Validation Study for the Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining Cytotoxicity Test Method with Triethanolamine (SIRC-CVS:TEA Test Method) as an Alternative to Eye Irritation Test Draft）

Study Report

Version 9.0

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SIRC-CVS:TEA Validation Management Team (VMT)

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Abbreviations

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

1 Abstract

The Statens Seruminstitut Rabbit Cornea-Cell-Crystal Violet Staining (SIRC-CVS) test method was developed as a simplified alternative to the Draize rabbit eye test for use in screening test chemicals used as ingredients in cosmetics and quasi-drugs for ocular irritation (Itagaki, 1991). The SIRC-CVS test method was validated in the 1990s under the Ministry of Health and Welfare (MHW) Project on alternatives to the Draize test (Itagaki, 1995; Ohno, 1999; Ohno, 2004; Tani, 1999), and a modified version of the SIRC-CVS test method that uses triethanlamine (TEA) as a relative control has been developed by Hagino (See Appendix 8.0). This validation study was implemented at three participating laboratories in accordance with the spirit of GLP to validate the SIRC-CVS:TEA test method for intra- and inter-laboratory reproducibility as well as usefulness in distinguishing non-irritants from irritants in a bottom up approach (Scot, 2010).

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

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

These results demonstrated that the test method:

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

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

2 Introduction

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

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

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

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

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

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

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

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

Data from the Japanese validation as reported by Tani et al. (1999) and the study reported by Hagino et al. (Appendix 8.0) were reanalyzed using a cut-off value for triethanolamine (TEA) as a reference in evaluating undiluted test chemicals. A Japanese Centre for the Validation of Alternative Methods (JaCVAM) peer review of the SIRC-CVS test method based on this data, which was obtained between 2009 and 2011, concluded that this test method was useful in identifying non-irritants, but that a validation using the modified SIRC-CVS:TEA test protocol was necessary.

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

3 Methods

3.1 Study Plan

3.1.1 Purpose

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

3.1.2 Organization

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

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

3.1.2.1 Chairperson

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

3.1.2.2 Chemical management group

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

3.1.2 3 Data analysis group

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

3.1.2.4 Record management group

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

3.1.2.5 Research laboratories

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

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

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

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

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

3.1.3 Study design

Validation of the SIRC-CVS:TEA test method was carried out in three phases, as detailed in Appendix 8.1.

3.1.3.1 Training of participating personnel

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

3.1.3.2 Phase I

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

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

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

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

3.1.3.3 Phase II

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

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

3.1.3.4 Phase III

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

3.1.3.5 Test chemicals

The test chemicals were selected to ensure that a variety of substances were represented, including various eye-irritant levels per GHS categories, physical states, chemical classes, and eye lesions produced.

Preference was given to substances for which high-quality in vivo data, especially data including results from individual animals, was available, such as substances listed in ICCVAM or EURL ECVAM Eye Irritation Validation Studies. All selected test chemicals are available commercially.

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

3.1.3.6 Study duration

Testing was performed from September 2011 until September 2013

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

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

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

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

3.1.4 Success criteria

Success criteria for intra- and inter-laboratory reproducibility was 80%, for accuracy was 80%, and for false negatives was less than 5%, as determined by the VMT prior to testing. Other acceptance criteria for the test protocol are described in section 3.2.9 Quality Control. The data file used at the participating laboratories was developed by the data analysis group, and entering data from test results automatically calculates values for IC_{50} using a dose-response plot in combination with several other quality control criteria, as described in protocol Ver. 2.13E (Appendix 8.2).

3.2 Summary of protocol

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

3.2.1 Cells

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

3.2.2 Determining solubility or suspensibility of test chemicals in the Medium

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

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

3.2.3 Preparing test chemicals

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

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

3.2.4 Preparing a cell suspension

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

3.2.5 Application of the test chemical

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

4. After mixing the test chemical and the cell suspension, allow to stand for 20 minutes on a clean bench. Once the cells adhere to the bottom of the wells, the microplate is moved to the incubator.

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

3.2.6 Crystal violet staining

1. After incubation, remove the Medium containing the test chemicals by gently but quickly turning the microplate upside down.

2. Add 200 µL of modified PBS and shake gently to rinse the cells, then remove the modified PBS by gently turning the microplate upside down. Perform this procedure twice.

3. Add 100 µL of crystal violet methanol solution to each well and allow to stand for 30 minutes.

4. After the staining, remove the crystal violet methanol solution by gently but quickly turning the microplate upside down. Wash the cells thoroughly with tap water and blotted away any residual water with a paper towel.

5. After drying, measure the optical absorbance at 588 nm with an automatic microplate reader. Any nearby wavelength for which equivalency can be demonstrated is suitable for measurements.

3.2.7 Calculating IC_{50}

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

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

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

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

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

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

In the Excel spreadsheet (Appendix 8.3), cell viability is rounded to the nearest tenth.

3.2.8 Quality control

Quality control of the SIRC cytotoxicity test is performed by taking six measurements, as shown in Appendix 8.4 Rational for Quality Control Ranges, which must satisfy the following criteria. Failure to satisfy the criteria means that the test substance must be retested. In particular, if a volatile test chemical fails to satisfy the criteria, it must be retested at a lower concentration.

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

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

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

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

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

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

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

During the validation study, all data was checked against these criteria using the format shown in Appendix 8.3.

3.2.9 Evaluation

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

3.3 Test chemicals

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

3.3.1.1 Test chemicals for Phase I

Transferability of the SIRC-CVS:TEA test was confirmed at the three participating laboratories using sodium dodecyl sulfate as a positive control, TEA as a relative control, and four un-coded test chemicals. The four un-coded substances were ethyl-2-methyl acetoacetate (water soluble), safflower oil (oil soluble), 3-chloropropionitrile (highly volatile and cytotoxic), and sodium dehydroacetate (cytotoxic), as shown in Table 4. One run was performed for each test chemical and the results from the three participating laboratories were then compared with data from the lead laboratory.

3.3.1.2 Test chemicals for Phase II

For Phase II, the chemical management group and the VMT selected 20 substances which had previously been assessed using the Draize eye test and classified under GHS, as shown in Table 5. The test chemicals were coded prior to distribution to the three participating laboratories, as shown in Appendix 8.5.

3.3.1.3 Test chemicals for Phase III

For the Phase III, the chemical management group and VMT selected 100 substances, as shown in Table 6. Each of the three participating laboratories were allocated a set of 10 common test chemicals and a set of 30 unique test chemicals, as shown in Table 2. One of these, 3,3-dithiodipropionic acid, was duplicated in distribution, so one entry was eliminated from the list. The test chemicals were coded prior to distribution to the three participating laboratories, as shown in Appendix 8.5.

3.3.2 Test chemicals selected for the validation study

The participating laboratories participated in VMT meetings as observers but did not take part in discussions related to selection of test chemicals. The 120 test chemicals listed in Tables 2-2 and 2-3 of Appendix 8.5 were selected for use in this validation study. As mentioned above, a duplication of 3,3-dithiodipropionic acid was excluded from the results. Furthermore, citric acid (P3-067) and potassium sorbate (P3-068) we also excluded from the results, since they lacked individual animal data from a clear source. Thus, a total of 117 test chemicals with individual animal data were used to evaluate intra- and inter-laboratory reproducibility. The physical state, chemical class, and classification per both GHS and EPA for each of the 117 test chemicals is shown in Table 4 of Appendix 8.5.

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

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

3.3.3 Purchase, coding, and distribution of test chemicals

All of the test chemicals used in Phases I, II, and III were obtained from commercial sources, as shown in Table 4 of Appendix 8.5. Test chemicals used in the Phases II and III were coded and distributed to the participating laboratories by JaCVAM.

3.4 Quality assurance

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

3.5 Record collection and analysis

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

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

4. Results

4.1 Data quality

All data sheets were analyzed by biostatisticians is shown in Appendix 8.6. Error found during quality checks are shown in Tables 7.1 and 7.2. The Quality Assurance group reviewed the records to assure that all tests were performed in the spirit of GLP.

4.1.1 Phase I

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

Testing during Phase I comprised three runs of four test chemicals, however there was a lack of awareness on the part of all three participating laboratories as to the need to perform testing in the spirit of GLP. Lab A submitted all data sheets and records for Phase I. Lab B submitted all records but only a portion of the data sheets. Therefore, they did not provide enough data to meet quality control criteria. Lab C submitted

all data sheets but none of the records. Thus, after Phase I, quality criteria for the negative, positive, and reference controls was developed.

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

Discrepancies in the test results led the VMT to direct Lab A to repeat the tests for all four test chemicals in Tables 9.1. The classification of sodium dehydroacetate at Lab A differed from that at the other two labs as well as from that at the lead lab. Investigation revealed that the cause was likely improper dilution of the test chemical, which prompted Lab A to offer to redo all Phase I testing, and the VMT accepted this offer. The results of the retest were not only more consistent, they also matched the classifications obtained by Lab B, Lab C, and the lead lab.

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

4.1.2 Phase II

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

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

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

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

Lab A submitted all data sheets and records for Phase II. Lab B submitted all data sheets and records for the testing of the test chemicals but failed to submit data sheets for preliminary set up, such as establishing solvents and concentrations. Lab C submitted all data sheets and records for the testing of the test chemicals but failed to submit any data sheet or records for preliminary set up. Unfortunately,

miscommunication between the VMT and the participating laboratories resulted in both Lab B and Lab C failing to submit all necessary records for Phase II testing.

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

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

1. Some volatile test chemicals were found to have affected the negative control. The VMT also thinks that the quality of the plate seal was also affected.
   P2-010: ethyl thioglycolate, P2-013: 1-bromo-4-chlorobutane, P2-014: sodium hydrogensulfite, P2-015: isobutyraldehyde

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

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

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

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

4.1.3 Phase III

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

obtained reagent bottles, which are significantly evaporation resistant, and redistributed the following test chemicals.

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

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

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

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

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

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

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

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

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

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

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

One of the two data sets for P3-95 (3,3-dithiodipropionic acid) was excluded from analysis of predictive capacity, due to duplication. Although no precipitation was found when P3-95 was tested at Lab A, Lab C

reported the presence of precipitation in medium. The VMT requested that Lab A retest P3-95, however, and no precipitation was observed. The IC_{50} values were similar at the two labs irrespective of the presence of precipitation after the 72-hour incubation period reported at Lab C. Therefore, the VMT decided to include the data for P3-23 from Lab C in the analysis.

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

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

Rather than using the Phase III record sheet version 2.2, which includes a column for recording solubility of the test chemical during the 72-hour exposure period, Lab C used the Phase II record sheet version 2.1, which did not contain such a column. The VMT decided to accept the submitted Phase II record sheet version 2.1 for analysis.

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

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

4.2 Transferability

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

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

4.3 Intra- and inter-laboratory reproducibility

4.3.1 Intra-laboratory reproducibility

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

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

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

4.3.2 Inter-laboratory reproducibility

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

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

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

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

4.4 Predictive capacity

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

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

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

The SIRC-CVS:TEA test method was developed primarily to identify ocular non-irritants in a bottom-up approach. Analysis in a top-down approach for identifying GHS Category 1 eye irritants was also performed as a part of this validation study in order to compare the results from a bottom-up approach to those from a top-down approach, as shown in Tables 15 and 16. In a bottom-up approach, the SIRC-CVS:TEA test method demonstrated an accuracy of 55% (64/116), a sensitivity of 60% (42/70), and a specificity of 48% (22/46), and in a top-down approach, demonstrated an accuracy of 53% (62/116), a sensitivity of 71% (20/28), and a specificity of 48% (42/88). Thus, the results were similar in either approach.

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

4.5 Applicability domain

Further analysis was conducted to reduce false negatives by delimiting the applicability domain to certain chemical classes and properties of interest. Chemical classes with at least six representative substances were examined: alcohols, carboxylic acids, esters, ethers, halogen compounds, heterocyclic compounds, hydrocarbons, ketones, organic salts, phenols, surfactants, and thiol compounds as shown in Appendix 8.7. Physical chemical properties of interest were molecular weight, physical state, purity, water solubility, distribution coefficient (log D), pKa, and vapor pressure. Criteria and rationale for selection of these properties of interest are shown in Table 17. These records were summarized in Table 18.

4.5.1 Chemical class

Table 19 shows these results of an analysis of chemical class based on Appendix 8.7. Chemical classes employed as applicability domains for the analysis are shown in the table:

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

4.5.2 Properties of interest

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

As can be seen in Table 20.2 and 20.3, molecular weight was the only property of interest to demonstrate improvement in false negatives and accuracy. The VMT analyzed a Shiseido proposal (Appendix 8.13) to use a combination of chemical category and molecular weight. It is difficult to evaluate the eye irritancy of test chemicals that have a molecular weight of less than 180 and are alcohols (The number of hydroxyl group≤2), esters, ethers, ketones, heterocyclic compounds, or carboxylic acids including salt. Incidentally, TEA that has three hydroxyl groups which is not excluded from applicability domain, though the molecular weight is less than 180. The VMT reviewed this analysis in the light of a pre-validation proposal from Shiseido, and excluded the test chemicals shown in Tables 21.1 to 21.6. The result was 8% (2/26) false negatives, 58% (18/31) false positives, and 65% (37/57) accuracy, as shown in Table 22. The two false negatives were GHS category 2B substances: P3-083 (toluene) and P3-023 (3,3-dithiodipropionic acid). Although this false negative rate did not meet the 5% target and the false positive rate was greater than 50%, the VMT considered this to be the most suitable applicability domain.

5. Discussion

5.1 Considerations for the validation study

In an earlier study performed in Japan (Ohno, 1999), the reproducibility and the predictive capacity of the SIRC-CVS test method was validated on the basis of assessing eye irritation potency for solutions or suspensions at a 10% concentration. In the present study, the SIRC-CVS:TEA test method was validated on the basis of assessing undiluted substances using TEA as a relative control. TEA was selected by Shiseido as a suitable control substance after a reanalysis of previous studies in which GHS No Category non-irritants were distinguished from Category 1 and 2 irritants. As shown in Appendix 8.9, TEA from different manufacturing lots provides consistent results. Also, differences in manufacturers or production lots of serum and SDS do not have any significant effect on test results. See Appendix 8.9 “Examination of difference by lot of triethanolamine and serum".

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

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

In response to a comment about the effects of different solvents, the VMT analyzed average ±standard deviations of the O.D. for each solvent. The negative control was 0.64 ±0.08 in the Medium (n = 52) and

0.66 ±0.08 in medium containing DMSO (n = 28), as calculated from Phase III data obtained at Lab A, and 0.97 ±0.09 in the Medium (n = 76) and 0.93 ±0.10 in medium containing ethanol (n = 4), as calculated from Phase III data obtained at Lab B. Neither Lab A nor Lab C used ethanol as a solvent, nor did Lab B use DMSO as solvent, as shown in Appendix 8.10 "Effect of solvents in the validation study." Actually, an investigation of the effects of different solvents was part of the previous Japanese validation study of the SIRC-CVS test. Also, the fact that the three participating laboratories were all naïve and that no practical training was given is another good indication of the robustness of the test method.

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

The database at the lead laboratory was not considered extensive enough to evaluate predictive capacity, and the VMT decided that data from at least 100 test substances would be needed. The 116 test chemicals selected for the analysis of predictive capacity comprised 28 from GHS Category 1, 42 from Category 2, and 46 from No Category. The VMT decided to validate the SIRC-CVS:TEA test method using as many test chemicals as possible and did not initially take into consideration as criteria for selection of test chemicals a proposal from Shiseido that the exclusion of alcohols, esters, ethers, or similar chemical classes would improve predictive capacity. Prediction of UN GHS classification by comparing the IC_{50} of the test chemicals with that of TEA as a preliminary step in a bottom-up approach yielded an accuracy of 55% (64/116), a sensitivity of 60% (42/70), and a specificity of 48% (22/46), as shown in Table 15. If a cut-off value of 1600 µg/mL is adopted instead of using TEA as a relative control, these values become 59% (66/112), 69% (48/68), and 43% (19/44), respectively, as shown in Table 16. In either case, the results are similar. Prediction of EPA classification by comparing the IC_{50} of the test chemicals with that of TEA yielded an accuracy of 54% (62/115), a sensitivity of 57% (50/88), and a specificity of 44% (12/27), as shown in Appendix 8.5. Thus, predictive capacity was similar for both UN GHS and EPA classification. These results show that the predictive capacity of the SIRC-CVS:TEA test method was not sufficient to permit its use as a preliminary step in a bottom-up approach. Nor was the predictive capacity good enough for use in a top-down approach, which yielded an accuracy of 53% (62/116), a sensitivity of 71% (20/28), and a specificity of 47% (8/28), as shown in Table 15. The VMT therefore concluded that revision of the applicability domain would be necessary for further improvement of predictive capacity.

5.2 Original applicability domain

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

Chemical class, physical state, molecular weight, purity, water solubility, distribution coefficient (log D), vapor pressure, and pKa were all studied as potential means of improving of predictive capacity. In this validation, chemical classes were defined by existence of functional group, as detailed in Appendix 8.7. Only surfactants were classified on the basis of function in accordance with the actual condition. Information on surfactants was obtained from the International Cosmetic Ingredient Dictionary (CTFA, 2006). The examination of finding applicability domain was performed in consideration of decreasing

false negative first and increasing accuracy second with end user in mind. Effective elements for decreasing false negatives were molecular weight and exclusion based on chemical classes such as alcohols (The number of hydroxyl group≤2), esters, ethers, ketones, heterocyclic compounds, and carboxylic acid. False positive rate did not have marked improvement for the selection of the applicability domain in consideration of decreasing false negative.

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

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

Reanalysis of validation test results suggested that the SIRC-CVS:TEA test was suitable for the identification of chemicals that were not ocular irritants when alcohols, esters, ethers, ketones, or other test chemicals with a molecular weight of less than 180 were excluded, as shown in appendix 8.7. In this validation, heterocyclic compounds and carboxylic acid compounds with a molecular weight of less than 180 were shown to be likely to cause false negatives. Excluding alcohols, esters, ethers, ketones, heterocyclic compounds, carboxylic acid compounds and similar chemical classes with a molecular weight of less than 180 improved the accuracy to 65% (37/57) and the false negative rate to 8% (2/26), which suggests that the predictive capacity of the SIRC-CVS:TEA test can be improved by delimiting the applicability domain. Toluene was one of the two false negatives and was > Category 2B per TSCA in vivo data, but was classified No Category, meaning “negative,” per ECETOC in vivo data.

The applicability domain was also reviewed using Shiseido’s in-house data in an attempt to find more test chemicals, as detailed in Appendix 8.7. Predictive capacity based on data from 57 test substances in this validation study and data from Shiseido on an additional 22 test chemicals yielded an accuracy of 65% (51/79), a sensitivity of 95% (35/37), and a specificity of 38% (16/42). Thus it is suggested that the SIRC-CVS:TEA test method is suitable for distinguishing ocular non-irritants and irritants, if the applicability domain is well defined.

Predictive capacity was further analyzed using data on 79 substances that conform to the applicability domain from this validation study and from Shiseido in-house data. Although false positives were unavoidable, the false negative rate was less than 10%. Thus, the VMT concluded that the SIRC-CVS:TEA test was a useful alternative to animal testing for distinguishing ocular non-irritants and irritants with a carefully defined applicability domain based on Appendix 8.11 “Analysis of predictive capacity by the data from this validation study and the additional data from Shiseido.”

5.3 Reanalysis of the original applicability domain

The original applicability domain for the SIRC-CVS:TEA test method was determined during the design of the validation study by analysis with a combination of chemical category and molecular weight. Additionally, upon completion of Phases I–III, we attempted to determine a more definite physicochemical basis for defining the applicability domain. We were unable, however, to overcome technical limitations affecting the results for test chemicals with poor solubility, high volatility, or color. As detailed in Appendix 8.15, we attempted to reduce false negatives by excluding (1) acids with an acid dissociation constant pKa of 4 or less or organic salts consisting of a weak acid and a strong base and (2) chemicals with a distribution coefficient (log P) of greater than -1.5 and less than 2. In this analysis, predictive capacity was calculated relative to Draize eye test reference data by Barroso et al, though the influence on the results was not significant (Appendix 8.14 and 8.15). Additional data from Shiseido were also used to analyze the predictive capacity as shown in Appendix 8.15. The SIRC-CVS:TEA test method finally demonstrated an accuracy of 62% (49/79), a sensitivity of 100% (25/25), and a specificity of 44% (24/54) with a false negative rate of 0% (0/25). Reanalysis of the test results using these criteria shows that the SIRC-CVS:TEA test is capable of distinguishing ocular non-irritants from irritants per UN GHS categories once test chemicals that are strong acids or alkalis, are amphiphilic substances with high cell membrane accessibility, or are cytotoxic have been excluded from the applicability domain. Even after considerable review of the test data, however, the VMT was unable to reach a consensus regarding a scientifically valid approach to achieving the requisite level of sensitivity and was unable to identify a scientifically valid applicability domain that would provide a high predictive capacity.

6 Conclusion

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

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

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

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Tables for SIRC-CVS:TEA validation version 9.2

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

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

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

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

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

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

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

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

Table 5. Twenty substances for Phase II study

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

Table 6. The 100 substances for the Phase III study

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

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

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

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

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

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

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

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

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

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

*N: Number of relative controls and positive controls

*IC_{50} in μg/mL.

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

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

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

* IC_{50} in μg/mL

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

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

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

* IC50 was expressed as μg/mL.

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

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

* N: Number of runs

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

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

* N: Negative, P: Positive

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

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

* N: Negative, P: Positive,

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

*N: Negative, P: Positive

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Property of interest	Inclusion criteria	Rationale for selection	References
Physical state	Solids and liquids only		
Molecular weight	≧180	The criteria were considered reasonable by the VMT.	Appendix 8.5
Purity	≧95%		
Water solubility	<1.0–10.0 g/L 10.0–100.0 g/L	Poorly or Somewhat soluble Soluble	SciFinder
Log D	≦2.88	generally less than 3.0	
Vapor pressure	≦6.0 kPa	Criteria used in SIRC-STE	ENV/JM/TG/RD (2013)19
PKa	<5.0		

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 21.1. Analysis of categories: Alcohol

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

Red: No correct predictive capacity with in vitro assay,

Table 21.2. Analysis of categories: Ester

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

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

Table 21.3. Analysis of categories:Ether

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

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

Table 21.4. Analysis of categories: Ketone

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

Red: No correct predictive capacity with in vitro assay,

Table 21.5. Analysis of categories: hetelocyclic compounds

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

Red: No correct predictive capacity with in vitro assay,

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

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

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

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

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

Figures for SIRC-CVS:TEA validation version 7.8

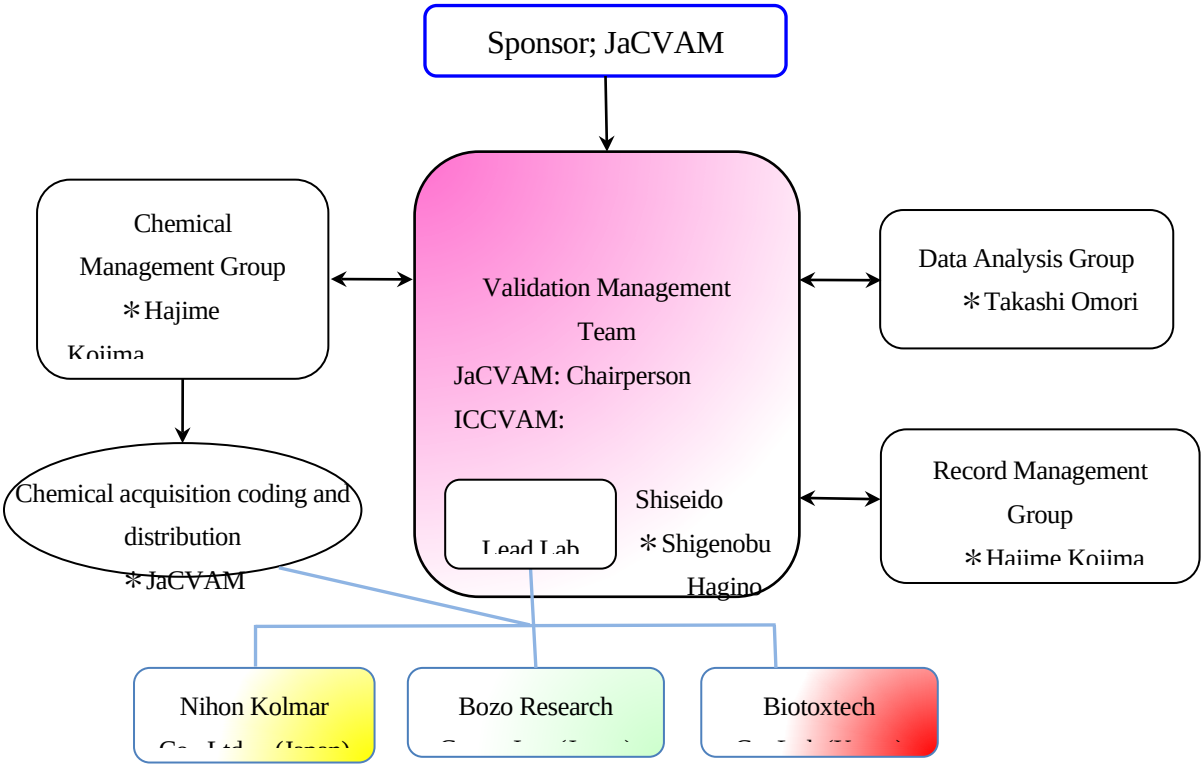


Fig. 1. Study organization for SIRC-CVS:TEA validation study

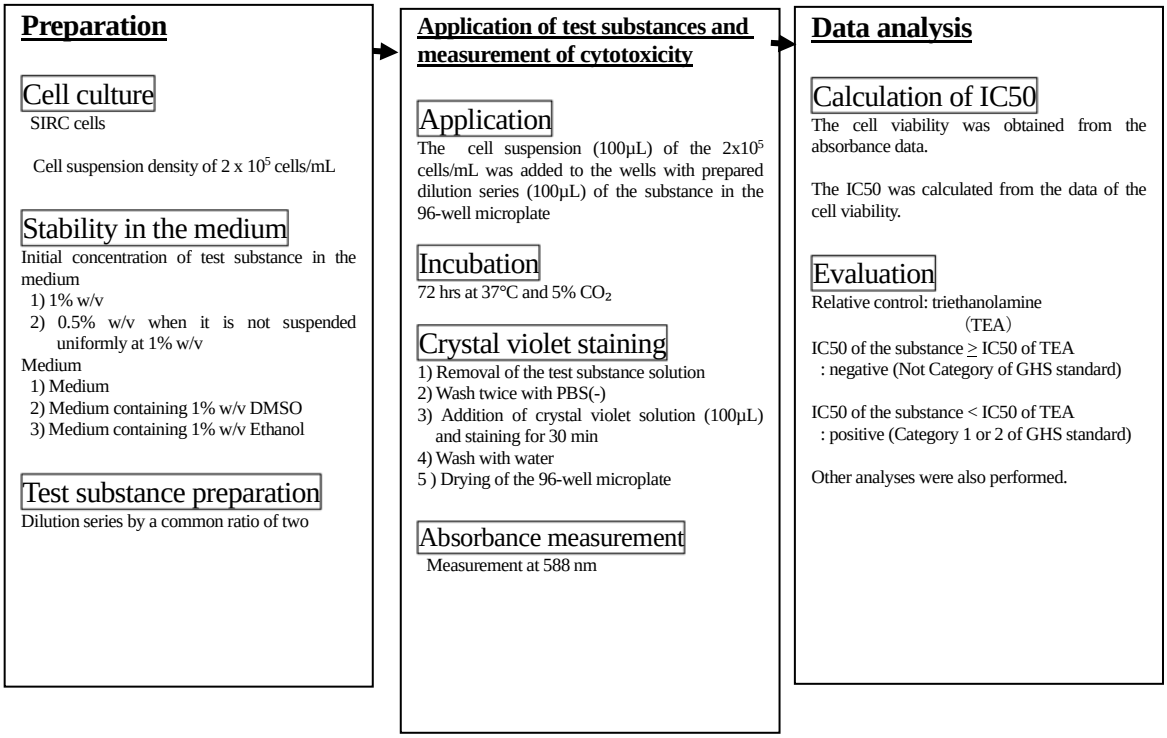


Fig. 2. SIRC-CVS:TEA test procedure

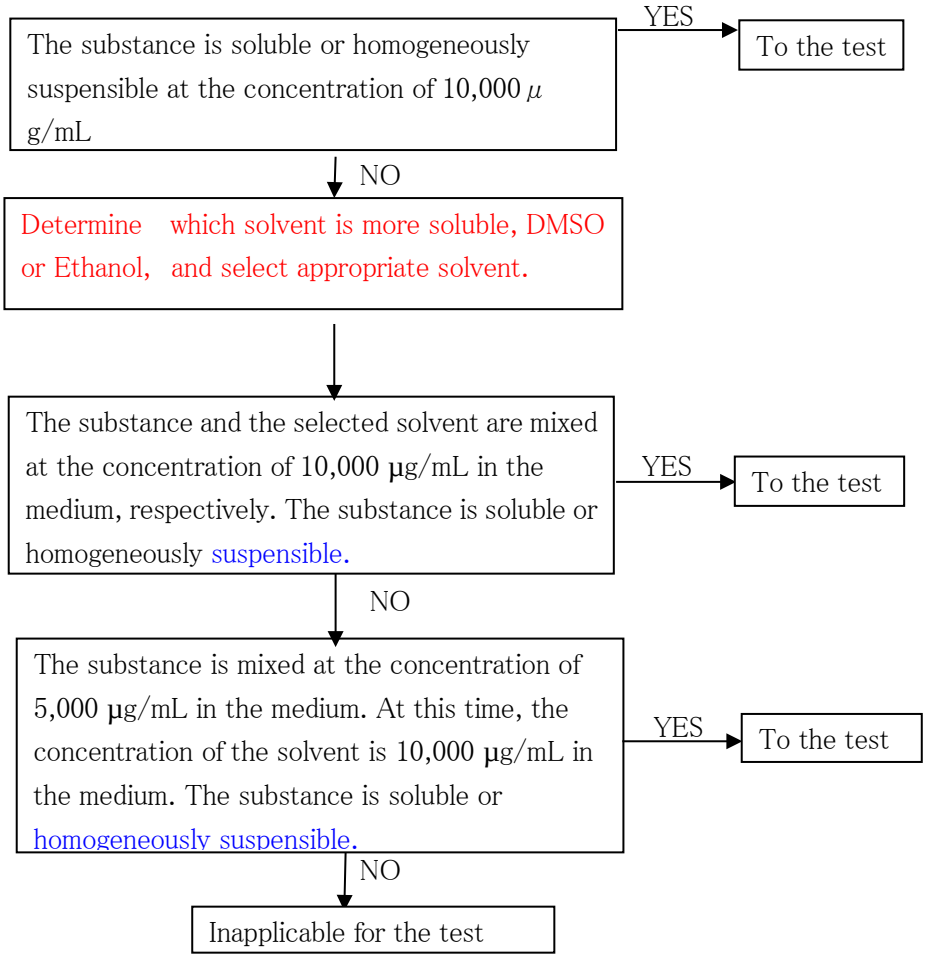


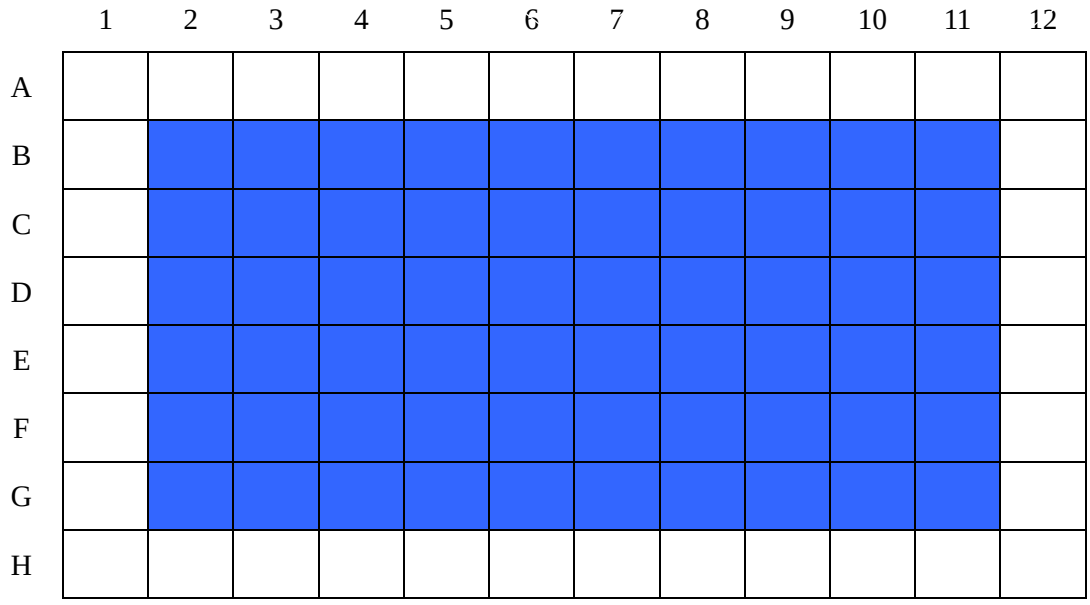
Fig. 3. Flow chart of examination of stability for the substance in the medium

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

Fig. 4.1. Layout of 96-well microplates

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

The dilution series of the test substance was made using medium, 10,000 µg/mL DMSO-medium solution or 10,000 µg/mL ethanol-medium solution. The dilution series of positive control and relative control was made using medium.



■ : Cell suspension (100 µL)

Fig. 4.2. Addition of cell suspension

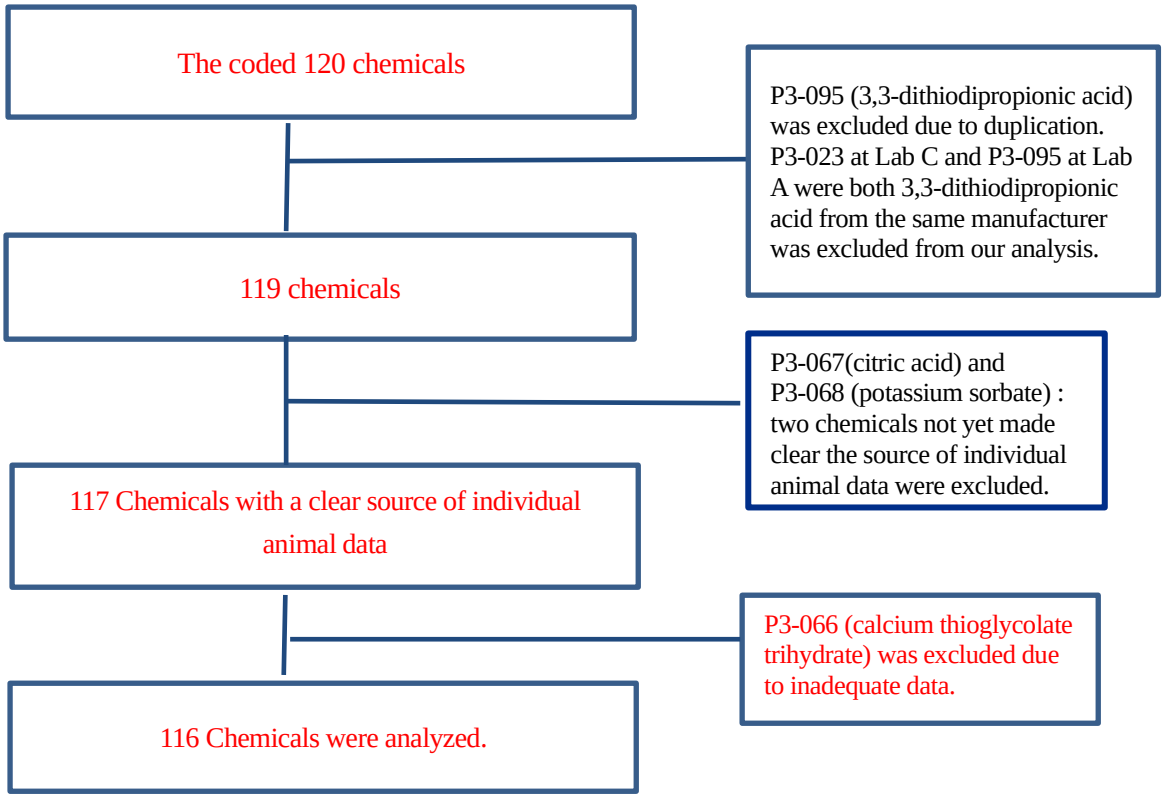


Fig.5. Evaluation of predictive capacity for the SIRC-CVS validation study

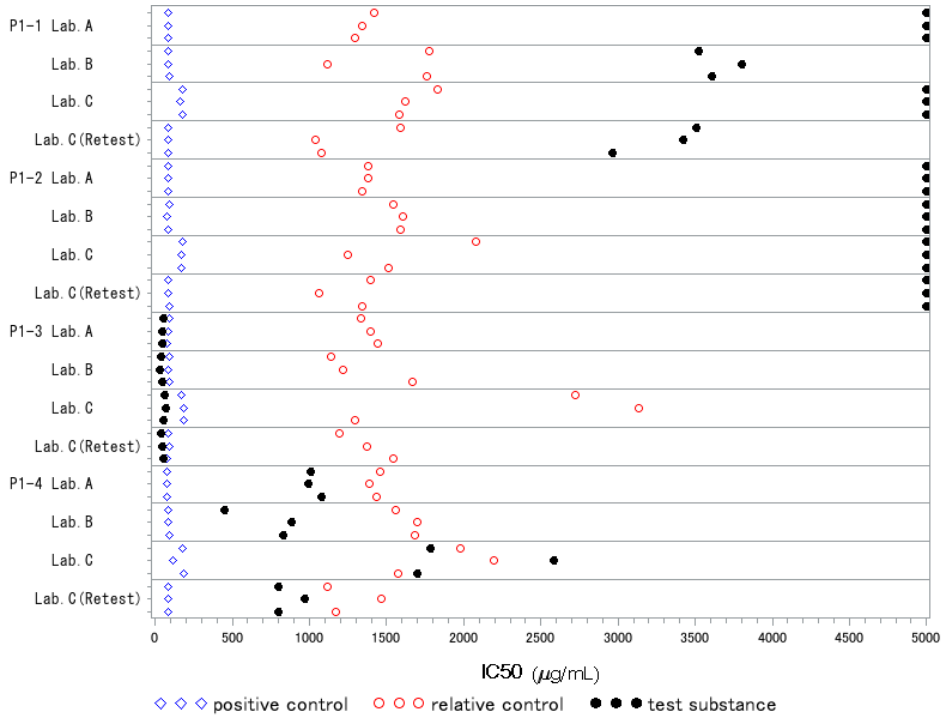
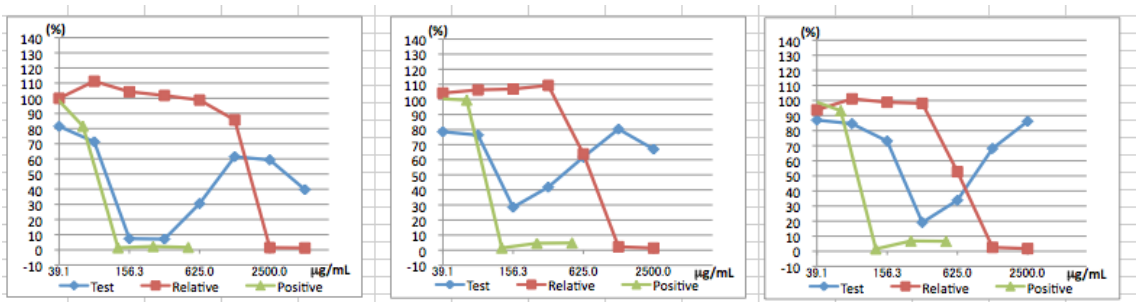


Fig. 6. A comparison of test substances, reference control, and positive control at the three participating laboratories

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

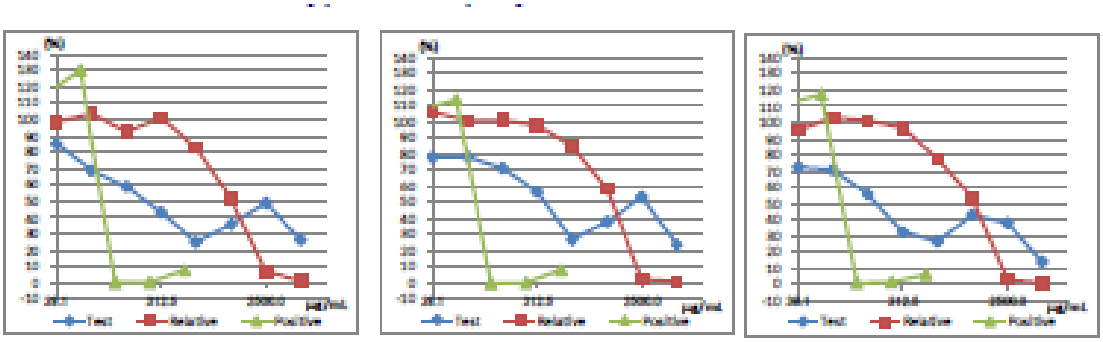
SA008 Solvent: DMSO

Run 1 Run 2 Run 3



SB001 Solvent: Medium

Run 1 Run 2 Run 3



SC006 Solvent: Medium

Run 1 Run 2 Run 3

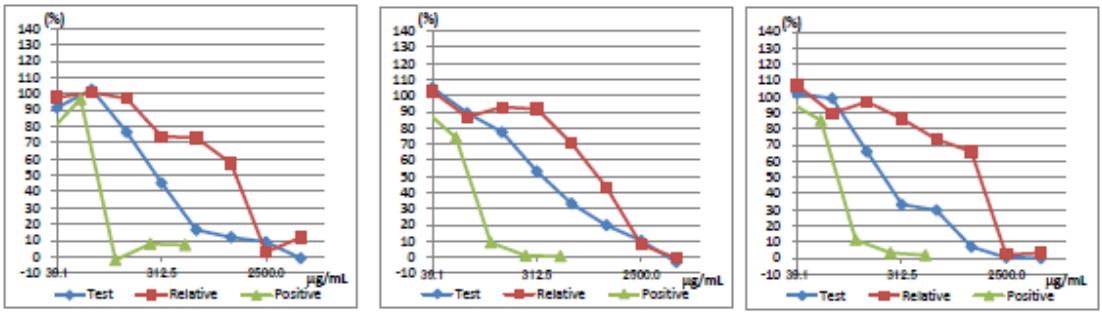
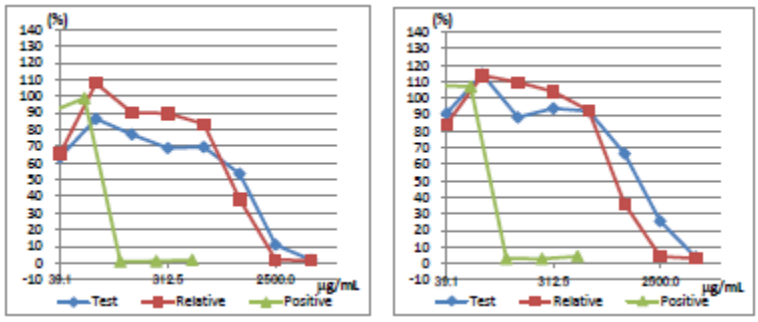


Fig.7. Dose response curves of P2-001

SA90

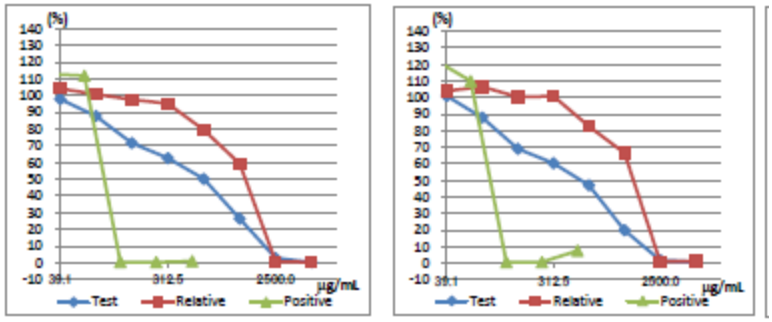
Run 1 Run 2



Solvent: Medium

SB71

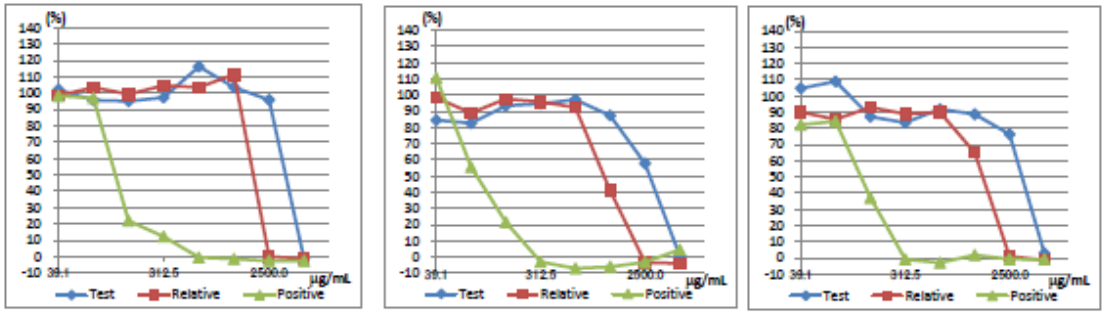
Run 1 Run 2



Solvent: Medium

SC63

Run 1 Run 2 Run 3

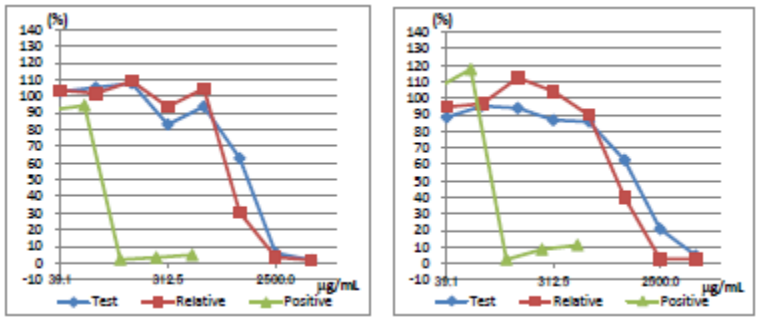


Solvent:

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

SA84

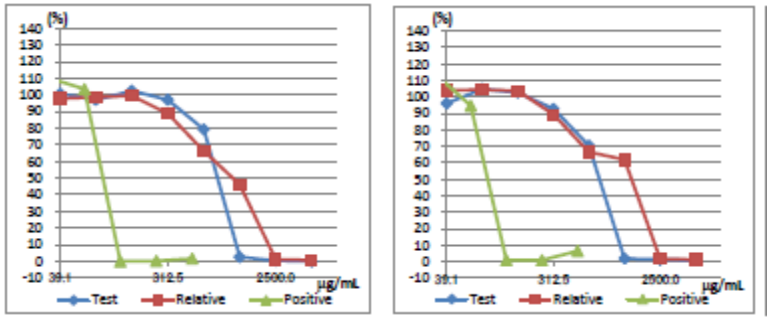
Run 1 Run 2



Solvent: Medium

SB77

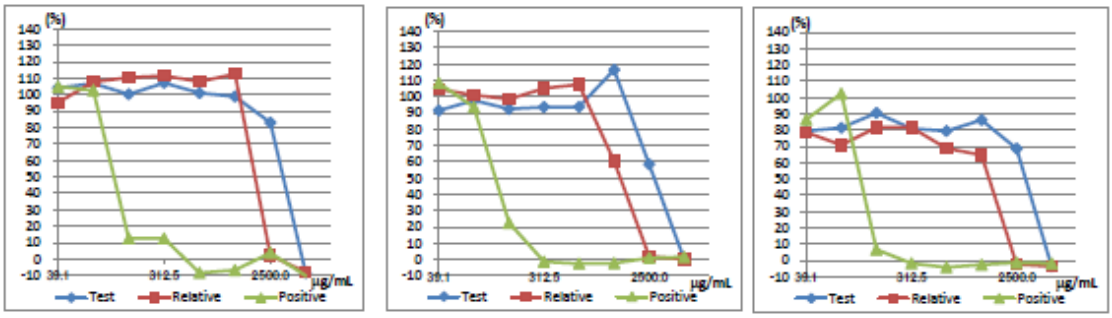
Run 1 Run 2



Solvent: Medium

SC64

Run 1 Run 2 Run 3

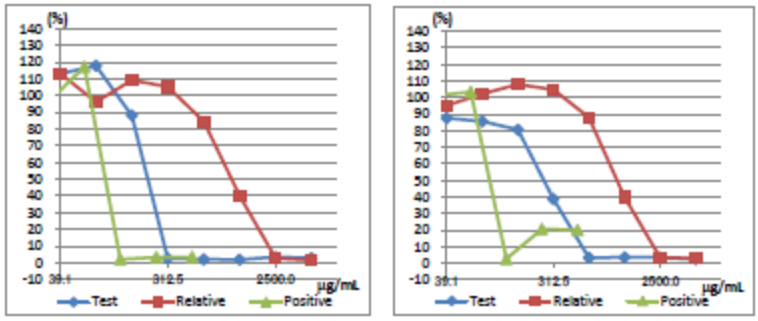


Solvent: Medium

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

SA82

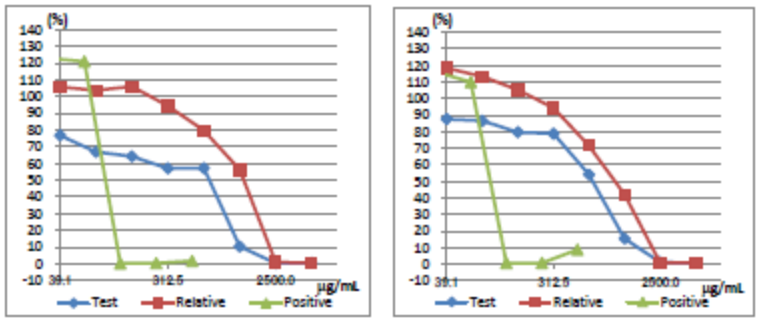
Run 1 Run 2



Solvent: Medium

SB79

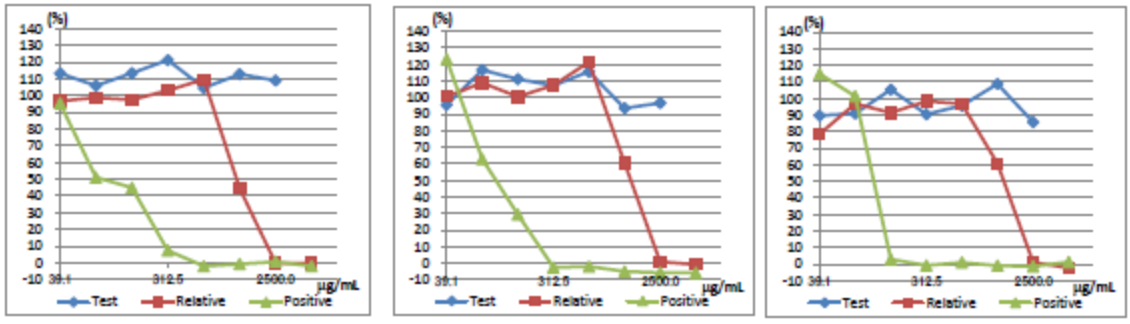
Run 1 Run 2



Solvent: Ethanol

SC61

Run 1 Run 2 Run 3



Solvent: DMSO

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

Run 1 Run 2

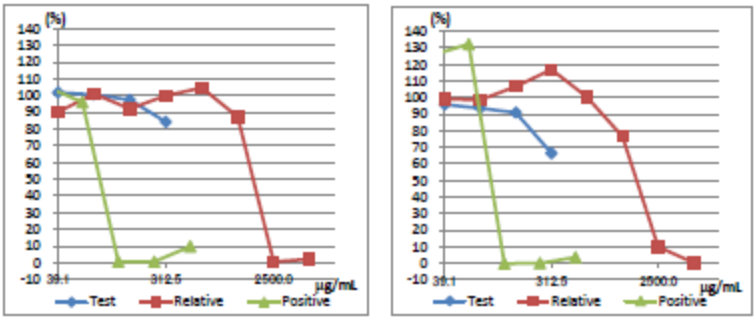


Fig.9. Dose response curves of P3-066 at Lab.B

P3-066: SB94 Solvent: Medium