | NA | 100% | Not Classified | Category IV | 1 |
| Rinse D | NA | Liquid | NA | 100% | Not Classified | Category III | 1 |
| Shampoo A | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Shampoo B | NA | Liquid | NA | 100% | Category 1 | Category I | 2 |
| Shampoo C | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |

| Test Substance | CASRN | Physical Form as Tested | Vapor Pressure (kPa 25°C)[b] | *In Vivo* Conc (%)[c] | GHS Consensus[d] | EPA Consensus[d] | Overall Consensus STE Rank[e] |
|---|---|---|---|---|---|---|---|
| Shampoo D | NA | Liquid | NA | 100% | Category 2A | Category II | 2 |
| Sodium 2-naphthalenesulfonate | 532-02-5 | Solid | NA | 100% | Not Classified | Category III | 2 |
| Sodium hydroxide | 1310-73-2 | Solid | 6.53E-22 | 10% | Category 1 | Category I | 3 |
| Sodium lauryl sulfate | 151-21-3 | Solid | 2.40E-13 | 100% | ≥Category 2A | Category III | 3 |
| Sodium lauryl sulfate (15%) | 151-21-3 | Solid | NA | 15% | Category 1 | Category I | 3 |
| Sodium monochloroacetate | 3926-62-3 | Solid | 4.23E-09 | 100% | Category 2B | Category III | 2 |
| Sodium polyoxyethylene(3) lauryl ether sulfate | 9004-82-4 | Liquid | 2.27E-13 | 100% | Category 1 | Category I | 3 |
| Sodium salicylate | 54-21-7 | Solid | 4.84E-12 | 100% | Category 1 | Category I | 1 |
| Sorbitan monolaurate | 1338-39-2 | Liquid | 1.25E-15 | 100% | Not Classified | Category IV | 2 |
| Stearyltrimethylammonium chloride | 112-03-8 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Styrene | 100-42-5 | Liquid | 0.673 | 100% | Not Classified | Category III | 1 |
| Sucrose fatty acid ester | NA | Solid | NA | 100% | ≥Category 2A | ≥Category II | 2 |
| Toluene | 108-88-3 | Liquid | 3.160 | 100% | ≥Category 2B | Category III | 1 |
| Triethanolamine | 102-71-6 | Liquid | 4.51E-07 | 100% | Not Classified | Category III | 1 |
| Triethanolamine polyoxyethylene(3.0) lauryl ether sulfate | 27028-82-6 | Liquid | 2.50E-10 | 100% | Category 1 | Category I | 3 |
| Triton X-100 | 9002-93-1 | Liquid | 0 | 100% | Category 1 | Category I | 3 |
| Tween 20 | 9005-64-5 | Liquid | 0 | 100% | Not Classified | Category III | 2 |
| Tween 80 | 9005-65-6 | Liquid | 0 | 100% | Not Classified | Category IV | 1 |
| Xylene | 1330-20-7 | Liquid | 0.883 | 100% | Not Classified | Category II | 1 |

Abbreviations: CASRN = CAS Registry Number® (American Chemical Society); EPA = U.S. Environmental Protection Agency; GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; JaCVAM = Japanese Center for the Validation of Alternative Methods; kPa = kilopascals; NA = not available; STE = short time exposure.

[a] A bottom-up approach is used to distinguish GHS Not Classified or EPA Category IV (minimal effects clearing in less than 24 hours) and STE Rank 1 from all other hazard categories (i.e., GHS Category 1, 2A, 2B; EPA Category I, II, III; or STE Rank 2 and 3).

[b] Vapor pressure is expressed in kilopascals at 25°C. Vapor pressures were found using the Hazardous Substances Data Bank (HSDB® [U.S. National Library of Medicine]), available at http://toxnet.nlm.nih.gov (accessed 2/25/2013) or from ChemSpider (available at www.chemspider.com [accessed 2/25/2013]). If actual values were not available, predicted values were obtained from the U.S. EPA EPI (Estimation Programs Interface) Suite™ for Microsoft® Windows, v. 4.11) or ACD/Labs' ACD/PhysChem Suite available at http://www.acdlabs.com/products/pc_admet/physchem/physchemsuite/ (accessed 2/25/2013). Data from the EPI Suite and ACD/PhysChem Suite programs were also available in ChemSpider.

[c] The concentration as tested in the rabbit eye test, based on NICEATM data. For substances tested at 100%, the starting material was tested neat/undiluted.

[d] The consensus classification of two or more studies. When there was no consensus using either the GHS (UN 2011) or EPA (EPA 2012) eye hazard classification system (e.g., one GHS Category 2A and one GHS Category 2B), the more hazardous classification (i.e., GHS Category 2A) was used as the consensus classification.

[e] STE rank scores from Kao Corporation were equated to the GHS or EPA classification of eye hazard (i.e., UN 2011 and EPA 2012) such that an STE rank of 3 was considered a severe eye irritant or corrosive (i.e., GHS Category 1 or EPA Category I); an STE rank of 2 was considered a moderate to mild eye irritant (i.e., GHS Category 2A or 2B or EPA Category II or III); and an STE rank of 1 was considered to be equivalent to GHS Not Classified or EPA Category IV (minimal effects clearing in less than 24 hours).

**Table 2-3 Test Substances Used to Evaluate STE Performance in a Bottom-Up Approach Within the Defined Applicability Domain[a,b]**

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25°C)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| 1-Bromo-4-chlorobutane | 6940-78-9 | 1,2 | Liquid | 1.640E-01 | 100% | Not classified | Category IV | 1 |
| 1-Dodecanaminium, N-(2-hydroxy-3-sulfopropyl)-N,N-dimethyl-, inner salt | 13197-76-7 | 1,2 | Liquid | 1.160E-21 | neat | Category 1 | Category I | 2 |
| 1-Methylpropyl benzene | 135-98-8 | 1,2 | Liquid | 1.760E-01 | 100% | Not classified | Category IV | 1 |
| 1-Octanol | 111-87-5 | 1,2 | Liquid | 1.320E-02 | 100% | Category 2A | Category II | 2 |
| 1,3-Di-isopropylbenzene | 99-62-7 | 1,2 | Liquid | 4.100E-02 | 100% | Not classified | Category IV | 1 |
| 1,9-Decadiene | 1647-16-1 | 1,2 | Liquid | 3.200E-01 | 100% | Not classified | Category IV | 1 |
| 2-Benzyloxyethanol | 622-08-2 | 1,2 | Liquid | 2.940E-04 | 100% | Category 2A | Category II | 2 |
| 2-Ethoxyethyl acetate (Cellosolve acetate) | 111-15-9 | 1,2 | Liquid | 3.970E-01 | 100% | Not classified | Category III | 1 |
| 2-Ethyl-1-hexanol | 104-76-7 | 1,2 | Liquid | 2.460E-02 | 100% | Category 2A | Category II | 2 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| 2-Ethylhexyl p-dimethylamino benzoate | 21245-02-3 | 1,2 | Liquid | 4.720E-06 | 100% | Not classified | Category IV | 1 |
| 2-Methyl-1-pentanol | 105-30-6 | 1,2 | Liquid | 1.910E-01 | 100% | Category 2B | Category III | 2 |
| 2-Methylbutyric acid | 116-53-0 | 1,2 | Liquid | 1.490E-01 | 100% | Category 1 | Category I | 2 |
| 2-Naphthalenesulfonic acid,6-hydroxy-,monosodium salt, polymer with formaldehyde and hydroxymethylbenzenesulfonic aid monosodium salt | 85255-76-1 | 1,2 | Liquid | NA | neat | Category 1 | Category II | 2 |
| 2,2-Dimethyl-3-pentanol | 3970-62-5 | 1,2 | Liquid | 4.130E-01 | 100% | Not classified | Category III | 1 |
| 2,4-Pentanediol | 625-69-4 | 1,2 | Liquid | 7.300E-03 | 100% | Not classified | Category IV | 1 |
| 3-Methoxy-1,2-propanediol | 623-39-2 | 1,2 | Liquid | 1.920E-03 | 100% | Not classified | Category IV | 1 |
| Acid red 92 | 18472-87-2 | 1 | Solid | 5.710E-24 | 100% | Category 1 | Category I | 3 |
| Acrylic acid homopolymer sodium salt | 9003-04-7 | 2 | Solid | 4.560E-04 | neat | Not classified | Category IV | 1 |
| Benzalkonium chloride | 8001-54-5 | 1,2 | Liquid | NA | 100% | Category 1 | Category I | 3 |
| Benzalkonium chloride (10%) | 63449-41-2 | 2 | Solid | NA | 10 | Category 1 | Category I | 3 |
| Benzene, 1,1'-oxybis-, tetrapropylene derivs., sulfonated, sodium salts | 119345-04-9 | 2 | Solid | NA | neat | Category 1 | Category I | 3 |
| Benzyl alcohol | 100-51-6 | 1,2 | Liquid | 7.140E-03 | 100% | Category 1 | Category I | 2 |
| Body shampoo A | NA | 1,2 | Liquid | NA | 100 | Category 2A | Category II | 2 |
| Butanol | 71-36-3 | 1,2 | Liquid | 1.040E+00 | 100% | Category 1 | Category I | 2 |
| Butyl acetate | 123-86-4 | 1,2 | Liquid | 1.587E+00 | 100% | Not classified | Category III | 1 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| Butyl cellosolve | 111-76-2 | 1,2 | Liquid | 6.330E-02 | 100% | Category 1 | Category II | 2 |
| Butylnaphthalenesulfonic acid sodium salt | 25638-17-9 | 2 | Solid | NA | neat | Category 1 | Category I | 2 |
| Butyrolactone | 96-48-0 | 1,2 | Liquid | 3.940E-02 | 100% | Category 2A | Category II | 2 |
| Cetylpyridinium bromide (10%) | 140-72-7 | 2 | Solid | 3.470E-07 | 10% | Category 1 | Category I | 3 |
| Cetylpyridinium chloride | 6004-24-6 | 2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Cetyltrimethylammonium bromide (10%) | 57-09-0 | 2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Calcium thioglycolate | 5793-98-6 | 1 | Solid | 4.200E-03 | 100% | Category 1 | Category I | 2 |
| Cyclohexanol | 108-93-0 | 1,2 | Liquid | 8.660E-02 | 100% | Category 1 | Category I | 2 |
| Cyclohexanone | 108-94-1 | 1,2 | Liquid | 5.390E-01 | 100% | Not classified | Category III | 2 |
| Cyclopentanol | 96-41-3 | 1,2 | Liquid | 3.070E-01 | 100% | Category 2B | Category II | 2 |
| Di-n-propyl disulphide | 629-19-6 | 1,2 | Liquid | 6.640E-02 | 100% | Not classified | Category IV | 1 |
| Di(2-Ethylhexyl) sodium sulfosuccinate | 577-11-7 | 1,2 | Solid | 1.630E-15 | 10% | Category 1 | Category I | 3 |
| Di(propylene glycol) propyl ether | 29911-27-1 | 1,2 | Liquid | 2.380E-04 | 100% | Category 2B | Category III | 2 |
| Diethylethanolamine | 100-37-8 | 1,2 | Liquid | 8.630E-02 | 100% | Category 1 | Category I | 2 |
| Diisobutyl ketone | 108-83-8 | 1,2 | Liquid | 2.870E-01 | 100% | Not classified | Category IV | 1 |
| Dimethyl sulfoxide | 67-68-5 | 1,2 | Liquid | 8.290E-02 | 100% | Not classified | Category III | 1 |
| Distearyldimethylammonium chloride | 107-64-2 | 1,2 | Solid | 2.550E-15 | 100% | Category 1 | Category I | 2 |
| Dodecane | 112-40-3 | 1,2 | Liquid | 3.150E-02 | 100% | Not classified | Category III | 1 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| Domiphen bromide | 538-71-6 | 1,2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Ethyl 2-methylacetoacetate | 609-14-3 | 1,2 | Liquid | 9.150E-02 | 100% | Category 2B | Category III | 2 |
| Ethyl trimethyl acetate | 3938-95-2 | 1,2 | Liquid | 2.240E+00 | 100% | Not classified | Category III | 1 |
| Ethylhexyl salicylate | 118-60-5 | 1,2 | Liquid | 9.510E-07 | 100% | Not classified | Category IV | 1 |
| Glycerol | 56-81-5 | 1,2 | Liquid | 1.060E-05 | 100% | Not classified | Category IV | 1 |
| Glycidyl methacrylate | 106-91-2 | 1,2 | Liquid | 8.290E-02 | 100% | Not classified | Category III | 2 |
| Imidazole | 288-32-4 | 1 | Solid | 5.780E-04 | 100% | Category 1 | Category I | 2 |
| Iso-octyl acrylate | 29590-42-9 | 1,2 | Liquid | 2.040E-02 | 100% | Not classified | Category IV | 1 |
| Isobutyl alcohol | 78-83-1 | 1,2 | Liquid | 1.780E+00 | 100% | Category 1 | Category I | 2 |
| Isopropyl myristate | 110-27-0 | 1,2 | Liquid | 1.080E-04 | 100% | Not classified | Category IV | 1 |
| Lactic acid | 50-21-5 | 1,2 | Liquid | 3.810E-03 | 100% | Category 1 | Category I | 2 |
| Lauric acid | 143-07-7 | 1,2 | Solid | 2.130E-09 | neat | ≥Category 2A | ≥Category II | 2 |
| Lauryldimethylamine oxide | 1643-20-5 | 2 | Solid | 1.680E-15 | neat | Category 1 | Category I | 3 |
| Lotion A | NA | 1,2 | Liquid | NA | 100 | Not classified | Category IV | 1 |
| m-Phenylene diamine | 108-45-2 | 1 | Solid | 2.510E-04 | 100% | Category 1 | Category I | 2 |
| Methoxyethyl acrylate | 3121-61-7 | 1,2 | Liquid | 5.980E-01 | 100% | Category 1 | ≥Category III | 2 |
| Methyl amyl ketone | 110-43-0 | 1,2 | Liquid | 6.550E-01 | 100% | Not classified | Category III | 1 |
| Methyl cyanoacetate | 105-34-0 | 1,2 | Liquid | 4.690E-02 | 100% | Category 2A | Category II | 2 |
| Methyl isobutyl ketone | 108-10-1 | 1,2 | Liquid | 2.900E+00 | 100% | Not classified | Category III | 1 |
| Methyl trimethyl acetate | 598-98-1 | 1,2 | Liquid | 4.760E+00 | 100% | Not classified | Category IV | 1 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| n-Hexanol | 111-27-3 | 1,2 | Liquid | 1.170E-01 | 100% | Category 2A | Category II | 2 |
| n-Hexyl bromide | 111-25-1 | 1,2 | Liquid | 5.410E-01 | 100% | Not classified | Category IV | 1 |
| n-Octyl bromide | 111-83-1 | 1,2 | Liquid | 6.910E-02 | 100% | Not classified | Category IV | 1 |
| Naphthalenesulfonic acid, butyl-, polymer with formaldehyde and 2-naphthalenesulfonic acid, sodium salt | 188070-49-7 | 2 | Solid | NA | neat | Category 2A | Category II | 2 |
| n,n-Dimethylguanidine sulfate | 598-65-2 | 1 | Solid | 4.040E+00 | 100% | Not classified | Category III | 1 |
| Polyethylene glycol 400 | 25322-68-3 | 1,2 | Liquid | NA | 100% | Not classified | Category IV | 1 |
| Polyethyleneglycol monolaurate (10 E.O.) | 9004-81-3 | 1,2 | Liquid | 0.000E+00 | 100% | Not classified | Category IV | 2 |
| Polyoxyethylene hydrogenated castor Oil (60E.O.) | 61788-85-0 | 2 | Solid | NA | 100% | Not classified | Category IV | 1 |
| Polyoxyethylene(10) polyoxypropylene(1.5) lauryl-myristyl ether | 68439-51-0 | 1,2 | Liquid | NA | neat | Category 1 | Category I | 3 |
| Polyoxyethylene(13) (mono-, di-, tri-)styrenated phenyl ether | 104376-75-2 | 1,2 | Liquid | NA | neat | Not classified | Category III | 3 |
| Polyoxyethylene(14) tribenzylated phenyl ether | 116998-28-8 | 1,2 | Liquid | NA | neat | Not classified | Category IV | 1 |
| Polyoxyethylene(160) sorbitan triisostearate | 54392-28-8 | 1,2 | Solid | NA | neat | Not classified | Category IV | 1 |
| Polyoxyethylene(19) (mono-, di-, tri-)styrenated phenyl ether | 104376-75-2 | 1,2 | Liquid | NA | neat | Not classified | Category II | 2 |
| Polyoxyethylene(20) hydrogenated tallow amine | 61790-82-7 | 1,2 | Solid | NA | neat | ≥Category 2A | ≥Category II | 3 |
| Polyoxyethylene(23) lauryl ether | 9002-92-0 | 2 | Solid | 2.030E-13 | neat | Category 2A | Category III | 2 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| Polyoxyethylene(40) hydrogenated castor oil | 61788-85-0 | 1,2 | Liquid | NA | neat | Not classified | Category IV | 1 |
| Potassium laurate | 10124-65-9 | 1,2 | Solid | 0.000E+00 | 10% | Category 1 | Category I | 3 |
| Potassium oleate | 143-18-0 | 2 | Solid | 4.930E-10 | neat | Not classified | Category III | 2 |
| Promethazine hydrochloride | 58-33-3 | 1 | Solid | 0.000E+00 | 100% | Category 1 | Category I | 3 |
| Propasol solvent P | 1569-01-3 | 1,2 | Liquid | 1.800E-01 | 100% | Category 2B | Category II | 2 |
| Propylene glycol | 57-55-6 | 1,2 | Liquid | 1.480E-02 | 100% | Not classified | Category IV | 1 |
| Pyridine | 110-86-1 | 1,2 | Liquid | 2.580E+00 | 100% | Category 1 | Category I | 2 |
| Rinse A | NA | 1,2 | Liquid | NA | 100 | Not classified | Category III | 2 |
| Rinse B | NA | 1,2 | Liquid | NA | 100 | Category 2B | Category III | 2 |
| Rinse C | NA | 1,2 | Liquid | NA | 100 | Not classified | Category IV | 1 |
| Rinse D | NA | 1,2 | Liquid | NA | 100 | Not classified | Category III | 1 |
| Shampoo A | NA | 1,2 | Liquid | NA | 100 | Category 2A | Category II | 2 |
| Shampoo B | NA | 1,2 | Liquid | NA | 100 | Category 1 | Category I | 2 |
| Shampoo C | NA | 1,2 | Liquid | NA | 100 | Category 2A | Category II | 2 |
| Shampoo D | NA | 1,2 | Liquid | NA | 100 | Category 2A | Category II | 2 |
| Sodium hydroxide | 1310-73-2 | 1 | Solid | 6.530E-22 | 10% | Category 1 | Category I | 3 |
| Sodium lauryl sulfate | 151-21-3 | 2 | Solid | 2.400E-13 | 100% | ≥Category 2A | Category III | 3 |
| Sodium lauryl sulfate (15%) | 151-21-3 | 2 | Solid | NA | 15 | Category 1 | Category I | 3 |
| Sodium polyoxyethylene(3) lauryl ether sulfate | 9004-82-4 | 1,2 | Liquid | 2.270E-13 | neat | Category 1 | Category I | 3 |

| Substance | CASRN | App Domain[b] | Physical Form as Tested | Vapor Pressure (kPa 25ºC)[c] | NICEATM *In Vivo* Conc[d] (%) | GHS-NICEATM Consensus[e] | EPA-NICEATM Consensus[e] | JaCVAM Overall Consensus STE Rank[f] |
|---|---|---|---|---|---|---|---|---|
| Sorbitan monolaurate | 1338-39-2 | 1,2 | Liquid | 1.250E-15 | neat | Not classified | Category IV | 2 |
| Stearyltrimethylammonium chloride | 112-03-8 | 1,2 | Solid | NA | 10% | Category 1 | Category I | 3 |
| Styrene | 100-42-5 | 1,2 | Liquid | 6.730E-01 | 100% | Not classified | Category III | 1 |
| Sucrose fatty acid ester | NA | 1,2 | Solid | NA | 100% | ≥Category 2A | ≥Category II | 2 |
| Toluene | 108-88-3 | 1,2 | Liquid | 3.160E+00 | 100% | ≥Category 2B | Category III | 1 |
| Triethanolamine | 102-71-6 | 1,2 | Liquid | 4.510E-07 | 100% | Not classified | Category III | 1 |
| Triethanolamine polyoxyethylene(3.0) lauryl ether sulfate | 27028-82-6 | 1,2 | Liquid | 2.500E-10 | neat | Category 1 | Category I | 3 |
| Triton X-100 | 9002-93-1 | 1,2 | Liquid | 0.000E+00 | 100% | Category 1 | Category I | 3 |
| Tween 20 | 9005-64-5 | 1,2 | Liquid | 0.000E+00 | 100% | Not classified | Category III | 2 |
| Tween 80 | 9005-65-6 | 1,2 | Liquid | 0.000E+00 | 100% | Not classified | Category IV | 1 |
| Xylene | 1330-20-7 | 1,2 | Liquid | 8.826E-01 | 100% | Not classified | Category II | 1 |

Abbreviations: App = applicability; CASRN = CAS Registry Number® (American Chemical Society); EPA = U.S. Environmental Protection Agency; GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; JaCVAM = Japanese Center for the Validation of Alternative Methods; kPa = kilopascals; NA = not available; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; STE = short time exposure.

[a] A bottom-up approach is used to distinguish GHS Not Classified or EPA Category IV (minimal effects clearing in less than 24 hours) and STE Rank 1 from all other hazard categories (i.e., GHS Category 1, 2A, 2B; EPA Category I, II, III; or STE Rank 2 and 3).

[b] There are two defined applicability domains (AD) for the STE test method. AD 1 (n=94 substances) includes all substances with vapor pressures ≤6 kilopascals but excludes solids that are alcohols, hydrocarbons, or salts. AD 2 (n = 101 substances) includes all liquids with vapor pressures ≤6 kilopascals and solid surfactants or surfactant-containing formulations (i.e., nonsurfactant solids and substances with vapor pressures >6 kilopascals are excluded).

[c] Vapor pressure is expressed in kilopascals at 25°C. Vapor pressures were found using the Hazardous Substances Data Bank (HSDB® [U.S. National Library of Medicine]), available at http://toxnet.nlm.nih.gov (accessed 2/25/2013) or from ChemSpider (available at www.chemspider.com [accessed 2/25/2013]). If actual values were not available, predicted values were obtained from the U.S. EPA EPI (Estimation Programs Interface) Suite™ for Microsoft® Windows, v. 4.11) or ACD/Labs' ACD/PhysChem Suite available at http://www.acdlabs.com/products/pc_admet/physchem/physchemsuite/ (accessed 2/25/2013). Data from the EPI Suite and ACD/PhysChem Suite programs were also available in ChemSpider.

[d] The concentration as tested in the rabbit eye test, based on NICEATM data.

[e] The consensus classification of two or more studies. When there was no consensus using either the GHS (UN 2011) or EPA (EPA 2012) eye hazard classification system (e.g., one GHS Category 2A and one GHS Category 2B), the more hazardous classification (i.e., GHS Category 2A) was used as the consensus classification.

[f] STE rank scores were equated to the GHS or EPA classification of eye hazard (i.e., UN 2011 and EPA 2012) such that an STE rank of 3 was considered a severe eye irritant or corrosive (i.e., GHS Category 1 or EPA Category I); an STE rank of 2 was considered a moderate to mild eye irritant (i.e., GHS Category 2A or 2B or EPA Category II or III); and an STE rank of 1 was considered to be equivalent to GHS Not Classified or EPA Category IV (minimal effects clearing in less than 24 hours).

## 3.0 STE Test Method Performance

Test method performance is typically evaluated by calculating the following (ICCVAM 2003):

- Accuracy (concordance): the proportion of correct outcomes (positive and negative) of a test method
- Sensitivity: the proportion of all positive substances that are classified correctly as positive
- Specificity: the proportion of all negative substances that are classified correctly as negative
- Positive predictivity: the proportion of correct positive responses among substances testing positive
- Negative predictivity: the proportion of correct negative responses among substances testing negative
- False positive rate: the proportion of all negative substances that are falsely identified as positive
- False negative rate: the proportion of all positive substances that are falsely identified as negative

The STE test method performance was evaluated for each study, and the data set is provided in **Supplement B**. An overall STE ocular irritation classification was assigned for each test substance in the database based on the majority of ocular irritation classification calls. When a test substance had an even number of different irritation classifications (e.g., two tests classified a substance as a moderate irritant and two tests classified a substance as a severe irritant), the more severe hazard classification was used for its overall classification (e.g., severe irritant). Using the consensus ocular irritation classification for each substance, the STE test method was evaluated in a top-down approach to distinguish ocular corrosives and severe irritants (i.e., GHS Category 1 or EPA Category I) from all other categories (i.e., GHS Category 2A, 2B, Not Classified or EPA Category II, III, IV). The STE test method was also evaluated in a bottom-up approach to identify GHS Not Classified substances or EPA Category IV (minimally irritant) substances from all other irritant categories (i.e., GHS Category 1, 2A, or 2B or EPA Category I, II, or III).

The overall accuracy of the STE test method in a top-down approach ranged from 70% to 96%, and the accuracy in a bottom-up approach ranged from 80% to 85% depending on the classification. The predictive capacity of the STE test method was assessed by identifying the chemical classes or physical properties that increased the false positive rate in a top-down approach and those that increased the false negative rate in a bottom-up approach. Excluding discordant chemical classes or physical properties optimized the applicability domain for a top-down or bottom-up approach.

### 3.1 GHS Classification System: STE Performance in a Top-Down Approach

The performance of the STE test method was evaluated for GHS ocular hazard classification in a top-down approach. STE accuracy, sensitivity, specificity, false positive rate, and false negative rate were determined based on available *in vivo* reference data for the test substances. Test substances that were identified as direct MTT reducers were removed from the analyses. These include two substances from Kojima et al. (Kao BRD), two substances from Takahashi et al. (2010), and one substance from the Kao in-house studies. These analyses were performed for each of the five studies as well as for 120 unique substances from these five studies that remained after duplicates were removed and consensus classifications were assigned (**Table 3-1**). The GHS classification for each test substance is listed in **Supplement B**.

**Table 3-1 STE Performance for GHS Classification in a Top-Down Approach**

| Data Source | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] |
| Kojima et al. (Kao BRD) | 30 | 70 | 21/30 | 10 | 1/10 | 100 | 20/20 | 0 | 0/20 | 90 | 9/10 |
| Sakaguchi et al. 2011 | 23 | 96 | 22/23 | 80 | 4/5 | 100 | 18/18 | 0 | 0/18 | 20 | 1/5 |
| Takahashi et al. 2009 | 37 | 84 | 31/37 | 65 | 11/17 | 100 | 20/20 | 0 | 0/20 | 35 | 6/17 |
| Takahashi et al. 2010 | 47 | 83 | 39/47 | 58 | 11/19 | 100 | 28/28 | 0 | 0/28 | 42 | 8/19 |
| Kao In-House | 22 | 96 | 21/22 | 0 | 0/1 | 100 | 21/21 | 0 | 0/21 | 100 | 1/1 |
| Kao New Surfactants | 39 | 69 | 27/39 | 45 | 9/20 | 95 | 18/19 | 5.3 | 1/19 | 55 | 11/20 |
| Unique Substances[d] | 120 | 85 | 102/120 | 53 | 19/36 | 99 | 83/84 | 1.2 | 1/84 | 47 | 17/36 |

Abbreviations: BRD = background review document; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] Substances from all of the above studies remaining after duplicates were removed and consensus classifications were assigned.

### 3.1.1 STE Discordant Results for GHS Classification in a Top-Down Approach

The STE results that were discordant with *in vivo* results were analyzed further. These analyses were performed on specific categories of chemicals, as well as on certain physicochemical properties potentially relevant to ocular toxicity testing (e.g., surfactants, pH, physical form).

Several trends were noted in STE performance among these subgroups of substances (**Table 3-2**). Only one of 84 substances was overpredicted (i.e., false positive) and slightly affected the overprediction of its constituent chemical classes (3.6% to 8.3% overprediction). The chemical categories of substances that were most consistently underpredicted (i.e., false negatives) by the STE test method were alcohols and carboxylic acids. Of the 17 underpredicted substances, 7 were alcohols, 4 were carboxylic acids, and 3 were salts. Additional chemical categories represented among the underpredicted substances were esters (2) and heterocyclic compounds (2).

With regard to the physical form of the substances underpredicted by the STE test method, 12 were liquids and 5 were solids. Considering the proportion of the total available database, solids (16%; 5/31) and liquids (13%; 12/89) were underpredicted at a similar rate by the STE test method.

**Table 3-2 STE False Positive and False Negative Rates by Chemical Category and Properties of Interest for GHS Classification in a Top-Down Approach**

| Category | N | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] |
| **Overall** | 120 | 1.2 | 1/84 | 47 | 17/36 |
| ***Chemical Category***[d] | | | | | |
| Alcohol | 39 | 3.6 | 1/28 | 64 | 7/11 |
| Amine/Amidine | 8 | 0 | 0/2 | 17 | 1/6 |
| Carboxylic acid | 21 | 0 | 0/14 | 57 | 4/7 |
| Ester | 17 | 0 | 0/14 | 67 | 2/3 |
| Ether/Polyether | 16 | 8.3 | 1/12 | 0 | 0/4 |
| Heterocyclic compound | 9 | 0 | 0/3 | 33 | 2/6 |
| Hydrocarbon | 23 | 5.0 | 1/20 | 33 | 1/3 |
| Ketone | 8 | 0 | 0/8 | - | 0/0 |
| Onium compound | 10 | 0 | 0/1 | 11 | 1/9 |
| Salt | 17 | 0 | 0/6 | 27 | 3/11 |
| ***Properties of Interest*** | | | | | |
| **Liquids** | 89 | 1.4 | 1/72 | 71 | 12/17 |
| **Solids** | 31 | 0 | 0/12 | 26 | 5/19 |
| **Surfactants – Total** | 44 | 4.2 | 1/24 | 20 | 4/20 |
| -nonionic | 14 | 8.3 | 1/12 | 0 | 0/2 |
| -anionic | 11 | 0 | 0/3 | 25 | 2/8 |
| -cationic | 7 | - | 0/0 | 0 | 0/7 |
| -ampholyic | 2 | - | 0/0 | 50 | 1/2 |
| **pH – Total**[e] | 27 | 0 | 0/10 | 41 | 7/17 |
| -acidic (pH < 7.0) | 19 | 0 | 0/8 | 36 | 4/11 |
| -basic (pH > 7.0) | 7 | 0 | 0/1 | 50 | 3/6 |
| -equals 7 | 1 | 0 | 0/1 | - | 0/0 |
| **Vapor Pressure – Total** | 90 | 0 | 0/66 | 58 | 14/24 |
| >6kPa | 13 | 0 | 0/13 | - | 0/0 |
| ≤6kPa | 77 | 0 | 0/53 | 58 | 14/24 |

Abbreviations: GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); kPa = kilopascals; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] One or more chemical categories were assigned to each test substance based on the chemical categories outlined in the tree structure provided for that chemical in the National Library of Medicine's Medical Subject Headings (MeSH®) for inorganic or organic chemicals when available (http://www.nlm.nih.gov/mesh) and on the presence of common organic functional groups (i.e., ketones) if that functional group was not available in the MeSH tree structure.

[e] Total number of substances with pH data available.

**Table 3-3** shows the STE test method performance in a top-down approach when problematic categories are excluded that gave the most discordant results in the GHS classification system. In general, exclusion of alcohols, ethers/polyethers, hydrocarbons, or nonionic surfactants individually reduced false positive rates to 0% and marginally reduced or slightly increased false negative rates. The performance of validated *in vitro* methods for GHS classification is included for comparison.

**Table 3-3 STE Performance for GHS Classification in a Top-Down Approach After Excluding Discordant Categories**

| Method Evaluated | Accuracy | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|
| | % | No.[c] | % | No.[c] | % | No.[c] |
| STE Overall | 85 | 102/120 | 1.2 | 1/84 | 47 | 17/36 |
| STE w/o Alcohols | 87 | 71/82 | 0 | 0/56 | 42 | 11/26 |
| STE w/o Ethers/Polyethers | 84 | 87/104 | 0 | 0/72 | 53 | 17/32 |
| STE w/o Hydrocarbons | 84 | 81/97 | 0 | 0/64 | 49 | 16/33 |
| STE w/o Nonionic surfactants | 84 | 89/106 | 0 | 0/73 | 50 | 17/34 |
| BCOP | 79 | 149/188 | 24 | 29/123 | 15 | 10/65 |
| ICE | 83 | 120/144 | 8 | 9/114 | 50 | 15/30 |
| CM | 90 | 74/82 | 2 | 1/48 | 21 | 7/34 |

Abbreviations: BCOP = bovine corneal opacity and permeability; CM = Cytosensor microphysiometer; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); ICE = isolated chicken eye; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

## 3.2 GHS Classification System: STE Performance in a Bottom-Up Approach

The performance of the STE test method was evaluated for GHS ocular hazard classification in a bottom-up approach. STE accuracy, sensitivity, specificity, false positive rate, and false negative rate were determined based on available *in vivo* reference data for the test substances. Test substances that were identified as direct MTT reducers and classified as STE nonirritants were removed from the bottom-up analysis, as these could be false negative. These include two substances from Kojima et al. (Kao BRD), two substances from Takahashi et al. (2010), and one substance from the Kao in-house studies. These analyses were performed for each of the five studies as well as for 129 unique substances from these five studies that remained after duplicates were removed and consensus classifications were assigned (**Table 3-4**). The GHS classification for each test substance is listed in **Supplement B**.

**Table 3-4 STE Performance for GHS Classification in a Bottom-Up Approach**

| Data Source | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] |
| Kojima et al. (Kao BRD) | 31 | 71 | 22/31 | 73 | 19/26 | 60 | 3/5 | 40 | 2/5 | 27 | 7/26 |
| Sakaguchi et al. 2011 | 24 | 88 | 21/24 | 77 | 10/13 | 100 | 11/11 | 0 | 0/11 | 23 | 3/13 |
| Takahashi et al. 2009 | 39 | 87 | 34/39 | 85 | 23/27 | 92 | 11/12 | 8.3 | 1/12 | 15 | 4/27 |
| Takahashi et al. 2010 | 52 | 83 | 43/52 | 85 | 28/33 | 79 | 15/19 | 21 | 4/19 | 15 | 5/33 |
| Kao In-House | 22 | 96 | 21/22 | 100 | 1/1 | 95 | 20/21 | 4.8 | 1/21 | 0 | 0/1 |
| Kao New Surfactants | 34 | 85 | 29/34 | 100 | 22/22 | 58 | 7/12 | 42 | 5/12 | 0 | 0/22 |
| Unique Substances[d] | 129 | 85 | 109/129 | 88 | 64/73 | 80 | 45/56 | 20 | 11/56 | 12 | 9/73 |

Abbreviations: BRD = background review document; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] Substances from all of the above studies remaining after duplicates were removed and consensus classifications were assigned.

### 3.2.1 STE Discordant Results for GHS Classification in a Bottom-Up Approach

The STE results that were discordant with *in vivo* results were analyzed further. These analyses were performed on specific categories of chemicals, as well as on certain physicochemical properties potentially relevant to ocular toxicity testing (e.g., surfactants, pH, physical form).

Several trends were noted in STE performance among these subgroups of substances (**Table 3-5**). The overall false positive rate was 20%. The chemical categories of substances that the STE test method most consistently underpredicted for GHS classification (i.e., false negatives) were salts (13%; 2/15), hydrocarbons (33%; 2/6), and alcohols (16%; 4/25).

With regard to the physical form of the substances underpredicted by the STE test method, four were liquids and five were solids. Considering the proportion of the total available database, solids (14%; 5/37) appear more likely than liquids (4.3%; 4/92) to be underpredicted by the STE test method.

**Table 3-5 STE False Positive and False Negative Rates by Chemical Category and Properties of Interest for GHS Classification in a Bottom-Up Approach**

| Category | N | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] |
| **Overall** | 129 | 20 | 11/56 | 12 | 9/73 |
| ***Chemical Category***[d] | | | | | |
| Alcohol | 41 | 31 | 5/16 | 16 | 4/25 |
| Amine/Amidine | 17 | 0 | 0/2 | 0 | 0/15 |
| Carboxylic acid | 33 | 28 | 3/11 | 9.1 | 2/22 |
| Ester | 18 | 46 | 5/11 | 14 | 1/7 |
| Ether/Polyether | 16 | 38 | 3/8 | 0 | 0/8 |
| Heterocyclic compound | 10 | 50 | 1/2 | 0 | 0/8 |
| Hydrocarbon | 24 | 17 | 3/18 | 33 | 2/6 |
| Ketone | 8 | 20 | 1/5 | 0 | 0/3 |
| Onium compound | 12 | - | 0/0 | 8.3 | 1/12 |
| Salt | 18 | 67 | 2/3 | 13 | 2/15 |
| ***Properties of Interest*** | | | | | |
| **Liquids** | 92 | 18 | 9/50 | 9.5 | 4/42 |
| **Solids** | 37 | 33 | 2/6 | 16 | 5/31 |
| **Surfactants – Total** | 49 | 41 | 7/17 | 0 | 0/32 |
| -nonionic | 16 | 46 | 5/11 | 0 | 0/5 |
| -anionic | 12 | 50 | 1/2 | 0 | 0/10 |
| -cationic | 8 | - | 0/0 | 0 | 0/8 |
| **pH – Total**[e] | 33 | 25 | 2/8 | 13 | 3/24 |
| -acidic (pH < 7.0) | 23 | 17 | 1/6 | 18 | 3/17 |
| -basic (pH > 7.0) | 8 | 0 | 0/1 | 0 | 0/7 |
| -equals 7 | 1 | 0 | 0/1 | - | 0/0 |
| **Vapor Pressure – Total** | 97 | 16 | 7/44 | 17 | 9/53 |
| >6kPa | 14 | 14 | 1/7 | 43 | 3/7 |
| ≤6kPa | 83 | 16 | 6/37 | 13 | 6/46 |

Abbreviations: GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); kPa = kilopascals; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = The proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] One or more chemical categories were assigned to each test substance based on the chemical categories outlined in the tree structure provided for that chemical in the National Library of Medicine's Medical Subject Headings (MeSH®) for inorganic or organic chemicals when available (http://www.nlm.nih.gov/mesh) and on the presence of common organic functional groups (i.e., ketones) if that functional group was not available in the MeSH tree structure.

[e] Total number of substances with pH data available.

**Table 3-6** shows the STE test method performance in a bottom-up approach when problematic categories are excluded that gave the most discordant results in the GHS classification system. In

general, exclusion of alcohols, hydrocarbons, salts, or solids individually resulted in small changes in assay performance. However, two applicability domains were evaluated based on excluding certain chemical and product classes, or physical characteristics. When substances with high vapor pressures, solid alcohols, hydrocarbons, and salts were excluded, the false negative rate was reduced to 2.0% (1/49). When substances with high vapor pressures and nonsurfactant solids were excluded, the false negative rate was reduced to 1.9% (1/54). The single false negative substance using the restricted applicability domains was toluene. In the NICEATM database, an *in vivo* study from the European Centre for Ecotoxicology and Toxicology of Chemicals classifies toluene as GHS Not Classified (ECETOC 1998), whereas a study submitted to the EPA under the Toxic Substances Control Act (TSCA) classifies it as GHS Category 2B (eye irritation data made available by the U.S. Environmental Protection Agency). These data suggest toluene is a mild ocular irritant and mitigates concern about the false negative classification. The performance of validated *in vitro* methods for GHS classification is included in **Table 3-6** for comparison.

**Table 3-6 STE Performance for GHS Classification in a Bottom-Up Approach After Excluding Discordant Categories**

| Method Evaluated | Accuracy | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|
| | % | No.[c] | % | No.[c] | % | No.[c] |
| STE Overall | 85 | 109/129 | 20 | 11/56 | 12 | 9/73 |
| STE w/o Alcohols | 89 | 78/89 | 15 | 6/40 | 10 | 5/49 |
| STE w/o Hydrocarbons | 86 | 90/105 | 21 | 8/38 | 10 | 7/67 |
| STE w/o Salts | 86 | 95/111 | 17 | 9/53 | 12 | 7/58 |
| STE w/o Solids | 86 | 79/92 | 18 | 9/50 | 9.5 | 4/42 |
| STE w/o Vapor Pressure >6kPa, solid alcohols, hydrocarbons, and salts | 90 | 85/94 | 18 | 8/45 | 2.0 | 1/49 |
| STE w/o Vapor Pressure >6kPa and Nonsurfactant Solids | 90 | 91/101 | 19 | 9/47 | 1.9 | 1/54 |
| BCOP | 66 | 125/188 | 69 | 63/91 | 0 | 0/97 |
| CM | 68 | 36/53 | 68 | 17/25 | 0 | 0/28 |

Abbreviations: BCOP = bovine corneal opacity and permeability; CM = Cytosensor microphysiometer; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); kPa = kilopascals; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

## 3.3 EPA Classification System: STE Performance in a Top-Down Approach

The performance of the STE test method was evaluated for EPA ocular hazard classification in a top-down approach. STE accuracy, sensitivity, specificity, false positive rate, and false negative rate were determined based on available *in vivo* reference data for the test substances. Test substances that were identified as direct MTT reducers were removed from the analyses. These include two substances from Kojima et al. (Kao BRD), two substances from Takahashi et al. (2010), one substance from the Kao in-house studies, and five substances from the combined overall data set. These analyses were performed for each of the five studies as well as for the overall data set of 120 test substances from all these studies (**Table 3-7**). The EPA classification for each test substance is listed in **Supplement B**.

**Table 3-7 STE Performance for EPA Classification in a Top-Down Approach**

| Data Source | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] |
| Kojima et al. (Kao BRD) | 30 | 70 | 21/30 | 11 | 1/9 | 95 | 20/21 | 4.8 | 1/21 | 89 | 8/9 |
| Sakaguchi et al. 2011 | 24 | 92 | 22/24 | 67 | 4/6 | 100 | 18/18 | 0 | 0/18 | 33 | 2/6 |
| Takahashi et al. 2009 | 38 | 82 | 31/38 | 61 | 11/18 | 100 | 20/20 | 0 | 0/20 | 39 | 7/18 |
| Takahashi et al. 2010 | 49 | 82 | 40/49 | 58 | 11/19 | 97 | 29/30 | 3.3 | 1/30 | 42 | 8/19 |
| Kao In-House | 21 | 100 | 21/21 | - | 0/0 | 100 | 21/21 | 0 | 0/21 | - | 0/0 |
| Kao New Surfactants | 39 | 72 | 28/39 | 47 | 9/19 | 95 | 19/20 | 5.0 | 1/20 | 53 | 10/19 |
| Overall | 120 | 87 | 104/120 | 58 | 19/33 | 98 | 85/87 | 2 | 2/87 | 42 | 14/33 |

Abbreviations: EPA = U.S. Environmental Protection Agency (EPA 2012); N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

### 3.3.1 STE Discordant Results for EPA Classification in a Top-Down Approach

The STE results that were discordant with *in vivo* results were analyzed further. These analyses were performed on specific categories of chemicals, as well as on certain physicochemical properties potentially relevant to ocular toxicity testing (e.g., surfactants, pH, physical form).

Several trends were noted in STE performance among these subgroups of substances (**Table 3-8**). Two of 87 substances were overpredicted (i.e., false positives) and affected its representative chemical classes, with false positive rates ranging from 4.8% to 12.5%. The chemical categories of substances that were most consistently underpredicted for EPA classification (i.e., false negatives) were alcohols (64%; 7/11) and carboxylic acids (50%; 3/6). Of the 14 underpredicted substances, seven were alcohols, three were carboxylic acids, two were heterocyclic compounds, and two were salts.

With regard to the physical form of the substances overpredicted by the STE test method, 1.4% (1/74) were liquids and 7.7% (1/13) were solids. With regard to the physical form of the substances underpredicted by the STE test method, 10 were liquids and 5 were solids. Considering the proportion of the total available database, solids (16%; 5/32) appear more likely than liquids (11%; 10/89) to be underpredicted by the STE test method.

**Table 3-8 STE False Positive and False Negative Rates by Chemical Category and Properties of Interest for EPA Classification in a Top-Down Approach**

| Category | N | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] |
| **Overall** | 120 | 2.3 | 2/87 | 42 | 14/33 |
| ***Chemical Category***[d] | | | | | |
| Alcohol | 40 | 6.9 | 2/29 | 64 | 7/11 |
| Amine/Amidine | 8 | 0 | 0/2 | 17 | 1/6 |
| Carboxylic acid | 20 | 0 | 0/14 | 50 | 3/6 |
| Ester | 16 | 0 | 0/15 | 0 | 0/1 |
| Ether/Polyether | 16 | 8.3 | 1/12 | 0 | 0/4 |
| Heterocyclic compound | 9 | 0 | 0/3 | 33 | 2/6 |
| Hydrocarbon | 24 | 4.8 | 1/21 | 33 | 1/3 |
| Ketone | 8 | 0 | 0/8 | - | 0/0 |
| Onium compound | 10 | 0 | 0/1 | 11 | 1/9 |
| Salt | 18 | 12.5 | 1/8 | 20 | 2/10 |
| ***Properties of Interest*** | | | | | |
| **Liquids** | 89 | 1.4 | 1/74 | 67 | 10/15 |
| **Solids** | 32 | 7.7 | 1/13 | 26 | 5/19 |
| **Surfactants – Total** | 45 | 7.7 | 2/26 | 16 | 3/19 |
| -nonionic | 14 | 8.3 | 1/12 | 0 | 0/2 |
| -anionic | 12 | 20 | 1/5 | 14 | 1/7 |
| -cationic | 7 | - | 0/0 | 0 | 0/7 |
| -ampholytic | 2 | - | 0/0 | 50 | 1/2 |
| **pH – Total**[e] | 28 | 10 | 1/10 | 44 | 8/18 |
| -acidic (pH < 7.0) | 20 | 13 | 1/8 | 42 | 5/12 |
| -basic (pH > 7.0) | 7 | 0 | 0/1 | 50 | 3/6 |
| -equals 7 | 1 | 0 | 0/1 | - | 0/0 |
| **Vapor Pressure – Total** | 91 | 1.5 | 1/68 | 57 | 13/23 |
| >6kPa | 13 | 0 | 0/12 | 100 | 1/1 |
| ≤6kPa | 78 | 1.8 | 1/56 | 55 | 12/22 |

Abbreviations: EPA = U.S. Environmental Protection Agency (EPA 2012); kPa = kilopascals; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] One or more chemical categories were assigned to each test substance based on the chemical categories outlined in the tree structure provided for that chemical in the National Library of Medicine's Medical Subject Headings (MeSH®) for inorganic or organic chemicals when available (http://www.nlm.nih.gov/mesh) and on the presence of common organic functional groups (i.e., ketones) if that functional group was not available in the MeSH tree structure.

[e] Total number of substances with pH data available.

**Table 3-9** shows the STE test method in a top-down approach when problematic categories are excluded that gave the most discordant results for the EPA classification system. Exclusion of

alcohols reduced the false positive rate from 2.3% (2/87) to 0% (0/58) with only a slight reduction in the false negative rate (42%, 14/33 to 38%, 9/24). In general, removal of other individual chemical classes with false positive rates produced higher false negative rates.

**Table 3-9 STE Performance for EPA Classification in a Top-Down Approach After Excluding Discordant Categories**

| Method Evaluated | Accuracy | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|
| | % | No.[c] | % | No.[c] | % | No.[c] |
| STE Overall | 87 | 104/120 | 2.3 | 2/87 | 42 | 14/33 |
| STE w/o Alcohols | 89 | 73/82 | 0 | 0/58 | 38 | 9/24 |
| STE w/o Ethers/Polyethers | 85 | 89/105 | 1.3 | 1/75 | 50 | 15/30 |
| STE w/o Hydrocarbons | 85 | 82/97 | 1.5 | 1/66 | 45 | 14/31 |
| STE w/o Salts | 86 | 89/103 | 1.3 | 1/79 | 54 | 13/24 |
| STE w/o Solids | 88 | 78/89 | 1.4 | 1/74 | 67 | 10/15 |

Abbreviations: EPA = U.S. Environmental Protection Agency (EPA 2012); STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

## 3.4 EPA Classification System: STE Performance in a Bottom-Up Approach

The performance of the STE test method was evaluated for EPA ocular hazard classification in a bottom-up approach to identify EPA Category IV substances (i.e., minimal effects clearing in less than 24 hours). STE accuracy, sensitivity, specificity, false positive rate, and false negative rate were determined based on available *in vivo* reference data for the test substances. Test substances that were identified as direct MTT reducers and classified as STE nonirritants were removed from bottom-up analysis, as these could be false negative. These include two substances from Kojima et al. (Kao BRD) and two substances from Takahashi et al. (2010). These analyses were performed for each of the five studies as well as for the overall data set of 129 test substances from all these studies (**Table 3-10**). The EPA classification for each test substance is listed in **Supplement B.**

**Table 3-10 STE Performance for EPA Classification in a Bottom-Up Approach**

| Data Source | N | Accuracy | | Sensitivity | | Specificity | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] | % | No.[c] |
| Kojima et al. (Kao BRD) | 31 | 77 | 24/31 | 75 | 21/28 | 100 | 3/3 | 0 | 0/3 | 25 | 7/28 |
| Sakaguchi et al. 2011 | 24 | 67 | 16/24 | 56 | 10/18 | 100 | 6/6 | 0 | 0/6 | 44 | 8/18 |
| Takahashi et al. 2009 | 39 | 80 | 31/39 | 75 | 24/32 | 100 | 7/7 | 0 | 0/7 | 25 | 8/32 |
| Takahashi et al. 2010 | 52 | 77 | 40/52 | 74 | 31/42 | 90 | 9/10 | 10 | 1/10 | 26 | 11/42 |
| Kao In-House | 22 | 68 | 15/22 | 22 | 2/9 | 100 | 13/13 | 0 | 0/13 | 78 | 7/9 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kao New Surfactants | 34 | 94 | 32/34 | 96 | 26/27 | 86 | 6/7 | 14 | 1/7 | 3.7 | 1/27 |
| Overall | 129 | 80 | 103/129 | 75 | 73/97 | 94 | 30/32 | 6.3 | 2/32 | 25 | 24/97 |

Abbreviations: BRD = background review document; EPA = U.S. Environmental Protection Agency (EPA 2012); N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

### 3.4.1 STE Discordant Results for EPA Classification in a Bottom-Up Approach

The STE results that were discordant with *in vivo* results were analyzed further. These analyses were performed on specific categories of chemicals, as well as on certain physicochemical properties potentially relevant to ocular toxicity testing (e.g., surfactants, pH, physical form).

Several trends were noted in STE performance among these subgroups of substances (**Table 3-11**). Two substances were overpredicted. The overall false positive rate was 6.3% (2/32), with alcohols (20% 2/10), esters (33%; 2/6), and heterocyclic compounds (50%; 1/2) overpredicted. The chemical categories of substances that the STE test method most consistently underpredicted for EPA classification (i.e., false negatives) were hydrocarbons, ketones, and esters. Of the 24 underpredicted substances, seven were alcohols, five were carboxylic acids, and six were hydrocarbons. Additional chemical categories represented among the underpredicted substances were amines/amidines (2), esters (3), ethers/polyethers (1), ketones (3), onium compounds (1), and salts (2).

With regard to the physical form of the substances in a bottom-up approach, two liquids were overpredicted (6.9%; 2/29), which was 2.2% (2/92) of the entire database. With regard to the physical form of the substances underpredicted by the STE test method, 18 were liquids and six were solids. Considering the proportion of the total available database, liquids (20%; 18/92) appear more likely than solids (16%; 6/37) to be underpredicted by the STE test method.

**Table 3-11 STE False Positive and False Negative Rates by Chemical Category and Properties of Interest for EPA Classification in a Bottom-Up Approach**

| Category | N | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|
| | | % | No.[c] | % | No.[c] |
| **Overall** | 129 | 6.3 | 2/32 | 25 | 24/97 |
| ***Chemical Category***[d] | | | | | |
| Alcohol | 41 | 20 | 2/10 | 23 | 7/31 |
| Amine/Amidine | 11 | - | 0/0 | 18 | 2/11 |
| Carboxylic acid | 33 | 0 | 0/5 | 18 | 5/28 |
| Ester | 18 | 33 | 2/6 | 25 | 3/12 |
| Ether/Polyether | 16 | 0 | 0/4 | 8.3 | 1/12 |
| Heterocyclic compound | 10 | 50 | 1/2 | 0 | 0/8 |
| Hydrocarbon | 24 | 0 | 0/11 | 46 | 6/13 |
| Ketone | 8 | - | 0/0 | 38 | 3/8 |
| Onium compound | 12 | - | 0/0 | 8.3 | 1/12 |
| Salt | 18 | 0 | 0/1 | 12 | 2/17 |

| ***Properties of Interest*** | | | | | |
|---|---|---|---|---|---|
| **Liquids** | 92 | 6.9 | 2/29 | 29 | 18/63 |
| **Solids** | 37 | 0 | 0/3 | 18 | 6/34 |
| **Surfactants – Total** | 49 | 18 | 2/11 | 2.6 | 1/38 |
| -nonionic | 16 | 25 | 2/8 | 0 | 0/8 |
| -anionic | 12 | 0 | 0/1 | 0 | 0/11 |
| -cationic | 8 | - | 0/0 | 0 | 0/8 |
| **pH – Total**[e] | 32 | 17 | 1/6 | 15 | 4/26 |
| -acidic (pH < 7.0) | 23 | 17 | 1/6 | 18 | 3/17 |
| -basic (pH > 7.0) | 8 | - | 0/0 | 12 | 1/8 |
| -equals 7 | 1 | - | 0/0 | 100 | 1/1 |
| **Vapor Pressure – Total** | 97 | 8.0 | 2/25 | 32 | 23/72 |
| >6kPa | 14 | 0 | 0/4 | 50- | 5/10 |
| <6kPa | 83 | 9.5 | 2/21 | 29 | 18/62 |

Abbreviations: EPA = U.S. Environmental Protection Agency (EPA 2012); kPa = kilopascals; N = number of substances; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

[d] One or more chemical categories were assigned to each test substance based on the chemical categories outlined in the tree structure provided for that chemical in the National Library of Medicine's Medical Subject Headings (MeSH®) for inorganic or organic chemicals when available (http://www.nlm.nih.gov/mesh) and on the presence of common organic functional groups (i.e., ketones) if that functional group was not available in the MeSH tree structure.

[e] Total number of substances with pH data available.

**Table 3-12** shows the STE test method performance in a bottom-up approach when problematic categories are excluded that gave the most discordant results in the EPA classification system. In general, exclusion of alcohols, hydrocarbons, salts, or solids individually resulted in small changes in assay performance. However, two applicability domains were evaluated based on excluding certain chemical and product classes, or physical characteristics. When substances with high vapor pressures, solid alcohols, hydrocarbons, and salts were excluded, the false negative rate was slightly reduced to 21% (14/68). When substances with high vapor pressures, and nonsurfactant solids were excluded, the false negative rate was slightly reduced to 18% (13/73).

**Table 3-12 STE Performance for EPA Classification in a Bottom-Up Approach After Excluding Discordant Categories**

| Method Evaluated | Accuracy | | False Positive Rate[a] | | False Negative Rate[b] | |
|---|---|---|---|---|---|---|
| | % | No.[c] | % | No.[c] | % | No.[c] |
| STE Overall | 80 | 103/129 | 6.3 | 2/32 | 25 | 24/97 |
| STE w/o Alcohols | 89 | 72/89 | 0 | 0/22 | 25 | 17/67 |
| STE w/o Hydrocarbons | 81 | 85/105 | 9.5 | 2/21 | 21 | 18/84 |
| STE w/o Solids | 78 | 72/92 | 6.9 | 2/29 | 29 | 18/63 |
| STE w/o Salts | 78 | 87/111 | 6.5 | 2/31 | 28 | 22/80 |
| STE w/o Vapor Pressure >6kPa, solid alcohols, hydrocarbons, and salts | 83 | 78/94 | 7.7 | 2/26 | 21 | 14/68 |
| STE w/o Vapor Pressure >6kPa and Nonsurfactant Solids | 85 | 86/101 | 7.1 | 2/28 | 18 | 13/73 |

Abbreviations: EPA = U.S. Environmental Protection Agency (EPA 2012); kPa = kilopascals; STE = short time exposure.

[a] False positive rate = the proportion of all negative substances that are falsely identified as positive *in vitro*.

[b] False negative rate = the proportion of all positive substances that are falsely identified as negative *in vitro*.

[c] Data used to calculate the percentage.

## 4.0 STE Test Method Reliability

Test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of an evaluation of assay performance (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test chemicals and indicates the extent to which a test method can be transferred successfully among laboratories. A reliability assessment includes (1) reviewing the rationale for selecting the substances used to evaluate test method reliability, (2) discussing the extent to which the substances tested represent the range of possible test outcomes and the properties of the various substances for which the test method is proposed for use, and (3) performing a quantitative and/or qualitative analysis of repeatability and intra- and interlaboratory reproducibility.

Background information, data, and the performance (i.e., accuracy and reliability) analyses of the STE test method conducted by Kao Corporation are provided in the BRD (**Supplement A)**. STE test data were available for replicates within individual experiments repeated three times for each test substance in two to five different laboratories. Coefficient of variation (CV) analyses were performed on within-experiment and between-laboratory STE data, using the cell viability value obtained for each test substance within each of the two to five testing laboratories.

The %CV values for intralaboratory reliability for substances classified as nonirritants ranged from 0.3% to 23.5% in the four studies evaluated. Substances classified *in vitro* as irritants tended to have greater %CV values, as expected, because the cell viability for these chemicals was often quite low. Further, the mean viability for the positive control, 0.01% sodium lauryl sulfate, was 41.7% (N = 71) with %CV of 24.7%.

In terms of interlaboratory agreement, the laboratories recorded 100% agreement for 83% to 100% of the substances for GHS classification and 87% to 100% of the substances for EPA classification.

## 5.0 Peer Review Summary

To ensure the completeness of the NICEATM STE performance review, NTP provided the STE Summary Review Document along with the original Kao BRD and other supporting documentation to four external scientific reviewers who were asked to:

- Comment on whether the protocol is complete and adequate
- Comment on the adequacy of the database used for evaluating STE
- Provide any additional published STE studies or data
- Comment on the adequacy of the test method reliability
- Comment on the adequacy of the performance evaluation
- Provide any additional comments on the protocol or analysis
- Provide comments for regulatory agencies considering using data from this test method

The reviewers commented that the evaluation of the STE test method performance was thoroughly conducted. The reviewers remarked that the evaluation not only examined performance of the test method based upon the entire set of chemicals but also had a secondary assessment of STE performance with select chemical classes removed from the applicability domain. Given the thoroughness of the review, the reviewers stated that no other analysis is necessary. As with the performance analysis, the reviewers commented that the reliability analysis was thorough with no need for additional analysis.

The reviewers commented that the STE database was adequate for its intended purpose and added that they were not aware of additional STE data that could be used in this evaluation. The reviewers did however suggest that the STE database would require further development if the test method were to be used in the evaluation of pesticides.

The reviewers made a number of comments directed towards regulatory agencies considering using data obtained from the STE method:

When compared to other *in vitro* or *in vivo* assays currently available for eye irritation assessment, STE has a number of advantages, including time and cost required to do the assay, the use of a cell line rather than *ex vivo* tissue, its ability to assess poorly water-soluble substances, a low false positive rate, and protocol simplicity

The analysis highlights that the performance of a test method is dependent upon the classification system to which it is being compared. Specifically, STE “false negatives”, after all poorly compatible substances are excluded, are EPA Cat III: mild irritants.

Acceptance and use of data from the STE method is suggested by the developers as part of an *in vitro* / *ex vivo* battery of tests designed to offer an alternative to the *in vivo* OECD Test Guideline 405 Acute Eye Irritation/Corrosion assay in rabbits. As such, results from the STE when considered alone would most often be seen as “screening data” and best interpreted as part of a systematic evaluation of hazard.

There are likely to be circumstances in which the predictive nature of the STE assay is unknown (i.e., new chemical domains, mixtures, etc.). Submission of useful STE data relies on careful and

consistent conduct of the assay and verification of the performance of the STE method when extended beyond the currently available chemical domain or space.

At present, there is no *in vitro* alternative test to definitively and accurately distinguish non-irritant and irritant chemicals. In a bottom-up approach aimed at identifying non-irritants, it is important to reduce the false negative substances as much as possible. The false negative rate of BCOP and ICE are 0%, but their false positive rates are high, 68 to 69%. While the false negative rate in STE is not 0%, when either applicability domain is adopted, the rate is 1.9 to 2%. Test systems that obtain high accuracy, low false positive rate, and low false negative rate are desirable and based upon its performance, STE is suitable for use in a bottom-up approach.

As a result of the reviewers comments, minor edits were made to the NICEATM SRD including updating OECD TG references and clarifying the evaluation of direct MTT reducers. The reviewers also had a number of comments and suggestions for the STE protocol that were compiled and provided to Kao Corporation.

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