Protocol for SIRC-CVS cytotoxicity test
Version 3.8

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1 Purpose

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

2 The principle of SIRC cytotoxicity test

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

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

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

3 Materials

3.1 Cell line

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

3.2 Equipment

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

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

3.3 Instruments

- 25-cm2 and 75-cm2 tissue culture flasks, such as the 353108 and 353136 from BD Falcon
- 96-well flat bottom tissue culture microtiter plates, such as the 353072 from BD Falcon
- Storage plates, such as the AB-0765 0.8-mL Storage plate from Thermo Scientific
- Multichannel pipettes, micropipettes
- Dispenser trays
- Tubes
- 1.5-mL cryotubes
- 15-mL and 50-mL centrifuge tubes
- 200-µL, 100-µL, and 5-mL tips for micropipettes
- Microplate sealing tape
- Paper towels, such as the 61000 Kim towel™ from Nippon Paper Crecia Co., Ltd
- Wrapping film, such as the Saran Wrap

3.4 Culture medium and reagents

- Minimum Essential Medium (MEM)
- Fetal Bovine Serum (FBS)
  The fetal bovine serum is to be inactivated before use. Inactivate by placing in a water bath at 56°C for 30 minutes. After cooling, store the serum in 56-mL or 28-mL tubes. The serum is stored at −70 or −20°C.
- Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
  (Antibiotic-Antimycotic 100×, GIBCO BRL)

- Modified PBS, comprising phosphate-buffered saline without calcium or magnesium
- 0.25% (w/v) Trypsin (1 mmol/L EDTA·4Na)
- Dimethyl Sulfoxide (DMSO, CAS Number 67-68-5)
  Measured per either weight or volume.
- Ethanol (CAS Number 64-17-5)
  Measured per either weight or volume.
- Crystal Violet (CAS Number 548-62-9)
- Methanol (CAS Number 67-56-1)
- Sodium Dodecyl Sulfate (SDS, CAS Number 151-21-3)
- Triethanolamine (CAS Number 102-71-6)
  Purity of 98% or higher.
- Hydrochloric Acid (CAS Number 7647-01-0)
- Sodium Hydroxide (CAS Number 1310-73-2)

Typical specifications and manufacturers for reagents are shown in Table 1.

3.5 Medium

The medium used in this test (the Medium) comprises MEM supplemented with10% inactivated FBS and about 1% antibiotic (P/S/F solution). For example, 500 mL of MEM is supplemented with 56 mL of FBS and 5.6 mL P/S/F. At this time, the concentrations of the antibiotics are 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of Amphotericin B.

3.6 Crystal violet solution

A 0.4% crystal violet solution is prepared using methanol.

3.7 Test chemicals

3.7.1 Determining solubility or suspensibility of test chemicals in the Medium

Confirm in advance the solubility or suspensibility of each test chemical in the Medium, using the procedure shown in Fig. 1. First, determine whether the test chemical can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL (1% w/v). Use a vortex mixer, water bath, or sonicator as necessary. If the test chemical cannot be dissolved or uniformly suspended in the Medium, the next step is to determine whether the test chemical is more easily dissolved in DMSO or ethanol. Next, dissolve or uniformly suspend the test substance in the more suitable solvent at a concentration of 10,000 μg/mL and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL. If not, dissolve or uniformly suspend the test substance in the more suitable solvent at a concentration of 5,000 μg/mL (0.5% w/v) and determine whether that solution can be dissolved or uniformly suspended in the Medium at a concentration of 10,000 μg/mL. If not, the test

substance is considered to be outside the applicability domain of the test. These judgments can all be performed by visually confirming the absence or presence of precipitate in the solution.

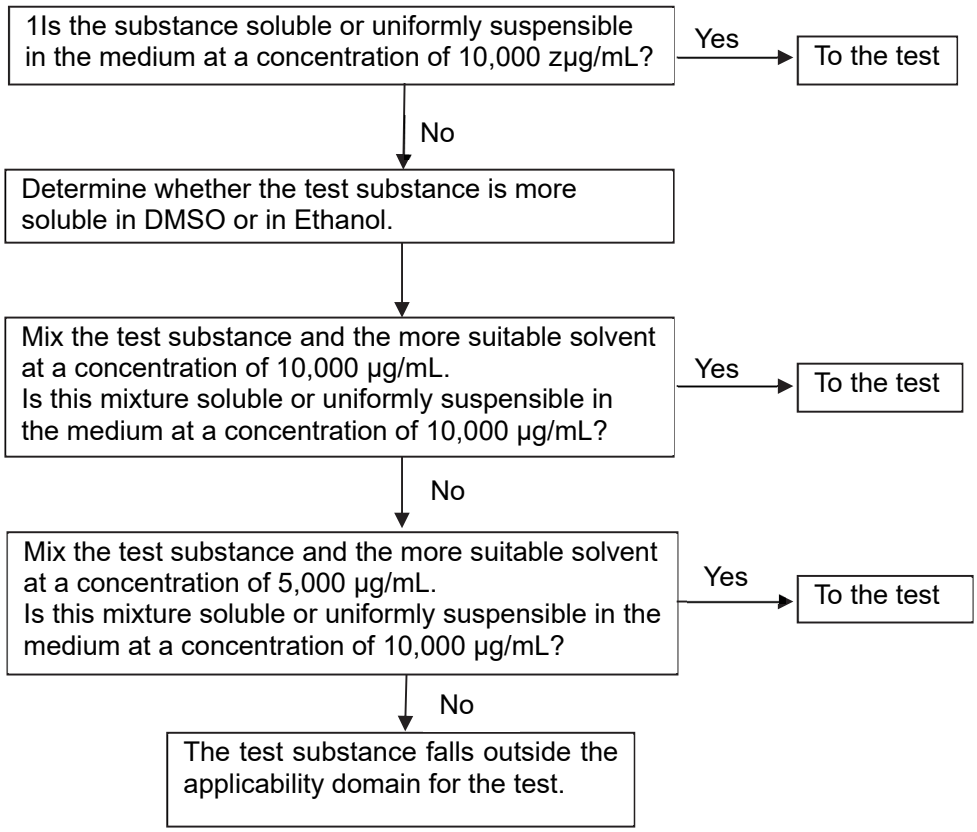

Figure 1: Determining solubility or suspensibility of test chemicals in the Medium

3.7.2 Preparing test chemicals

After determining an appropriate concentration for each test chemical per the procedure described in section 3.7.1. When the maximal concentration of a stock test chemical dilution series is 10,000 μg/mL, once the test chemical dilution series in the microplate is mixed with the Medium containing the SIRC cells, as described in section 4.3, the final maximal concentration is halved to 5,000 μg/mL (0.5% w/v). When either DMSO or ethanol is used as a solvent, the final maximal concentration is 5,000 μg/mL (0.5% w/v).

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

3.7.3 Preparing test chemical dilution series

Prepare in duplicate on the microplate an eight-well, two-fold serial dilution for each test chemical, as shown in Fig. 2: Layout of 96-well microplate.

3.8 Reference substances

3.8.1 Positive control

Use a solution of SDS at a final concentration of 1,000 μg/mL in the Medium as the positive control.

3.8.2 Relative control

Use a solution of triethanolamine at a final concentration of 10,000 μg/mL in the Medium as the relative control.

3.8.3 Negative control

Use the Medium, a DMSO-medium solution at a final concentration 10,000 μg/mL, or an ethanol-medium solution at a final concentration of 10,000 μg/mL as the negative control. The negative control should match the solvent used to dissolve or uniformly suspend the test chemical.

4 Test procedure

4.1 Passaging SIRC cells

Cell culture

1. Culture SIRC cells in MEM supplemented with 10% FBS and 1% P/S/F (the Medium) at 37°C in a humidified incubator at 5% CO2 in air. The concentrations of the antibiotics are 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of Amphotericin B.
2. Remove the Medium from the culture flask, then rinse the SIRC cells twice with 10 mL of modified PBS to remove the serum, which is a trypsin inhibiter.
3. Remove the modified PBS, then add and ensure that all the cells in the culture flask are exposed to 1.5 to 2.0 mL of 0.25% trypsin solution.
4. Remove the 0.25% trypsin solution, then incubate the cells as is for two or three minutes at 37°C. Detach the cells from the inside surface of the flask by tapping. Collect the cells in an appropriate volume of MEM (10% FBS). Count the cells and prepare a cell suspension at a density of 6 to 8 $\times 10^5$ cells in 15 to 30 mL of medium. Use this culture to passage the cells.

Freezing and preserving cells

1. Prepare a mixture of medium and 10% DMSO for freezing and preserving cells. Commercially available cell preservation solution such as Cellbanker 1or 2 (Juji Field, Inc.) may be used, and the solution may be either a serum type or non-serum type.

2. Add a solution at a density of 1×10^6 cells/mL to a stock tube and slowly lower the temperature until frozen. For example, cool the stock tube for 5 minutes in ice, 50 minutes at about −20°C, and 12 hours at about −70°C before placing it in liquid nitrogen. Commercially available freezing vessels such as Bicell (Nihon Freezer Co., Ltd) may be used to hold the tube.
3. The tube containing the cells is then preserved in liquid nitrogen.

Thawing of frozen cells

1. Immerse the stock tube in hot water at a temperature of 37°C to thaw the frozen cells.
2. Add 10 mL of the Medium to the cell suspension and centrifuge at 1,000 rpm for 5 minutes.
3. Remove the supernatant, then add the Medium to prepare the cell suspension. Passage the preserved cells at least once to confirm appropriate growth.

4.2 Preparing a cell suspension

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

4.3 Exposing the cells to a test chemical

1. Prepare 100 µL of modified PBS and the negative control as well as 100 µL of the serial dilutions of the test chemical, positive control, and relative control in a 96 well microplate, as shown in Fig. 1.
2. Add 100 µL of the 2×10^5 cells/mL cell suspension to the wells, as shown in Fig. 2.
3. Seal the microplate to prevent contamination from volatile test chemicals. Wrapping film may be used for this purpose. The six measurements described in steps (1)–(6) of section 4.6 Quality Control are to be used to verify that there is no contamination of other wells by volatile test chemicals. The criterion for toxic effect is the same as that for quality control. If contamination is found, the test is to be redone at a lower concentration.
4. After mixing the test chemical and the cell suspension, allow to stand for 20 minutes on a clean bench. Once the cells adhere to the bottom of the wells, the microplate is moved to the incubator.
5. Incubate for about 72 hours at 37°C and 5% CO_2 in air.

4.4 Crystal violet staining

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

4.5 Calculating IC_{50}

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

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

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

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

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

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

In the Excel spreadsheet, cell viability is rounded to the nearest tenth.

4.6 Quality control

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

1. The absolute OD obtained from the negative control is an index of the normal proliferation of SIRC cells seeded at a concentration of 2×10^4 cells/well and incubated for 72 hours. The mean

OD of the negative control (right and left wells) must be greater than 0.4 for the test data to be considered valid.

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

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

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

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

6. The two test results adopted for making a prediction must be checked for equality. The higher of the two IC_{50} values of the two positive controls (SDS) must be no more than twice as large as the lower of the two values. (The higher value ÷ the lower value ≦ 2)

4.7 Evaluation

Eye irritation potency of the test chemical is predicted using triethanolamine as a relative control (See Annex 1.). Triethanolamine is classified No Category under GHS, and using this as a reference, a test chemical is identified as negative (No Category) when the IC_{50} is higher than or equal to that of triethanolamine and is identified as positive (Category 1 or 2) when the IC_{50} is lower than that of triethanolamine. The test is performed twice. If the results of the two tests are different, a third test is performed and the data of the two tests with the same result are adopted for evaluating. If discrepancies between three results must be reviewed, the test is repeated three times.

5 References

1. Scott, L. et al., Toxicol. In Vitro, 24(1), 1-9 (2010).
2. Hagino, S. et al., ATLA, 36, 641-652 (2008)
3. Hagino, S. et al., ATLA, 38, 139-152 (2010)
4. Itagaki, H. et al., Toxicol. in Vitro, 5, 139-143 (1991).
5. Ohno, Y. et al., Toxicol. in Vitro, 13, 73 (1999).
6. Saotome, K. et al., Toxicol. in Vitro, 3, 317-321 (1989).

7. Itagaki, H., AATEX, 3, 182-190 (1995).
8. Ohno, Y et al., AATEX, 3, 123 (1995).
9. Tani, N., Toxicol. in Vitro, 13,175 (1999).
10. Guidance for evaluation of eye irritation of cosmetic ingredients using alternative method (Draft document by the study team supported by Ministry of Health and Welfare), AATEX,5,Suppl., Guideline Draft1-3 (1998).
11. Ohno, Y., ATLA, 32, Supplement 1, 643-655, 2004.

6 List of abbreviations and acronyms

°C	degrees Centigrade
ATCC	American Type Culture Collection
DMSO	Dimethyl Sulfoxide
EPA	United States Environmental Protection Agency
FBS	Fetal Bovine Serum
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
IC_{50}	half maximal inhibitory concentration
I	Irritant
JaCVAM	Japanese Center for the Validation of Alternative Methods
MEM	Minimum Essential Medium
NI	Non Irritant
OD	Optical density
Modified PBS	Phosphate-Buffered Saline without calcium or magnesium
SDS	Sodium Dodecyl Sulfate
SIRC cells	Statens Seruminstitut Rabbit Corneal cells
SIRC-CVS	Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining

7 Revision history

(1) The revision of ver.1 – ver.1.71 that is the same as ver.1.71j and ver.1.71e, is shown by the green character in ver. 2.13. The protocol of the ver.1 was used for evaluating 68 chemicals at Shiseido Research Center in 2009-2010 and was subjected to the peer review by JaCVAM.

(2) The revision after ver.1.71 is shown by the blue character in ver. 2.13.

(3) The revision from ver.2.07 to ver.2.08:

In　4.7.(4), “The difference between two dilution series of the substance should be confirmed. The IC50s of the first series and the second series should be within + 20% of the mean IC50 of two series （the mean + 20%）, respectively.” was changed to “The difference between two dilution series of the substance should be confirmed. The IC50s of the first series and the second series should be within + 20% of the mean IC50 of two series(the mean of the first IC50 and the second IC50), respectively.”

(4) The revision form ver.2.08 to ver.2.09:

In 4.3.(3), “The five measurements (4.7.(1)-(5)) of the quality control should be used for checking wether the volatile substance has an effect on other wells. The criterion of the toxic effect is the same as that of the quality control. When the volatile substance has an effect on other wells, the retest should be performed using dilution.”

(5) The revision from ver.2.09 to ver.2.11:

“SIRC cytotoxicity test” was changed to “SIRC-CVS cytotoxicity test”. “Table 1” of 3.7.1. was changed to “Figure 1”.

(6) The revision from ver.2.11 to ver.2.12:

The version was added to title. SIRC-CVS was added to list of abbriviations and acronyms of 6. “Figure 1. Layout of 96 well microplate“ was changed to “Figure 2. Layout of 96 well microplate”. “Figure 2. Addition of cell suspension” was changed to “Figure 3. Addition of cell suspension”

(7)The version from ver.2.12 to ver.2.13:

In 3.7.2., “Futhermore, the data of the well with precipitation or so on at anytime after the mix of the substance and the cell should be reject for unsuitable suspension” was changed to “

Furthermore, the data of the well with precipitation or so on at anytime after the mix of the substance and the cell, especially after 72 hr incubation, should be rejected for unsuitable suspension”.

The address of the author, “2-12-1, Fukuura, Kanazawa-ku, Yokohama-shi, 236-8643 Japan” was changed to “2-2-1, Hayabuchi, Tsuzuki-ku, Yokohama-shi, 224-8558, Japan”.

(8)The revision from ver.2.12 from ver.3.1:

The version and the date were renewed.

In 1., “and United States Environmental Protection Agency (EPA)” was deleted.

In 3.1, “（e.g. When the cell culture starts at passage number of 435 and is maintained by two passages per one week, it should be used within passage number of 458）” was added.

In 3.4., ”The manufacturer and so on of the reagent is shown in the Table 1.” was changed to “The example of the manufacturer and so on of the reagent is shown in the Table 1.”.

In 3.7.1., “The solubility of the substance in the medium should be confirmed in advance.” was changed to “The solubility of each substance in the medium should be confirmed in advance, using the procedure shown in Fig. 1.”.

In figure 1, “Select the solvent on the basis of the examination of the solubility in DMSO or Ethanol.” was changed to “Examine which solvent is more soluble, DMSO or Ethanol，and select appropriate solvent.

In 4.6., “For EPA standard, the test substance is judged as negative (Category 4) when the IC50 is higher than that of triethanolamine, and is judged as positive (Category 1-3) when the IC50 is lower than or equal to that of triethanolamine （see table 3 and 4 of annex 1）.” was deleted.

In table 1., “The manufacturer and so on of the reagent” was changed to “The example of the manufacturer and so on of the reagent” . Also, lot numbers were deleted from remarks.

In annex 1, “The same examination that the in vivo evaluation is performed by EPA classification is shown in table 2. The in vivo results are discriminated between category 4 and others (category 1-3) of the EPA standard. Triethanolamine is classified as category 3. Therefore, when the IC50 of future test substance is higher than that of triethanolamine, it should be evaluated as a non irritant. If the IC50 of the test substance is lower than or equal to that of triethanolamine, it should be evaluated as an irritant.” was deleted.

Table 2 and Table 4 of annex 1 were deleted and table number was moved up.

(8)The revision from ver.3.1 to ver.3.2:

In 4.7. (4), “Treatment of IC50 expressed with inequality sign is performed in the same manner.” was added.

(9)The revision from ver.3.2 to ver.3.3:

In “1.Purpose”, “as a bottom up approach（Scott et al., 2010）” was added.

In “2. The principle of SIRC cytotoxicity test”, ”The relative control was additionally used to obtain the invariable results (Ohno, 2004).“ was added.

In triethanolamine of “3.4. Culture medium and reagent”, “It should be used that of Purity>=98.0%.” was added.

In 3.7.2., overlap with 3.7.1 was removed. “The test substance is solved or uniformly suspended with medium at a concentration of 10,000 μg/mL (1% w/v). It is solved or uniformly suspended using vortex mixer, waterbath and sonicator when it finds necessary. DMSO or ethanol is used for solving or suspending if needed. The concentration of DMSO or ethanol is 10,000μg/mL (1% w/v) in the initial substance solution. The solvent selection is medium, DMSO in medium and ethanol in medium in order. In addition, the concentration of the substance is decreased to 5,000 μg/mL (0.5% w/v) for suspending when it finds necessary. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test.”was deleted. And, “The test substance is solved or uniformly suspended at appropriate concentration by the procedure described in 3.7.1.” was added.

In “4.6.Evaluation”, “on the basis of the in vivo data by Ohno et al. “ was deleted.

In “5.References” and the text, the reference numbers were added. The format of reference was changed. The references of the following paper were added.

Ohno, Y., ATLA, 32, Supplement 1, 643-655, 2004.

Scott, L. et al., Toxicol. In Vitro, 24(1), 1-9 (2010).

The reference of the following paper was deleted.

Ohno, Y. et al., In Vitro Toxicol.,7, 89 (1994)

Table 2 of annex 1 and the related sentences were deleted.

In reference of annex 1, the format of references was changed.

(10)The revision from ver.3.3 to ver.3.4:

In 2., “The application time of 72hr is set with consideration of time (within 72hr) from ocular instillation to maximal eye irritation for general chemicals except acid or alkali, on the basis of in vivo data at the previous research project5).” was added.

In 2., “The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet.” was changed to “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane and stains biological macromolecules.”

In 2., “On the other hand, the disadvantage of this method is to be confined to test substances which are solved or uniformly suspended in the medium.” was added.

In 4.5., “If the multiple concentrations showing 50% cell viability were obtained from one substance, the lowest IC50 should be adopted.” was added.

(11)The revision from ver.3.4 to ver.3.5:

In 2., “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane and stains biological macromolecules” was changed to “The SIRC cytotoxicity test is based on the measurement of viable cells stained by crystal violet, which penetrates via cell membrane treated with methanol and stains biological macromolecules.”.

In 3.1., “The cells should be used during 3 months after the start of cultivation” was changed to “The cells should be used within 35 passages from their purchased stock”.

In 3.4., the explanation of Phosphate-Buffered Saline（-）,“Calcium and magnesium are removed from PBS” was added.

4.4 was reinstated from mistaken deletion in ver.3.4.

In (1) of 4.7., “seeded at the concentration of 1x104 cells/well” was changed to “seeded at the concentration of 2x104 cells/well”.

(12)The revision from ver 3.5 to ver.3.6:

In 4.5., “Treatment of IC50 expressed with inequality sign is performed in the same manner.” was added.

The order of 4.6.Evaluation and 4.7. Quality control was changed to that of 4.6. Quality control and 4.7. Evaluation.

In (4) of 4.6., “The treatment to IC50 expressed with equality sign are only used at the calculation for quality control.” was added.

(13) The revision from ver.3.6 to ver.3.7:

In 4.7, “ of GHS standard” was revised to “of UN GHS classification system”.

In 3.7.1., “and has no test.” was revised to “and is not judged as testable.”

(14) The revision form ver.3.7 to ver 3.8

In 3.8, many minor revisions for readability were made by a native-English speaker. (see Word file)

Table 1. Typical reagents and their manufacturers

Reagent or Medium	Manufacturer	Catalog number		Notes
MEM (Minimum Essential Medium)	GIBCO	Code No.	11095	
Fetal Bovine Serum	GIBCO	REF No.	26140-079	
Penicillin-Streptomycin-Amphotericin (100x)	GIBCO/BRL	REF No.	15240-062	
Phosphate-Buffered Saline (modified PBS)	Nissui	Code No.	05913	
0.25% (w/v) Trypsin (1mmol/L EDTA·4Na)	Wako	Cat No.	209-16941	
Dimethyl sulfoxide (DMSO)	Kanto	Cat No.	2950-1B	
Ethanol	Wako	Cat No.	057-00456	
Crystal violet	Wako	Cat No.	031-04852	
Methanol	Wako	Cat No.	131-01826	
Sodium Dodecyl Sulfate	Wako	Cat No.	191-07145	
Triethanolamine	Kanto	Cat No.	40268-00	

Products of the same specification from the specified manufacturer may be used for Minimum Essential Medium, Fetal Bovine Serum, Penicillin-Streptomycin-Amphotericin, Sodium Dodecyl Sulfate, and Triethanolamine. Equivalent products from other manufacturers are acceptable for other reagents.

Nissui: Nissui Pharmaceutical Co., Ltd

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: Kanto Chemical, Co., Inc.

Figure 2: Layout of the 96-well microplate

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

PBS: 200 µL of modified PBS

NC: The Medium, a DMSO-Medium solution at a concentration of 10,000 µg/mL, or an ethanol-Medium solution at a concentration of 10,000 µg/mL

S: Eight-well, two-fold serial dilution of the test chemical (100 µL per well)

R: Eight-well, two-fold serial dilution of the relative control (100 µL per well)

P: Eight-well, two-fold serial dilution of the positive control (100 µL per well).

Serial dilution of the test chemical is made using the Medium, a 10,000-µg/mL concentration of DMSO-Medium solution, or a 10,000-µg/mL concentration of ethanol-Medium solution. Serial dilution of the positive and relative controls are made using the Medium.

Figure 3. Addition of cell suspension

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

Cell suspension (100 µL)

Annex 1 Rational for using triethanolamine as a reference control

Triethanolamine was selected as a relative control substance of the SIRC cytotoxicity test for distinguishing between ocular non-irritants (NI) and irritants (I) per the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). It is one of the substances used in the previous validation study that was performed by a Research Grant for Health Sciences, MHW and Japanese Cosmetic Industry Association, and reported by Ohno et al.[1] and Tani et al.[2] Also, it is readily available commercially, is soluble in the Medium, and has been studied extensively, with plenty of useful cytotoxicity and in vivo data available for evaluating eye irritation potency.

In selecting the relative control substance, accuracy and other characteristics for distinguishing between NI and I were checked for every available substance from previous validations. As a result of this comparative study, triethanolamine was selected as a relative control substance because of a relatively low instance of false negatives, a high accuracy except for substances which for which cytotoxicity could not be quantified (10000 < etc.), and clarity of categorization (for example, alcohol) for substances yielding false negative, as shown in table 1. Identification of substances likely to result in false negatives is important from the perspective of minimizing the risk of eye irritation.

References

1) Ohno, Y. et al., Toxicology in Vitro 13, 73-98 (1999)

2) Tani, N. et al., Toxicology in Vitro 13, 175-187(1999)

Table 1 The correlative evaluation on the basis of the previous validation study data for selecting relative control substance
- GHS classification -

Key: TN: true negative, FN: false negative, TP: true positive, FP: false positive

Substances	GHS classification based on In vivo testing[NB1]	SIRC cytotoxicity: IC_{50} (μg/mL)[NB2]	Ranking	TN	FN	TP	FP	Acc (%)
Polyethylene glycol 400	NI	35300 <	35300	1	0	27	6	82
Silicic anhydride	NI	14800 <	14800	2	0	26	6	82
Glycerin	NI	11600	11600	3	0	25	6	82
Isotonic sodium chloride solution	NI	10000 <	10000	4	1	24	5	82
Ethanol	1 or 2A	10000 <	10000	4	1	24	5	82
Isopropyl myristate	NI	9330 <	9330	5	1	24	4	85
Butanol*	1 or 2A	8880 <	8880	5	2	23	4	82
Triethanolamine	NI	2090	2090	6	2	23	3	85
Lactic acid	1	1230	1230	6	3	22	3	82
Benzyl alcohol	1 or 2A	1190	1190	6	4	21	3	79
Polyoxyethylene sorbitan monooleate (20E.O.)	NI	963	963	7	4	21	2	82
Sodium salicylate	1 or 2A	952	952	7	5	20	2	79
Glycolic acid*	1 or 2A	868	868	7	6	19	2	76
Acetic acid*	1 or 2A	721	721	7	7	18	2	74
Diisopropanolamine*	1, 2A, or2B	699	699	7	8	17	2	71
2-Ethylhexyl p-dimethylamino benzoate	NI	474	474	8	8	17	1	74
Calcium thioglycolate	1	392	392	8	9	16	1	71
Acid red 92	1 or 2A	297	297	8	10	15	1	68

Sucrose fatty acid ester	1 or 2A	286	286	8	11	14	1	65
m-Phenylenediamine	1 or 2A	218	218	8	12	13	1	62
Methyl p-hydroxybenzoate	NI	207	207	9	12	13	0	65
Di (2-ethylhexyl) sodium sulfosuccinate*	1 or 2A	181	181	9	13	12	0	62
Sodium lauryl sulfate*	1 or 2A	168	168	9	14	11	0	59
Sodium hydrogenated tallow L-glutamate*	1 or 2A	140	140	9	15	10	0	56
Potassium laurate*	1 or 2A	120 (Data from 4 labs)	120	9	16	9	0	53
Chlorhexidine gluconate (20% solution)*	1 or 2A	67.6	67.6	9	17	8	0	50
Polyoxyethylene octylphenylether (10 E.O.)*	1 or 2A	38.4	38.4	9	18	7	0	47
Distearyldimethylammonium chloride	1	37.8	37.8	9	19	6	0	44
Benzalkonium chloride*	1 or 2A	19.0	19	9	20	5	0	41
Domiphen bromide*	1 or 2A	12.1	12.1	9	21	4	0	38
Monoethanolamine*	1 or 2A	9.62	9.62	9	22	3	0	35
Cetyltrimethylammonium bromide*	1 or 2A	2.59 (Data from 4 labs)	2.59	9	23	2	0	32
Cetylpyridinium chloride*	1	1.67	1.67	9	24	1	0	29
Stearyltrimethylammonium chloride*	1	1.58	1.58	9	25	0	0	26

NB1 The Draize eye test results do not always discriminate between GHS Category 1 and Category 2 when data was recorded on day 21. Data was recorded on day 14.

NB2 Data for the SIRC cytotoxicity test are the mean IC_{50} (ug/mL) from five or more laboratories, except for one part.

* The in vivo results for “as is” applications were predicted from data taken with 10% concentrations.

Annex 2 The basis for the set IC50 range of triethanolamine as a reference control

The IC_{50} range of triethanolamine, 1,000–2,500 µg/mL, is based on the mean +2 standard deviations from the data (n=144).