Appendix 8.2 Protcol

Protocol for SIRC cytotoxicity test
January 18, 2012.
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1. Purpose

This test method is used to measure cytotoxicity of chemicals using SIRC cell and to discriminate between non irritant and irritant in the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

2. The principle of SIRC cytotoxicity test

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

3. Materials

3.1. Cell

The SIRC cell (Statens Seruminstitut rabbit corneal cell: ATCC NO. CCL-60), the cell line from the rabbit cornea is obtained from ATCC (American Type Culture Collection) through Dainippon Sumitomo Pharma Co. Ltd. as an agency. The cell is frozen and kept in liquid nitrogen. The cell should be confirmed the absence of mycoplasma (e.g., Venor GeM Mycoplasma Detection Kit、Minerva Biolabs GmbH、11-1025)。

3.2.Technical Equipment

- CO_2 Incubator (e.g., SANYO Electric Co., Ltd., MCO-17AIC)
- Clean bench (e.g., Hitachi, Ltd., CCV1300E)
- Microplate reader (e.g., Bio-Rad Laboratories, Benchmark Plus ™)
- Inverse phase contrast microscope (e.g., Nikon, ECLIPSE TS100)
- Autoclave (e.g., TOMY SEIKO CO.,LTD, BS-325 and SS-320)
- Centrifuge (e.g., Kubota Corporation, 5800)
- Water bath
- Electronic chemical balance
- Ultrasonic bath sonicator
- Hemocytometer (e.g. ERMA INC, 03-303-5) or Cell counter

3.3. Experimental instrument

- Tissue culture flasks (75 cm^2, 25 cm^2) (e.g., BD Falcon 353136 and 353112)
- 96-well flat bottom tissue culture microtiter plates (e.g., BD Falcon, 353072)
- Storage plate (e.g., Thermo Scientific、0.8 mL Storage plate、AB-0765)
- Multichannel pipette, micropipette
- Dispenser tray
- Tubes
- Cryotube(1.5 mL)
- Centrifuge tube (15 mL, 50 mL)
- Tip for micropepett (200 μL, 100uL, 5 mL)
- Microplate sealing tape

・Vortex mixer
・pH paper (e.g. Whatman 2613991)
・Magnetic stirrer
・Paper towel (e.g., NIPPON PAPER CRECIA Co. LTD, Kim towel™, 61000)
・Wrap film (e.g., Saran Wrap)

3.4.Culture medium and reagent

Minimum Essential Medium (MEM)
Fetal Bovine Serum (FBS)

・Inactivated fetal bovine serum should be used. Inactivation is performed by 56℃, 30 min in the water bath. After fall in temperature, the serum is taken 56 mL or 28 mL in each tube. The serum is stored at −70℃ or at −20℃.

Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
(Antibiotic-Antimycotic 100x ; GIBCO BRL)
Phosphate-Buffered Saline(−) (PBS(−))
0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)
Dimethyl Sulfoxide (DMSO; CAS Number 67-68-5)
Ethanol (EtOH; CAS Number 64-17-5)
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)
Hydrochloric Acid (CAS Number 7647-01-0)
Sodium Hydroxide (CAS Number 1310-73-2)
The manufacturer and so on of the reagent is shown in the Table 1.

3.5.Medium

MEM is supplemented with 10% FBS (inactivated) and 1% appropriate 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 Penicillin 100 U/mL, Streptomycin 100 μg/mL and Amphotericin B 250 ng/mL, respectively.

3.6.Crystal violet solution

0.4% Crystal violet solution is prepared using methanol.

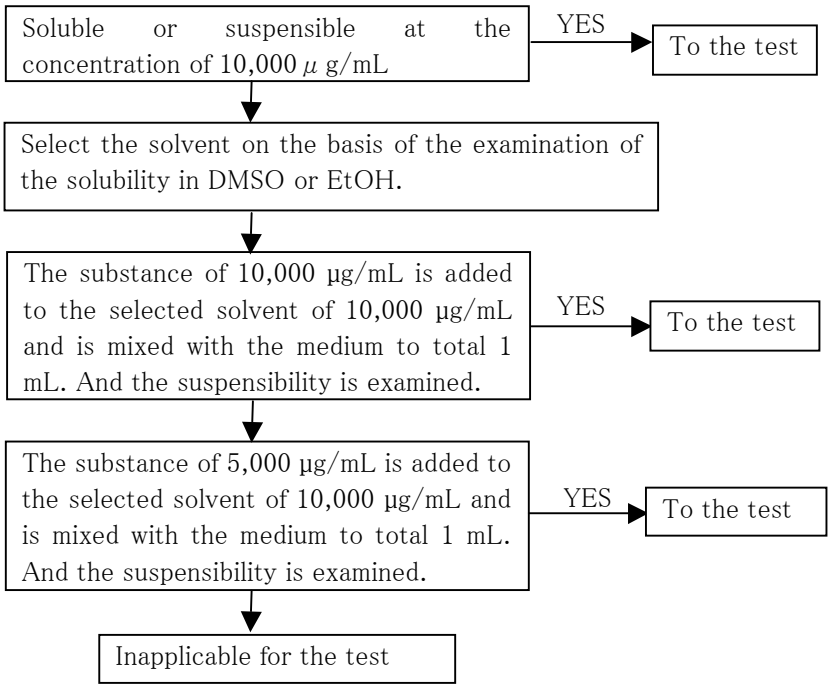
3.7. Test substances

Test substance is prepared.

3.7.1. Examination of stability for the substance in the medium

The solubility of the substance in the medium should be confirmed in advance. Firstly, the solubility or suspensibility of the substance is examined at the concentration of 10,000 μg/mL in the medium. When it is not suspended uniformly, the solubility to DMSO or ethanol is examined as a next step. The substance of 10,000 μg/mL is added to the selected solvent of 10,000 μg/mL and is mixed with the medium to total 1 mL. The suspensibility is examined after sonicaiton and so on. When it is not suspended uniformly at the concentration of 10,000 μg/mL, the suspensibility is examined at the concentration of 5,000 μg/mL. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test. These judges are performed macroscopically.

Flow chart of examination of stability for the substance in the medium



3.7.2. Preparation of test substances

The test substance is solved or uniformly suspended with medium at a concentration of 10,000 μg/mL. Vortex mixer, waterbath and sonicator can be used for solving and suspending. Also, DMSO or EtOH of the concentration of 10,000 μg/mL can be used for solving or suspending. The solvent selection is medium, DMSO in medium and ethanol in medium in order. The maximal concentration of the substance can be decreased to 5,000 μg/mL for suspending. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test. The final maximal concentrations of the test substances and the recommended solvents are 5,000 μg/mL, respectively. The final maximal concentration of the test substance may be 2,500 μg/mL. In addition, the data of the well with precipitation or so on should be reject for unsuitable suspension.

If the neutralization of the substance is needed for the test, 0.1N HCl or 0.1N NaOH and so on is used for the nutralizaiton.

3.7.3. Dilution of substance solution

The dilution step of substance should be eight by a common ratio of two. The test of the same concentration should be performed in duplicate on the plate.

3.8. Reference substance

3.8.1. Positive control substance

SDS should be used as a positive control. The SDS solution (in medium) is prepared at the concentration of 1,000 μg/mL.

3.8.2. Relative control substance

Triethanolamine should be used as a relative control. The triethanolamine solution (in medium)

is prepared at the concentration of 10,000 μg/mL.

3.8.3. Negative control substance

Medium, 10,000 μg/mL DMSO-Medium solution or 10,000 μg/mL EtOH-Medium solution should be used as a negative control. It should be selected on the basis of what kind of solvent solve or suspend test substance.

4. Procedure

4.1. Passage culture of cell

・cell culture

(1)The SIRC cell is cultured with MEM supplemented 10% FBS, 1% P/S/F and so on at 37 ℃ in a humidified incubator containing 5% (v/v) CO_2 in air. The concentrations of the penicillin, streptomycin and amphotericin B are 100 U/mL, 100 μg/mL and 250 ng/mL, respectively.

(2)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(3)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(4)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2 or 3 minutes at 37℃. The cells are detached from the surface of the flask, tapping it. They are collected by the appropriate amount of the MEM (10% FBS) from the culture flask. After counting the cells, the cell suspension is prepared at the density of 6-8x10^5cells in 15-30 mL of the medium. The passage culture is performed using it.

・Freeze preservation of cell

(1) The medium contained 10% DMSO is prepared as a medium for the freeze preservation of cell. The commercial available freeze preservative solution (e.g., Juji Field Inc., Cell banker 1or 2) can be used. Both serum type and non serum type can be used.

(2) The cell of 1x10^6 cells/mL is add to stock tube. It is froze slowly. For example, the freeze is performed at 5 minutes in the ice, 50 minutes at -20℃ and 12 hours in the -80℃ and in the liquid nitrogen in order. The commercial available freezing vessel (e.g., Nihon Freezer Co. LTD., BICELL) can be used. In this case, the vessel containing the tube for the cell is frozen at -80℃ and in the liquid nitrogen after 3 hours - overnight.

(3) The tube containing the cell is preserved in the liquid nitrogen.

・Melting of cell

(1)The stock tube is dipped in the water of 37℃ for melting.

(2)The cell suspension added the medium of 10 mL is centrifuged at 1,000 rpm x5 min.

(3)After removing the supernatant, the cell suspension is prepared by the medium.

・The freeze preservative cell is passaged one or more times and should be confirmed the appropriate growth.

4.2. Preparation of cell suspension

(1)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(2)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(3)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2 or 3 minutes at 37℃.

(4)The cells are detached from the surface of the flask, tapping it.

(5)The cells are collected by the appropriate amount of the MEM (10% FBS) and pepetting.

(6)After counting the cells, the cell suspension is prepared at the density of 2x10^5 cells/mL.

4.3.Application of test substance

(1)PBS(-), negative control and dilution series of positive control and relative control are prepared in the 96 well microplate. The layout of the microplate is shown in fig. 1.

(2)One tenth mL of the $2x10^5$ cells/mL cell suspension is added to the wells as shown in fig.2.

(3)Microplate sealer is used to avoid the effect of volatile toxicant. Moreover, wrap film can be used. When the test substance has an effect on other wells, the test can be performed using dilution.

(4)The microplate added the test substance and the cell suspension is still standing for 20 minutes in the clean bench. The cell is adhered to the bottom of the microplate. After that, the microplate is moved to the CO_2 incubator.

(5)The microplate is incubated for about 72 hr at the condition of 37℃ and 5% CO_2,

4.4. Crystal violet staining

(1)After the incubation, the medium containing the test substances is removed by gentle turnover of the microplate.

(2)PBS(-) of 200 μL is added. After gentle shake, PBS(-) is removed by the turnover of the microplate. This procedure is repeated twice.

(3)One hundred μL of crystal violet methanol solution is added to each well of the microplate, and stain the cells for 30 minutes.

(4)After the staining, the crystal violet methanol solution is removed by gentle turnover of the microplate. It is washed by tap water sufficiently, and is blotted water with the paper towel.

(5)After drying sufficiently, the absorbance at 588 nm is measured with an automatic microplate reader.

4.5.Calculation of IC50

The absorbance of control wells, which contained no test substance, is regarded as 100%, and the percentage absorbance for each well is calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC50) is obtained from the next formula using two concentrations around the predicted concentration of 50% cell viability.

$LogIC50=[(50-y1)logx2-(50-y2)logx1]/(y2-y1)$

(x 1 : Low concentration, x2 : High concentration, y1: cell viability at low concentration, y2 : cell viability at high concentration, Log means common logarithm.)

If the cell viability is >50% at the maximal concentration of 5,000 μg/mL, the result of the test substance is IC50>5,000 μg/mL. Also, if the cell viability is <50% at the concentration of 39.1 μg/mL, the result of the test substance is IC50<39.1 μg/mL.

In a spreadsheet software (Excel), the cell viability value is rounded to the nearest tenths.

4.6.Evaluation

The eye irritation of the test substance is evaluated by using triethanolamine as a relative control. The test substance is judged as negative when the IC50 is higher than or equal to that of triethanolamine, and is judged as positive when the IC50 is lower than that of triethanolamine. If the two decisions are different, the two results with the same decision are adopted after the third test. If the confirmation of the deviation is needed with three results, the test is repeated three times.

4.7.Quality control

The quality control of the SIRC cytotoxicity test is performed by the three measurements. It needs to satisfy the next criterion. If it is not satisfy the criterion, the retest of the microplate

should be performed. Especially, if the toxic effect of the volatile substance causes unacceptable values, the retest should be performed at the lower concentration.

(1)The absolute OD obtained from the negative control is index of normal proliferation of SIRC cell seeded at the concentration of $1x10^4$ cells/well and incubated 72hr. The mean OD of the negative control (the right and left wells) should be >0.4 for complete test.
(2) Sodium dodecyl sulfate (SDS) is used as a positive control. The IC50 of SDS should be 77.7-258.7 μg/mL when it is tested by the standard protocol. It needs for complete test.
(3) Triethanolamine (TEA) is used as a relative control. The IC50 of TEA should be 1,000-5,000 μg/mL when it is tested by the standard protocol. It needs for complete test.
(4) The difference between left and right wells of the negative control should be confirmed to check the systematic quality. The mean ODs of left and right wells should be within ±15% of the mean OD of all negative control wells (the mean ± 15%), respectively.
(5) The difference between the two test results adopted should be confirmed to check the equality of them. The IC50 values of two tests of positive control (SDS) should be lower or equal to twice. (The high value/the low value≦2)

References

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Y.Ohno, T.Kaneko, H.Itagaki et al., Toxicol. *in Vitro*, 13,73 (1999).
N.Tani, H.Itagaki, Y.Ohno et al., Toxicol. *in Vitro*, 13,175 (1999).

Table 1. The manufacturer and so on of the reagent

Reagent or Medium	Manufacturer	Catalog number	
MEM (Minimum Essential Medium)	GIBCO	Code#:	11095
Fetal Bovine Serum	GIBCO	Cat#:	Under consideration
Penicillin-Streptomycin-Amphotericin (100x)	GIBCO/BRL	REF#:	15240-062
Phosphate-Buffered Saline (PBS(-))	Nissui	Code#:	05913
0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)	Wako	Cat#:	209-16941
Dimethyl sulfoxide (DMSO)	Kanto	Cat#	2950-1B
Ethanol (EtOH)	Wako	Cat#:	057-00456
Crystal violet	Wako	Cat#:	031-04852
Methanol	Wako	Cat#:	131-01826
Sodium Dodecyl Sulfate	Wako	Cat#:	191-07145
Triethanolamine	Kanto	Cat#:	40268-00

Nissui: NISSUI PHARMACEUTICAL CO., LTD

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: KANTO CHEMICAL, CO., INC.

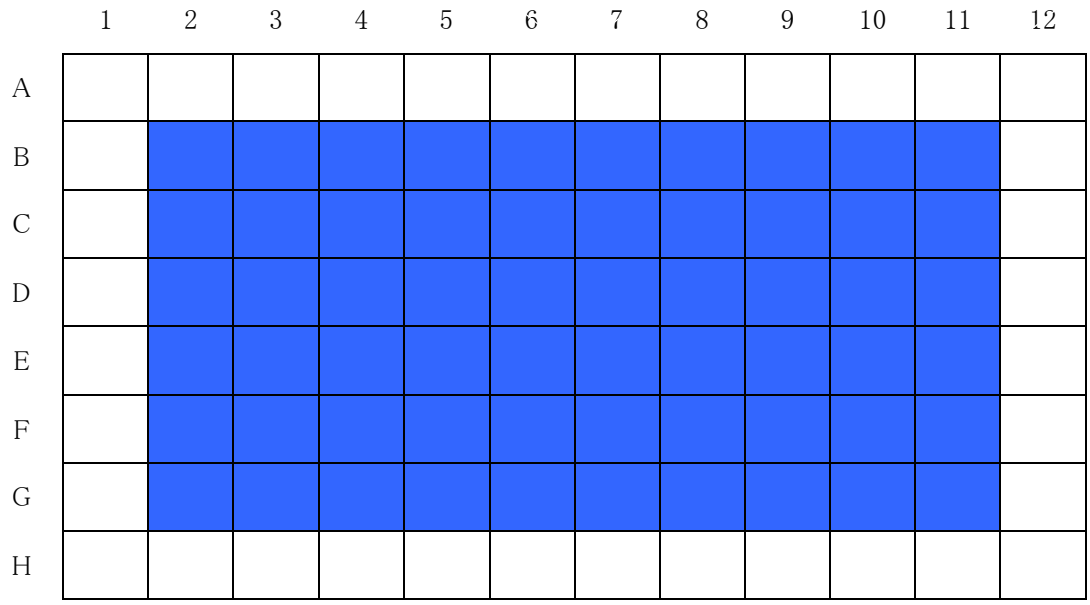
Fig. 1 Layout of 96 well microplate

	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:PBS(-) of 200 µL, NC:Medium, 10,000 µg/mL DMSO-Medium solution or 10,000 µg/mL EtOH-Medium solution of 100 µL, S:Dilution series of the test substances by a common ratio of two (100 µL), R:Dilution series of the relative control by a common ratio of two (100 µL), P: Dilution series of the positive control by a common ratio of two (100 µL).

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

Fig. 2 Addition of cell suspension



■ : Cell suspension (100 µL)

Protocol for SIRC-CVS cytotoxicity test (Version 2.12)
August 27, 2012.
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1. Purpose

This test method is used to measure cytotoxicity of chemicals using SIRC cell and to discriminate between non ocular irritant (NI) and ocular irritant (Others). The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. The principle of SIRC cytotoxicity test

The cytotoxicity has been known as an index for evaluating the eye irritation by chemicals, as the corneal damage that has a greater impact on the total eye irritation is related to that of the corneal epithelium cell. The cytotoxicity test is reported to be useful for identifying the non ocular irritants that has almost no effect on the cornea. The cell line used comes from rabbit cornea.
The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the tested microplate can be stored. The results can be confirmed by using the stored microplates in any time. No other method can match it.

3. Materials

3.1. Cell

The SIRC cell (Statens Seruminstitut rabbit corneal cell: ATCC NO. CCL-60), the cell line from the rabbit cornea is obtained from ATCC (American Type Culture Collection). The cells can be frozen and kept in liquid nitrogen. The cells should be confirmed the absence of mycoplasma(e.g., Venor GeM Mycoplasma Detection Kit、Minerva Biolabs GmbH、11-1025). The cells should be used during 3 months after the start of cultivation. They should be checked on the basis of the quality control of 4.7.

3.2.Technical Equipment

- CO_2 Incubator (e.g., SANYO Electric Co., Ltd., MCO-17AIC)
- Clean bench (e.g., Hitachi, Ltd., CCV1300E)
- Microplate reader (e.g., Bio-Rad Laboratories, Benchmark Plus ™)
- Inverse phase contrast microscope (e.g., Nikon, ECLIPSE TS100)
- Autoclave (e.g., TOMY SEIKO CO.,LTD, BS-325 and SS-320)
- Centrifuge (e.g., Kubota Corporation, 5800)
- Water bath
- Electronic chemical balance
- Ultrasonic bath sonicator
- Vortex mixer
- Magnetic stirrer
- Hemocytometer (e.g. ERMA INC, 03-303-5) or Cell counter

3.3. Experimental instrument

- Tissue culture flasks (75 cm^2, 25 cm^2) (e.g., BD Falcon 353136 and 353112)

・96-well flat bottom tissue culture microtiter plates (e.g., BD Falcon, 353072)
・Storage plate (e.g., Thermo Scientific、0.8 mL Storage plate、AB-0765)
・Multichannel pipette, micropipette
·Dispenser tray
・Tubes
・Cryotube(1.5 mL)
・Centrifuge tube (15 mL, 50 mL)
・Tip for micropepett (200 μL, 100uL, 5 mL)
·Microplate sealing tape
・Paper towel (e.g., NIPPON PAPER CRECIA Co. LTD, Kim towel™, 61000)
・Wrap film (e.g., Saran Wrap)

3.4.Culture medium and reagent

Minimum Essential Medium (MEM)
Fetal Bovine Serum (FBS)
・Inactivated fetal bovine serum should be used. Inactivation is performed by 56℃, 30 min in the water bath. After fall in temperature, the serum is taken 56 mL or 28 mL in each tube. The serum is stored at -70℃ or less or at -20℃ or less.
Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
(Antibiotic-Antimycotic 100x ; GIBCO BRL)
Phosphate-Buffered Saline(-) (PBS(-))
0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)
Dimethyl Sulfoxide (DMSO; CAS Number 67-68-5)
・the weighing can be converted to volume on the basis of specific gravity.
Ethanol (CAS Number 64-17-5)
・the weighing can be converted to volume on the basis of specific gravity.
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)
Hydrochloric Acid (CAS Number 7647-01-0)
Sodium Hydroxide (CAS Number 1310-73-2)
The manufacturer and so on of the reagent is shown in the Table 1.

3.5.Medium

MEM is supplemented with 10% FBS (inactivated) and 1% appropriate 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 Penicillin 100 U/mL, Streptomycin 100 μg/mL and Amphotericin B 250 ng/mL, respectively.

3.6.Crystal violet solution

0.4% Crystal violet solution is prepared using methanol.

3.7. Test substances

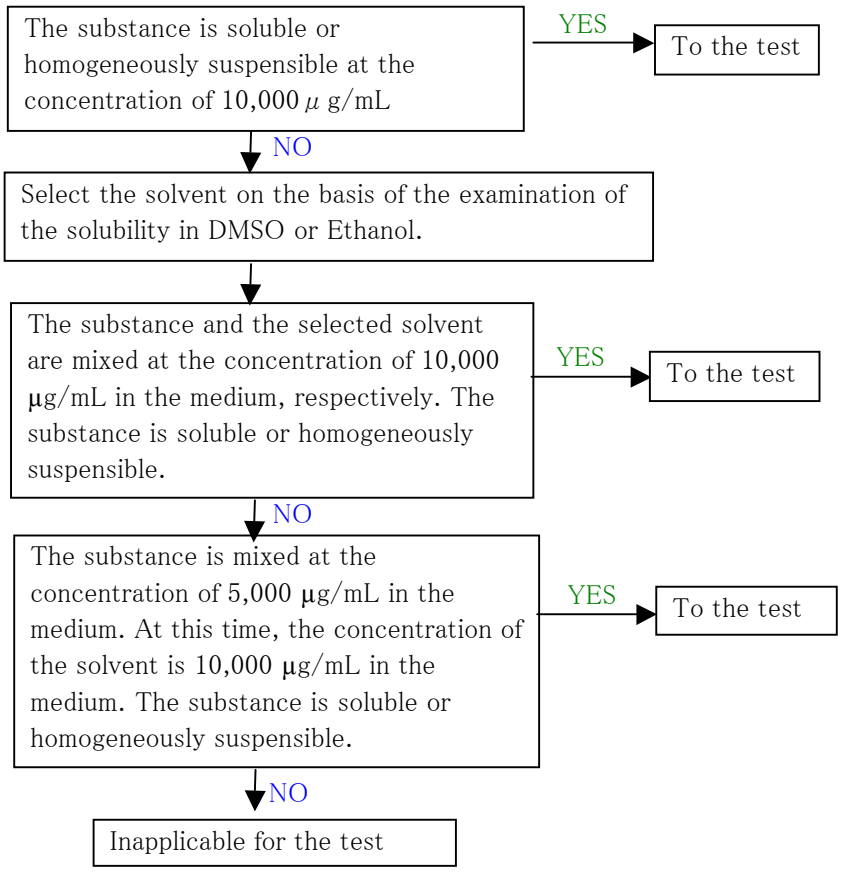
Test substance is prepared.

3.7.1. Examination of stability for the substance in the medium

The solubility of the substance in the medium should be confirmed in advance. The solubility or suspensibility of the substance is examined at the concentration of 10,000 μg/mL (1 w/v%)

in the medium. It is solved or uniformly suspended using vortex mixer, waterbath and sonicator when it finds necessary. When it is not suspended uniformly, the solubility to DMSO and ethanol is examined as a next step. The substance and the selected solvent are mixed at the concentration of 10,000 μg/mL (1 w/v%) in the medium, respectively. The suspensibility is examined after sonicaiton and so on. When it is not suspended uniformly at the concentration of 10,000 μg/mL (1 w/v%), the suspensibility is examined using the substance of 5,000 μg/mL (0.5 w/v%) containing the selected solvent of 10,000 μg/mL (1 w/v%) in the medium. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test, and has no test. These judges are performed macroscopically.

Figure 1. Flow chart of examination of stability for the substance in the medium



3.7.2. Preparation of test substances

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. The final maximal concentrations of the substances and the recommended solvents are 5,000 μg/mL (0.5 w/v%), respectively, after diluted by the medium containing the SIRC cells as described in 4.3. The final maximal concentration of the substances and recommended slovents are 2,500 μg/mL (0.25 w/v%) and 5,000 μg/mL (0.5 w/v%), respectively, when the low concentration of the substance is selected . 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.

3.7.3. Dilution of substance solution

The dilution step of substance should be eight by a common ratio of two. The test of the same concentration should be performed in duplicate on the plate.

3.8. Reference substance

3.8.1. Positive control substance

SDS should be used as a positive control. The SDS solution is prepared at the concentration of 1,000 μg/mL in the medium.

3.8.2. Relative control substance

Triethanolamine should be used as a relative control. The triethanolamine solution is prepared at the concentration of 10,000 μg/mL in the medium.

3.8.3. Negative control substance

Medium, 10,000 μg/mL DMSO-medium solution or 10,000 μg/mL ethanol-medium solution should be used as a negative control. It should be selected on the basis of what kind of solvent solve or suspend test substance.

4. Procedure

4.1. Passage culture of cell

・cell culture

(1)The SIRC cell is cultured with MEM supplemented 10% FBS, 1% P/S/F and so on at 37 ℃ in a humidified incubator containing 5% (v/v) CO_2 in air. The concentrations of the penicillin, streptomycin and amphotericin B are 100 U/mL, 100 μg/mL and 250 ng/mL, respectively.

(2)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(3)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(4)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2-3 minutes at 37℃. The cells are detached from the surface of the flask, tapping it. They are collected by the appropriate amount of the MEM (10% FBS) from the culture flask. After counting the cells, the cell suspension is prepared at the density of 6-8x10^5cells in 15-30 mL of the

medium. The passage culture is performed using it.

・Freeze preservation of cell

(1) The medium contained 10% DMSO is prepared as a medium for the freeze preservation of cell. The commercial available freeze preservative solution (e.g., Juji Field Inc., Cell banker 1or 2) can be used. Both serum type and non serum type can be used.

(2) The cell of $1x10^6$ cells/mL is add to stock tube. It is froze slowly. For example, the freeze is performed at 5 minutes in the ice, 50 minutes at about -20℃ and 12 hours in about -70℃ and in the liquid nitrogen in order. The commercial available freezing vessel (e.g., Nihon Freezer Co. LTD., BICELL) can be used. In this case, the vessel containing the tube for the cell is frozen at about -70℃ and in the liquid nitrogen after 3 hours - overnight.

(3) The tube containing the cell is preserved in the liquid nitrogen.

・Melting of cell

(1)The stock tube is dipped in the water of 37℃ for melting.

(2)The cell suspension added the medium of 10 mL is centrifuged at 1,000 rpm x5 min.

(3)After removing the supernatant, the cell suspension is prepared by the medium.

・The freeze preservative cell is passaged one or more times and should be confirmed the appropriate growth.

4.2. Preparation of cell suspension

(1)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(2)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(3)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2-3 minutes at 37℃.

(4)The cells are detached from the surface of the flask, tapping it.

(5)The cells are collected by the appropriate amount of the MEM (10% FBS) and pepetting.

(6)After counting the cells, the cell suspension is prepared at the density of $2x10^5$ cells/mL.

4.3.Application of test substance

(1)PBS(-), negative control, and dilution series of test substance, positive control and relative control are prepared in the 96 well microplate. The layout of the microplate is shown in fig. 1.

(2)One tenth mL of the $2x10^5$ cells/mL cell suspension is added to the wells as shown in fig.2.

(3)Microplate sealer is used to avoid the effect of volatile toxicant. Moreover, wrap film can be used. 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.

(4)The microplate added the test substance and the cell suspension is still standing for 20 minutes in the clean bench. The cell is adhered to the bottom of the microplate. After that, the microplate is moved to the CO_2 incubator.

(5)The microplate is incubated for about 72 hr at the condition of 37℃ and 5% CO_2,

4.4. Crystal violet staining

(1)After the incubation, the medium containing the test substances is removed by gentle turnover of the microplate.

(2)PBS(-) of 200 μL is added. After gentle shake, PBS(-) is removed by the turnover of the

microplate. This procedure is repeated twice.

(3)One hundred μL of crystal violet methanol solution is added to each well of the microplate, and stain the cells for 30 minutes.

(4)After the staining, the crystal violet methanol solution is removed by gentle turnover of the microplate. It is washed by tap water sufficiently, and is blotted water with the paper towel.

(5)After drying sufficiently, the absorbance at 588 nm is measured with an automatic microplate reader. The mesurement at the near wavelength that the equivalency was demonstrated can be used.

4.5.Calculation of IC_{50}

The absorbance of control wells, which contained no test substance, is regarded as 100%, and the percentage absorbance for each well is calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) is obtained from the next formula using two concentrations around the predicted concentration of 50% cell viability.

$LogIC_{50}=[(50-y1)logx2-(50-y2)logx1]/(y2-y1)$

(x 1 : Low concentration, x2 : High concentration, y1: cell viability at low concentration, y2 : cell viability at high concentration, Log means common logarithm.)

If the cell viability is >50% at the maximal concentration of 5,000 μg/mL, the result of the test substance is IC_{50}>5,000 μg/mL. Also, if the cell viability is <50% at the concentration of 39.1 μg/mL, the result of the test substance is IC_{50}<39.1 μg/mL.

In a spreadsheet software (Excel), the cell viability value is rounded to the nearest tenths.

4.6.Evaluation

The eye irritation of the test substance is evaluated by using triethanolamine as a relative control (see annex 1). The classification of the eye irritation of triethanolamine (100%) is "Not Category" of GHS standard and "Category 3" of EPA standard on the basis of the in vivo data by Ohno et al. For the GHS standard, the test substance is judged as negative (Not Category) when the IC_{50} is higher than or equal to that of triethanolamine, and is judged as positive (Category 1 or 2) when the IC_{50} is lower than that of triethanolamine. For EPA standard, the test substance is judged as negative (Category 4) when the IC_{50} is higher than that of triethanolamine, and is judged as positive (Category 1-3) when the IC_{50} is lower than or equal to that of triethanolamine (see table 3 and 4 of appendix 1). If the two decisions are different, the two results with the same decision are adopted after the third test. If the confirmation of the deviation is needed with three results, the test is repeated three times.

4.7.Quality control

The quality control of the SIRC cytotoxicity test is performed by the six measurements. It needs to satisfy the next criterion. If it is not satisfy the criterion, the retest of the microplate should be performed. Especially, if the toxic effect of the volatile substance causes unacceptable values, the retest should be performed at the lower concentration.

(1)The absolute OD obtained from the negative control is index of normal proliferation of SIRC cell seeded at the concentration of $1x10^4$ cells/well and incubated 72hr. The mean OD of the negative control (the right and left wells) should be >0.4 for complete test.

(2) Sodium dodecyl sulfate (SDS) is used as a positive control. The IC_{50} of SDS should be 77.7-258.7 μg/mL when it is tested by the standard protocol. It needs for complete test.

(3) Triethanolamine is used as a relative control (see appendix 1). The IC_{50} range of triethanolamine should be 1,000-2,500 μg/mL when it is tested by the standard protocol (see appendix 2). It needs for complete test.

(4) The difference between two dilution series of the substance should be confirmed. The IC_{50}s of the first series and the second series should be within ± 20% of the mean IC_{50} of two series(the mean of the first IC50 and the second IC50), respectively. It needs for complete test. If the IC_{50} is lower than 39.1μg/mL, it is calculated using the IC_{50}=39.1μg/mL. If the IC_{50} is higher than 5000μg/mL, it is calculated using the IC_{50}=5000μg/mL.

(5) The difference between left and right wells of the negative control should be confirmed to check the systematic quality. The mean ODs of left and right wells should be within ±15% of the mean OD of all negative control wells (the mean ± 15%), respectively. It needs for complete test.

(6) The difference between the two test results adopted should be confirmed to check the equality of them. The IC_{50} values of two tests of positive control (SDS) should be lower or equal to twice. (The high value/the low value≦2)

5.References

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6.List of abbriviations and acronyms

℃	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}	50% Inhibitory Concentration
JaCVAM	Japanese Center for the Validation of Alternative Methods
MEM	Minimum Essential Medium
NI	Non Irritant
OD	Optical density
PBS(-)	Phosphate-Buffered Saline(-)
SDS	Sodium Dodecyl Sulfate
SIRC cell	Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS	Statens Seruminstitut Rabbit Cornea -Crystal Violet Staining

7.About the revision of this protocol

(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. 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.

(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 version 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"

Table 1. The manufacturer and so on of the reagent

No.	Reagent or Medium	Manufacturer	Catalog number	Remarks
1	MEM (Minimum Essential Medium)	GIBCO	Code#: 11095	
2	Fetal Bovine Serum	GIBCO	REF#: 26140-079	Lot.1073767
3	Penicillin-Streptomycin-Amphotericin (100x)	GIBCO/BRL	REF#: 15240-062	
4	Phosphate-Buffered Saline (PBS(-))	Nissui	Code#: 05913	
5	0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)	Wako	Cat#: 209-16941	
6	Dimethyl sulfoxide (DMSO)	Kanto	Cat# 2950-1B	Lot.211U1463
7	Ethanol	Wako	Cat#: 057-00456	
8	Crystal violet	Wako	Cat#: 031-04852	Lot.LAK5122
9	Methanol	Wako	Cat#: 131-01826	
10	Sodium Dodecyl Sulfate	Wako	Cat#: 191-07145	Lot.SDF8154
11	Triethanolamine	Kanto	Cat#: 40268-00	Lot.DCK3718

The products of the same specification are used for the reagents of No.1, 2, 3, 10 and 11. The equivalents regardless of manufacturer are acceptable for other reagents.

Nissui: NISSUI PHARMACEUTICAL CO., LTD

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: KANTO CHEMICAL, CO., INC.

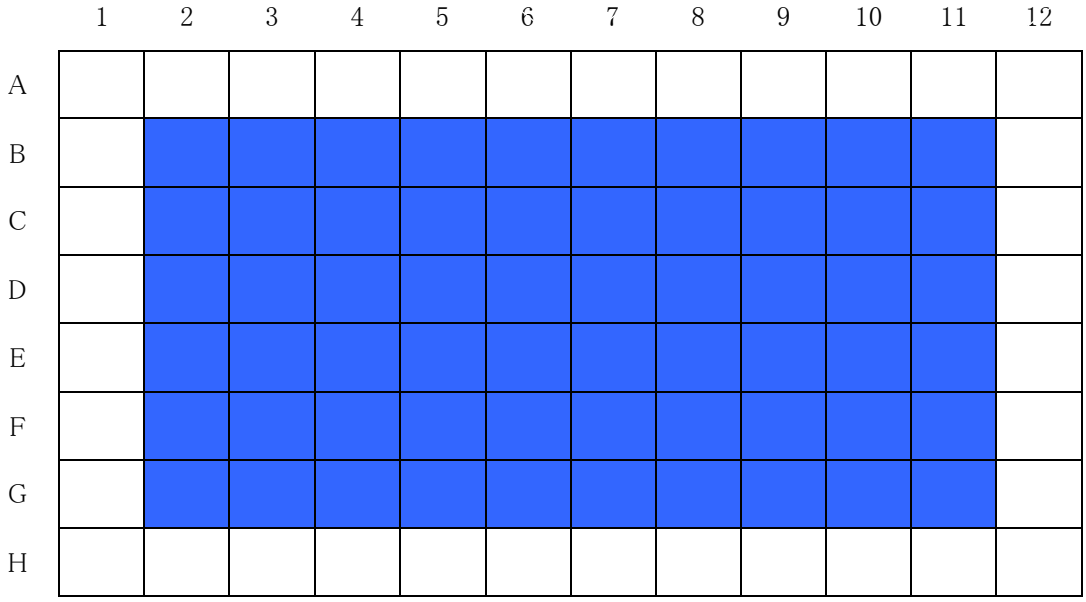
Figure 2. Layout of 96 well microplate

	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:PBS(-) of 200 μL, NC:Medium, 10,000 μg/mL DMSO-Medium solution or 10,000 μg/mL ethanol-Medium solution of 100 μL, S:Dilution series of the test substances by a common ratio of two (100 μL), R:Dilution series of the relative control by a common ratio of two (100 μL), P: Dilution series of the positive control by a common ratio of two (100 μL).

The dilution series of the test substance should be 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 should be made using medium.

Figure 3.　Addition of cell suspension



■ : Cell suspension (100 μL)

Appendix 1 The reason for selecting triethanolamine as a reference control

Triethanolamine was selected as a relative control substance of the SIRC cytotoxicity test for discriminating between non irritant (NI) (= not classified) and irritant in 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 has the ready availability as a commercial reagent, the solvability in the medium, and the useful cytotoxicity and in vivo data for evaluating the eye irritancy.

For selecting the relative control substance, the accuracy and others for discriminating between non irritant and irritant was checked using every available substances in the previous validation with the intention of selecting each substance as a relative control substance. As a result of the comparative study using GHS classification, the triethanolamine was selected as a relative control substance because of the relatively small false negative, the highest accuracy except for substances which could not be obtained the cytotoxicity (10000< etc.), and the clarity about the category (for example, alcohol) of false negative substances, as shown in table 1. The identificaiton of the group of the false negative substances is important to avoid the risk of eye irritation.

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 IC_{50} of future test substance is higher than that of triethanolamine, it should be evaluated as a non irritant. If the IC_{50} of the test substance is lower than or equal to that of triethanolamine, it should be evaluated as an irritant.

The relationship between the classification of the in vivo standard and the classification predicted by the SIRC cytotoxcity test is shown in table 3 for GHS and table 4 for EPA, respectively.

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

Substances	In vivo Evaluation by GHS[$]	The SIRC cytotoxicity : IC_{50} (ug/mL)[#]	The values for ranking	True Negative	False Negative	True Positive	False Positive	Accuracy (%)
Polyethylene glycol 400	NI	35300<	35300	1	0	27	6	82
Silicic anhydride	NI	14800<	14800	2	0	26	6	82
Glycerin	NI	11600	11600	3	0	25	6	82
Isotonic sodium chloride solution	NI	10000<	10000	4	1	24	5	82
Ethanol	1or2A	10000<	10000	4	1	24	5	82
Isopropyl myristate	NI	9330<	9330	5	1	24	4	85
Butanol*	1or2A	8880<	8880	5	2	23	4	82
Triethanolamine	NI	2090	2090	6	2	23	3	85
Lactic acid	1	1230	1230	6	3	22	3	82
Benzyl alcohol	1or2A	1190	1190	6	4	21	3	79
Polyoxyethylene sorbitan monooleate (20E.O.)	NI	963	963	7	4	21	2	82
Sodium salicylate	1or2A	952	952	7	5	20	2	79
Glycolic acid*	1or2A	868	868	7	6	19	2	76
Acetic acid*	1or2A	721	721	7	7	18	2	74
Diisopropanolamine*	1, 2Aor2B	699	699	7	8	17	2	71
2-Ethylhexyl p-dimethylamino benzoate	NI	474	474	8	8	17	1	74
Calcium thioglycolate	1	392	392	8	9	16	1	71
Acid red 92	1or2A	297	297	8	10	15	1	68
Sucrose fatty acid ester	1or2A	286	286	8	11	14	1	65
m-Phenylenediamine	1or2A	218	218	8	12	13	1	62
Methyl p-hydroxybenzoate	NI	207	207	9	12	13	0	65
Di (2-ethylhexyl) sodium sulfosuccinate*	1or2A	181	181	9	13	12	0	62
Sodium lauryl sulfate*	1or2A	168	168	9	14	11	0	59
Sodium hydrogenated tallow L-glutamate*	1or2A	140	140	9	15	10	0	56
Potassium laurate*	1or2A	120 (Data from 4 labs)	120	9	16	9	0	53
Chlorhexidine gluconate (20% solution)*	1or2A	67.6	67.6	9	17	8	0	50
Polyoxyethylene octylphenylether (10 E.O.)*	1or2A	38.4	38.4	9	18	7	0	47
Distearyldimethylammonium chloride	1	37.8	37.8	9	19	6	0	44
Benzalkonium chloride*	1or2A	19.0	19	9	20	5	0	41
Domiphen bromide*	1or2A	12.1	12.1	9	21	4	0	38
Monoethanolamine*	1or2A	9.62	9.62	9	22	3	0	35
Cetyltrimethylammonium bromide*	1or2A	2.59 (Data from 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

$: The Draize eye test results couldn't always discriminate between 1 and 2 of GHS classification for no observation data on day 21. The observation was performed to day 14.

: The data of SIRC cytotoxicity test were the mean of IC_{50} (ug/mL) from more than 5 laboratory except for one part.

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

Table 2 The correlative evaluation on the basis of the previous validation study data for selecting relative control substance – EPA classification –

Substances	In vivo Evaluation by EPA[$]	The SIRC cytotoxicity : IC_{50} (ug/mL)[#]	The values for ranking	True Negative	False Negative	True Positive	False Positive	Accuracy (%)
Polyethylene glycol 400	4	35300<	35300	1	0	27	6	82
Silicic anhydride	4	14800<	14800	2	0	27	5	85
Glycerin	4	11600	11600	3	0	27	4	88
Isotonic sodium chloride solution	4	10000<	10000	4	0	27	3	91
Ethanol	1or2	10000<	10000	4	1	26	3	88
Isopropyl myristate	4	9330<	9330	5	1	26	2	91
Butanol*	1or2	8880<	8880	5	2	25	2	88
Triethanolamine	3	2090	2090	5	3	24	2	85
Lactic acid	1or2	1230	1230	5	4	23	2	82
Benzyl alcohol	1or2	1190	1190	5	5	22	2	79
Polyoxyethylene sorbitan monooleate (20E.O.)	4	963	963	6	5	22	1	82
Sodium salicylate	1or2	952	952	6	6	21	1	79
Glycolic acid*	1, 2or3	868	868	6	7	20	1	76
Acetic acid*	1or2	721	721	6	8	19	1	74
Diisopropanolamine*	1, 2or3	699	699	6	9	18	1	71
2-Ethylhexyl p-dimethylamino benzoate	4	474	474	7	9	18	0	74
Calcium thioglycolate	1or2	392	392	7	10	17	0	71
Acid red 92	1or2	297	297	7	11	16	0	68
Sucrose fatty acid ester	1or2	286	286	7	12	15	0	65
m-Phenylenediamine	1or2	218	218	7	13	14	0	62
Methyl p-hydroxybenzoate	3	207	207	7	14	13	0	59
Di (2-ethylhexyl) sodium sulfosuccinate*	1or2	181	181	7	15	12	0	56
Sodium lauryl sulfate*	1,2or3	168	168	7	16	11	0	53
Sodium hydrogenated tallow L-glutamate*	1or2	140	140	7	17	10	0	50
Potassium laurate*	1or2	120 (Data from 4 labs)	120	7	18	9	0	47
Chlorhexidine gluconate (20% solution)*	1,2or3	67.6	67.6	7	19	8	0	44
Polyoxyethylene octylphenylether (10 E.O.)*	1or2	38.4	38.4	7	20	7	0	41
Distearyldimethylammonium chloride	1or2	37.8	37.8	7	21	6	0	38
Benzalkonium chloride*	1or2	19.0	19	7	22	5	0	35
Domiphen bromide*	1or2	12.1	12.1	7	23	4	0	32
Monoethanolamine*	1, 2or3	9.62	9.62	7	24	3	0	29
Cetyltrimethylammonium bromide*	1or2	2.59 (Data from 4 labs)	2.59	7	25	2	0	26
Cetylpyridinium chloride*	1or2	1.67	1.67	7	26	1	0	24
Stearyltrimethylammonium chloride*	1or2	1.58	1.58	7	27	0	0	21

$: The Draize eye test results couldn't always discriminate between 1 and 2 of GHS classification for no observation data on day 21. The observation was performed to day 14.

: The data of SIRC cytotoxicity test were the mean of IC_{50} (ug/mL) from more than 5 laboratory except for one part.

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

Table 3 The relationship between the GHS classification and the classification predicted by the SIRC cytotoxcity test

GHS classication	The classification predicted by the SIRC cytotoxicity test
1	Irritant
2A	
2B	
Not classified	Non irritant (NI)

Table 4 The relationship between the EPA classification and the classification predicted by the SIRC cytotoxcity test

EPA classification	The classification predicted by the SIRC cytotoxicity test
1	Irritant
2	
3	
4	Non irritant (NI)

Appendix 2 The basis for the set IC_{50} range of triethanolamine as a reference control

The IC_{50} range of triethanolamine, 1,000-2,500 μg/mL is based on the mean $\pm$ 2 x standard deviation from the data (n=144).

Protocol for SIRC-CVS cytotoxicity test　(Version 2.13E)
February 16, 2013.
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1. Purpose

This test method is used to measure cytotoxicity of chemicals using SIRC cell and to discriminate between non ocular irritant (NI) and ocular irritant (Others). The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. The principle of SIRC cytotoxicity test

The cytotoxicity has been known as an index for evaluating the eye irritation by chemicals, as the corneal damage that has a greater impact on the total eye irritation is related to that of the corneal epithelium cell. The cytotoxicity test is reported to be useful for identifying the non ocular irritants that has almost no effect on the cornea. The cell line used comes from rabbit cornea.
The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the tested microplate can be stored. The results can be confirmed by using the stored microplates in any time. No other method can match it.

3. Materials

3.1. Cell

The SIRC cell (Statens Seruminstitut rabbit corneal cell: ATCC NO. CCL-60), the cell line from the rabbit cornea is obtained from ATCC (American Type Culture Collection). The cells can be frozen and kept in liquid nitrogen. The cells should be confirmed the absence of mycoplasma (e.g., Venor GeM Mycoplasma Detection Kit、Minerva Biolabs GmbH、11-1025). The cells should be used during 3 months after the start of cultivation. They should be checked on the basis of the quality control of 4.7.

3.2.Technical Equipment

- CO_2 Incubator　(e.g., SANYO Electric Co., Ltd., MCO-17AIC)
- Clean bench　(e.g., Hitachi, Ltd., CCV1300E)
- Microplate reader (e.g., Bio-Rad Laboratories, Benchmark Plus ™)
- Inverse phase contrast microscope (e.g., Nikon, ECLIPSE TS100)
- Autoclave (e.g., TOMY SEIKO CO.,LTD, BS-325 and SS-320)
- Centrifuge (e.g., Kubota Corporation, 5800)
- Water bath
- Electronic chemical balance
- Ultrasonic bath sonicator
- Vortex mixer
- Magnetic stirrer
- Hemocytometer (e.g. ERMA INC, 03-303-5) or Cell counter

3.3. Experimental instrument

- Tissue culture flasks (75 cm^2, 25 cm^2)　(e.g., BD Falcon 353136 and 353112)

・96-well flat bottom tissue culture microtiter plates (e.g., BD Falcon, 353072)
・Storage plate (e.g., Thermo Scientific、0.8 mL Storage plate、AB-0765)
・Multichannel pipette, micropipette
·Dispenser tray
・Tubes
・Cryotube(1.5 mL)
・Centrifuge tube (15 mL, 50 mL)
・Tip for micropepett (200 μL, 100uL, 5 mL)
·Microplate sealing tape
・Paper towel (e.g., NIPPON PAPER CRECIA Co. LTD, Kim towel™, 61000)
・Wrap film (e.g., Saran Wrap)

3.4.Culture medium and reagent

Minimum Essential Medium (MEM)

Fetal Bovine Serum (FBS)

・Inactivated fetal bovine serum should be used. Inactivation is performed by 56℃, 30 min in the water bath. After fall in temperature, the serum is taken 56 mL or 28 mL in each tube. The serum is stored at -70℃ or less or at -20℃ or less.

Penicillin/Streptomycin/Amphotericin B (P/S/F) solution
(Antibiotic-Antimycotic 100x ; GIBCO BRL)

Phosphate-Buffered Saline(-) (PBS(-))

0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)

Dimethyl Sulfoxide (DMSO; CAS Number 67-68-5)

・the weighing can be converted to volume on the basis of specific gravity.

Ethanol (CAS Number 64-17-5)

・the weighing can be converted to volume on the basis of specific gravity.

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)

Hydrochloric Acid (CAS Number 7647-01-0)

Sodium Hydroxide (CAS Number 1310-73-2)

The manufacturer and so on of the reagent is shown in the Table 1.

3.5.Medium

MEM is supplemented with 10% FBS (inactivated) and 1% appropriate 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 Penicillin 100 U/mL, Streptomycin 100 μg/mL and Amphotericin B 250 ng/mL, respectively.

3.6.Crystal violet solution

0.4% Crystal violet solution is prepared using methanol.

3.7. Test substances

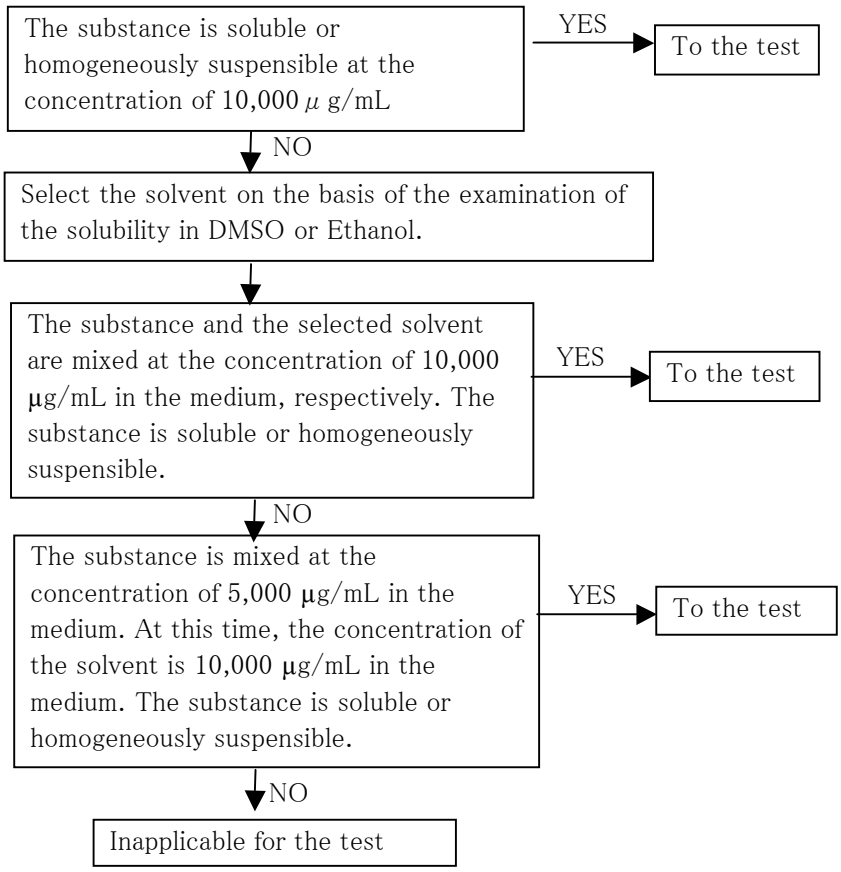
Test substance is prepared.

3.7.1. Examination of stability for the substance in the medium

The solubility of the substance in the medium should be confirmed in advance. The solubility or suspensibility of the substance is examined at the concentration of 10,000 μg/mL (1 w/v%)

in the medium. It is solved or uniformly suspended using vortex mixer, waterbath and sonicator when it finds necessary. When it is not suspended uniformly, the solubility to DMSO and ethanol is examined as a next step. The substance and the selected solvent are mixed at the concentration of 10,000 μg/mL (1 w/v%) in the medium, respectively. The suspensibility is examined after sonicaiton and so on. When it is not suspended uniformly at the concentration of 10,000 μg/mL (1 w/v%), the suspensibility is examined using the substance of 5,000 μg/mL (0.5 w/v%) containing the selected solvent of 10,000 μg/mL (1 w/v%) in the medium. The substance that is not suspended homogeneously is judged as an inapplicable substance for this test, and has no test. These judges are performed macroscopically.

Figure 1. Flow chart of examination of stability for the substance in the medium



3.7.2. Preparation of test substances

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. The final maximal concentrations of the substances and the recommended solvents are 5,000 μg/mL (0.5 w/v%), respectively, after diluted by the medium containing the SIRC cells as described in 4.3. The final maximal concentration of the substances and recommended slovents are 2,500 μg/mL (0.25 w/v%) and 5,000 μg/mL (0.5 w/v%), respectively, when the low concentration of the substance is selected. 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

3.7.3. Dilution of substance solution

The dilution step of substance should be eight by a common ratio of two. The test of the same concentration should be performed in duplicate on the plate.

3.8. Reference substance

3.8.1. Positive control substance

SDS should be used as a positive control. The SDS solution is prepared at the concentration of 1,000 μg/mL in the medium.

3.8.2. Relative control substance

Triethanolamine should be used as a relative control. The triethanolamine solution is prepared at the concentration of 10,000 μg/mL in the medium.

3.8.3. Negative control substance

Medium, 10,000 μg/mL DMSO-medium solution or 10,000 μg/mL ethanol-medium solution should be used as a negative control. It should be selected on the basis of what kind of solvent solve or suspend test substance.

4. Procedure

4.1. Passage culture of cell

・cell culture

(1)The SIRC cell is cultured with MEM supplemented 10% FBS, 1% P/S/F and so on at 37 ℃ in a humidified incubator containing 5% (v/v) CO_2 in air. The concentrations of the penicillin, streptomycin and amphotericin B are 100 U/mL, 100 μg/mL and 250 ng/mL, respectively.

(2)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(3)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(4)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2-3 minutes at 37℃. The cells are detached from the surface of the flask, tapping it. They are collected by the appropriate amount of the MEM (10% FBS) from the culture flask. After counting the

cells, the cell suspension is prepared at the density of 6-8x10^5cells in 15-30 mL of the medium. The passage culture is performed using it.

・Freeze preservation of cell

(1) The medium contained 10% DMSO is prepared as a medium for the freeze preservation of cell. The commercial available freeze preservative solution (e.g., Juji Field Inc., Cell banker 1or 2) can be used. Both serum type and non serum type can be used.

(2) The cell of 1x10^6 cells/mL is add to stock tube. It is froze slowly. For example, the freeze is performed at 5 minutes in the ice, 50 minutes at about -20℃ and 12 hours in about -70℃ and in the liquid nitrogen in order. The commercial available freezing vessel (e.g., Nihon Freezer Co. LTD., BICELL) can be used. In this case, the vessel containing the tube for the cell is frozen at about -70℃ and in the liquid nitrogen after 3 hours - overnight.

(3) The tube containing the cell is preserved in the liquid nitrogen.

・Melting of cell

(1)The stock tube is dipped in the water of 37℃ for melting.

(2)The cell suspension added the medium of 10 mL is centrifuged at 1,000 rpm x5 min.

(3)After removing the supernatant, the cell suspension is prepared by the medium.

・The freeze preservative cell is passaged one or more times and should be confirmed the appropriate growth.

4.2. Preparation of cell suspension

(1)After the medium is removed from culture flask, the SIRC cells are rinsed 2 times by PBS(-) 10 mL for removing serum as a trypsin inhibiter.

(2)After PBS(-) is removed, 0.25% trypsin solution (1.5-2 mL) is added contacting the all cells in the culture flask.

(3)After 0.25% trypsin solution is removed halfway, the cells are incubated for 2-3 minutes at 37℃.

(4)The cells are detached from the surface of the flask, tapping it.

(5)The cells are collected by the appropriate amount of the MEM (10% FBS) and pepetting.

(6)After counting the cells, the cell suspension is prepared at the density of 2x10^5 cells/mL.

4.3.Application of test substance

(1)PBS(-), negative control, and dilution series of test substance, positive control and relative control are prepared in the 96 well microplate. The layout of the microplate is shown in fig. 1.

(2)One tenth mL of the 2x10^5 cells/mL cell suspension is added to the wells as shown in fig.2.

(3)Microplate sealer is used to avoid the effect of volatile toxicant. Moreover, wrap film can be used. 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.

(4)The microplate added the test substance and the cell suspension is still standing for 20 minutes in the clean bench. The cell is adhered to the bottom of the microplate. After that, the microplate is moved to the CO_2 incubator.

(5)The microplate is incubated for about 72 hr at the condition of 37℃ and 5% CO_2,

4.4. Crystal violet staining

(1)After the incubation, the medium containing the test substances is removed by gentle turnover of the microplate.

(2)PBS(-) of 200 μL is added. After gentle shake, PBS(-) is removed by the turnover of the microplate. This procedure is repeated twice.

(3)One hundred μL of crystal violet methanol solution is added to each well of the microplate, and stain the cells for 30 minutes.

(4)After the staining, the crystal violet methanol solution is removed by gentle turnover of the microplate. It is washed by tap water sufficiently, and is blotted water with the paper towel.

(5)After drying sufficiently, the absorbance at 588 nm is measured with an automatic microplate reader. The mesurement at the near wavelength that the equivalency was demonstrated can be used.

4.5.Calculation of IC_{50}

The absorbance of control wells, which contained no test substance, is regarded as 100%, and the percentage absorbance for each well is calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) is obtained from the next formula using two concentrations around the predicted concentration of 50% cell viability.

$LogIC_{50}=[(50-y1)logx2-(50-y2)logx1]/(y2-y1)$

(x 1 : Low concentration, x2 : High concentration, y1: cell viability at low concentration, y2 : cell viability at high concentration, Log means common logarithm.)

If the cell viability is >50% at the maximal concentration of 5,000 μg/mL, the result of the test substance is IC_{50}>5,000 μg/mL. Also, if the cell viability is <50% at the concentration of 39.1 μg/mL, the result of the test substance is IC_{50}<39.1 μg/mL.

In a spreadsheet software (Excel), the cell viability value is rounded to the nearest tenths.

4.6.Evaluation

The eye irritation of the test substance is evaluated by using triethanolamine as a relative control (see annex 1). The classification of the eye irritation of triethanolamine (100%) is "Not Category" of GHS standard and "Category 3" of EPA standard on the basis of the in vivo data by Ohno et al. For the GHS standard, the test substance is judged as negative (Not Category) when the IC_{50} is higher than or equal to that of triethanolamine, and is judged as positive (Category 1 or 2) when the IC_{50} is lower than that of triethanolamine. For EPA standard, the test substance is judged as negative (Category 4) when the IC_{50} is higher than that of triethanolamine, and is judged as positive (Category 1-3) when the IC_{50} is lower than or equal to that of triethanolamine (see table 3 and 4 of appendix 1). If the two decisions are different, the two results with the same decision are adopted after the third test. If the confirmation of the deviation is needed with three results, the test is repeated three times.

4.7.Quality control

The quality control of the SIRC cytotoxicity test is performed by the six measurements. It needs to satisfy the next criterion. If it is not satisfy the criterion, the retest of the microplate should be performed. Especially, if the toxic effect of the volatile substance causes unacceptable values, the retest should be performed at the lower concentration.

(1)The absolute OD obtained from the negative control is index of normal proliferation of SIRC cell seeded at the concentration of $1x10^4$ cells/well and incubated 72hr. The mean OD of the negative control (the right and left wells) should be >0.4 for complete test.

(2) Sodium dodecyl sulfate (SDS) is used as a positive control. The IC_{50} of SDS should be 77.7-258.7 μg/mL when it is tested by the standard protocol. It needs for complete test.

(3) Triethanolamine is used as a relative control (see appendix 1). The IC_{50} range of triethanolamine should be 1,000-2,500 μg/mL when it is tested by the standard protocol

(see appendix 2). It needs for complete test.

(4) The difference between two dilution series of the substance should be confirmed. The IC_{50}s of the first series and the second series should be within ± 20% of the mean IC_{50} of two series(the mean of the first IC50 and the second IC50), respectively. It needs for complete test. If the IC_{50} is lower than 39.1μg/mL, it is calculated using the IC_{50}=39.1μg/mL. If the IC_{50} is higher than 5000μg/mL, it is calculated using the IC_{50}=5000μg/mL.

(5) The difference between left and right wells of the negative control should be confirmed to check the systematic quality. The mean ODs of left and right wells should be within ±15% of the mean OD of all negative control wells (the mean ± 15%), respectively. It needs for complete test.

(6) The difference between the two test results adopted should be confirmed to check the equality of them. The IC_{50} values of two tests of positive control (SDS) should be lower or equal to twice. (The high value/the low value≦2)

5.References

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6.List of abbriviations and acronyms

℃	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}	50% Inhibitory Concentration
JaCVAM	Japanese Center for the Validation of Alternative Methods
MEM	Minimum Essential Medium
NI	Non Irritant
OD	Optical density
PBS(-)	Phosphate-Buffered Saline(-)
SDS	Sodium Dodecyl Sulfate
SIRC cell	Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS	Statens Seruminstitut Rabbit Cornea -Crystal Violet Staining

7.About the revision of this protocol

(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. 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.

(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 version 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”.

Table 1. The manufacturer and so on of the reagent

No.	Reagent or Medium	Manufacturer	Catalog number	Remarks
1	MEM (Minimum Essential Medium)	GIBCO	Code#: 11095	
2	Fetal Bovine Serum	GIBCO	REF#: 26140-079	Lot.1073767
3	Penicillin-Streptomycin-Amphotericin (100x)	GIBCO/BRL	REF#: 15240-062	
4	Phosphate-Buffered Saline (PBS(-))	Nissui	Code#: 05913	
5	0.25% (w/v) Trypsin (1mmol/L EDTA・4Na)	Wako	Cat#: 209-16941	
6	Dimethyl sulfoxide (DMSO)	Kanto	Cat# 2950-1B	Lot.211U1463
7	Ethanol	Wako	Cat#: 057-00456	
8	Crystal violet	Wako	Cat#: 031-04852	Lot.LAK5122
9	Methanol	Wako	Cat#: 131-01826	
10	Sodium Dodecyl Sulfate	Wako	Cat#: 191-07145	Lot.SDF8154
11	Triethanolamine	Kanto	Cat#: 40268-00	Lot.DCK3718

The products of the same specification are used for the reagents of No.1, 2, 3, 10 and 11. The equivalents regardless of manufacturer are acceptable for other reagents.

Nissui: NISSUI PHARMACEUTICAL CO., LTD

Wako: Wako Pure Chemical Industries, Ltd.

Kanto: KANTO CHEMICAL, CO., INC.

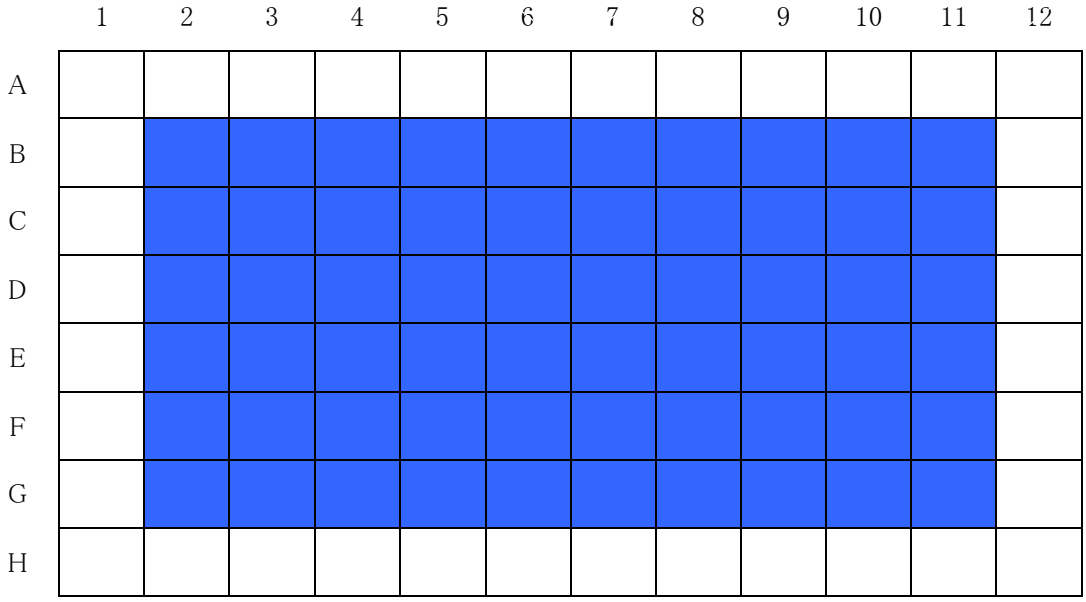
Figure 2. Layout of 96 well microplate

	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:PBS(-) of 200 μL, NC:Medium, 10,000 μg/mL DMSO-Medium solution or 10,000 μg/mL ethanol-Medium solution of 100 μL, S:Dilution series of the test substances by a common ratio of two (100 μL), R:Dilution series of the relative control by a common ratio of two (100 μL), P: Dilution series of the positive control by a common ratio of two (100 μL).

The dilution series of the test substance should be 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 should be made using medium.

Figure 3.　Addition of cell suspension



■ : Cell suspension (100 μL)

Appendix 1 The reason for selecting triethanolamine as a reference control

Triethanolamine was selected as a relative control substance of the SIRC cytotoxicity test for discriminating between non irritant (NI) (= not classified) and irritant in 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 has the ready availability as a commercial reagent, the solvability in the medium, and the useful cytotoxicity and in vivo data for evaluating the eye irritancy.

For selecting the relative control substance, the accuracy and others for discriminating between non irritant and irritant was checked using every available substances in the previous validation with the intention of selecting each substance as a relative control substance. As a result of the comparative study using GHS classification, the triethanolamine was selected as a relative control substance because of the relatively small false negative, the highest accuracy except for substances which could not be obtained the cytotoxicity (10000< etc.), and the clarity about the category (for example, alcohol) of false negative substances, as shown in table 1. The identificaiton of the group of the false negative substances is important to avoid the risk of eye irritation.

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 IC_{50} of future test substance is higher than that of triethanolamine, it should be evaluated as a non irritant. If the IC_{50} of the test substance is lower than or equal to that of triethanolamine, it should be evaluated as an irritant.

The relationship between the classification of the in vivo standard and the classification predicted by the SIRC cytotoxcity test is shown in table 3 for GHS and table 4 for EPA, respectively.

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

Substances	In vivo Evaluation by GHS[$]	The SIRC cytotoxicity : IC_{50} (ug/mL)[#]	The values for ranking	True Negative	False Negative	True Positive	False Positive	Accuracy (%)
Polyethylene glycol 400	NI	35300<	35300	1	0	27	6	82
Silicic anhydride	NI	14800<	14800	2	0	26	6	82
Glycerin	NI	11600	11600	3	0	25	6	82
Isotonic sodium chloride solution	NI	10000<	10000	4	1	24	5	82
Ethanol	1or2A	10000<	10000	4	1	24	5	82
Isopropyl myristate	NI	9330＜	9330	5	1	24	4	85
Butanol*	1or2A	8880<	8880	5	2	23	4	82
Triethanolamine	NI	2090	2090	6	2	23	3	85
Lactic acid	1	1230	1230	6	3	22	3	82
Benzyl alcohol	1or2A	1190	1190	6	4	21	3	79
Polyoxyethylene sorbitan monooleate (20E.O.)	NI	963	963	7	4	21	2	82
Sodium salicylate	1or2A	952	952	7	5	20	2	79
Glycolic acid*	1or2A	868	868	7	6	19	2	76
Acetic acid*	1or2A	721	721	7	7	18	2	74
Diisopropanolamine*	1, 2Aor2B	699	699	7	8	17	2	71
2-Ethylhexyl p-dimethylamino benzoate	NI	474	474	8	8	17	1	74
Calcium thioglycolate	1	392	392	8	9	16	1	71
Acid red 92	1or2A	297	297	8	10	15	1	68
Sucrose fatty acid ester	1or2A	286	286	8	11	14	1	65
m-Phenylenediamine	1or2A	218	218	8	12	13	1	62
Methyl p-hydroxybenzoate	NI	207	207	9	12	13	0	65
Di (2-ethylhexyl) sodium sulfosuccinate*	1or2A	181	181	9	13	12	0	62
Sodium lauryl sulfate*	1or2A	168	168	9	14	11	0	59
Sodium hydrogenated tallow L-glutamate*	1or2A	140	140	9	15	10	0	56
Potassium laurate*	1or2A	120 (Data from 4 labs)	120	9	16	9	0	53
Chlorhexidine gluconate (20% solution)*	1or2A	67.6	67.6	9	17	8	0	50
Polyoxyethylene octylphenylether (10 E.O.)*	1or2A	38.4	38.4	9	18	7	0	47
Distearyldimethylammonium chloride	1	37.8	37.8	9	19	6	0	44
Benzalkonium chloride*	1or2A	19.0	19	9	20	5	0	41
Domiphen bromide*	1or2A	12.1	12.1	9	21	4	0	38
Monoethanolamine*	1or2A	9.62	9.62	9	22	3	0	35
Cetyltrimethylammonium bromide*	1or2A	2.59 (Data from 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

$: The Draize eye test results couldn't always discriminate between 1 and 2 of GHS classification for no observation data on day 21. The observation was performed to day 14.

: The data of SIRC cytotoxicity test were the mean of IC_{50} (ug/mL) from more than 5 laboratory except for one part.

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

Table 2　The correlative evaluation on the basis of the previous validation study data for selecting relative control substance　– EPA classification –

Substances	In vivo Evaluation by EPA[$]	The SIRC cytotoxicity : IC_{50} (ug/mL)[#]	The values for ranking	True Negative	False Negative	True Positive	False Positive	Accuracy (%)
Polyethylene glycol 400	4	35300<	35300	1	0	27	6	82
Silicic anhydride	4	14800<	14800	2	0	27	5	85
Glycerin	4	11600	11600	3	0	27	4	88
Isotonic sodium chloride solution	4	10000<	10000	4	0	27	3	91
Ethanol	1or2	10000<	10000	4	1	26	3	88
Isopropyl myristate	4	9330<	9330	5	1	26	2	91
Butanol*	1or2	8880<	8880	5	2	25	2	88
Triethanolamine	3	2090	2090	5	3	24	2	85
Lactic acid	1or2	1230	1230	5	4	23	2	82
Benzyl alcohol	1or2	1190	1190	5	5	22	2	79
Polyoxyethylene sorbitan monooleate (20E.O.)	4	963	963	6	5	22	1	82
Sodium salicylate	1or2	952	952	6	6	21	1	79
Glycolic acid*	1, 2or3	868	868	6	7	20	1	76
Acetic acid*	1or2	721	721	6	8	19	1	74
Diisopropanolamine*	1, 2or3	699	699	6	9	18	1	71
2-Ethylhexyl p-dimethylamino benzoate	4	474	474	7	9	18	0	74
Calcium thioglycolate	1or2	392	392	7	10	17	0	71
Acid red 92	1or2	297	297	7	11	16	0	68
Sucrose fatty acid ester	1or2	286	286	7	12	15	0	65
m-Phenylenediamine	1or2	218	218	7	13	14	0	62
Methyl p-hydroxybenzoate	3	207	207	7	14	13	0	59
Di (2-ethylhexyl) sodium sulfosuccinate*	1or2	181	181	7	15	12	0	56
Sodium lauryl sulfate*	1,2or3	168	168	7	16	11	0	53
Sodium hydrogenated tallow L-glutamate*	1or2	140	140	7	17	10	0	50
Potassium laurate*	1or2	120 (Data from 4 labs)	120	7	18	9	0	47
Chlorhexidine gluconate (20% solution)*	1,2or3	67.6	67.6	7	19	8	0	44
Polyoxyethylene octylphenylether (10 E.O.)*	1or2	38.4	38.4	7	20	7	0	41
Distearyldimethylammonium chloride	1or2	37.8	37.8	7	21	6	0	38
Benzalkonium chloride*	1or2	19.0	19	7	22	5	0	35
Domiphen bromide*	1or2	12.1	12.1	7	23	4	0	32
Monoethanolamine*	1, 2or3	9.62	9.62	7	24	3	0	29
Cetyltrimethylammonium bromide*	1or2	2.59 (Data from 4 labs)	2.59	7	25	2	0	26
Cetylpyridinium chloride*	1or2	1.67	1.67	7	26	1	0	24
Stearyltrimethylammonium chloride*	1or2	1.58	1.58	7	27	0	0	21

$: The Draize eye test results couldn't always discriminate between 1 and 2 of GHS classification for no observation data on day 21. The observation was performed to day 14.

: The data of SIRC cytotoxicity test were the mean of IC_{50} (ug/mL) from more than 5 laboratory except for one part.

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

Table 3 The relationship between the GHS classification and the classification predicted by the SIRC cytotoxcity test

GHS classication	The classification predicted by the SIRC cytotoxicity test
1	Irritant
2A	
2B	
Not classified	Non irritant (NI)

Table 4 The relationship between the EPA classification and the classification predicted by the SIRC cytotoxcity test

EPA classification	The classification predicted by the SIRC cytotoxicity test
1	Irritant
2	
3	
4	Non irritant (NI)

Appendix 2 The basis for the set IC_{50} range of triethanolamine as a reference control

The IC_{50} range of triethanolamine, 1,000-2,500 μg/mL is based on the mean $\pm$ 2 x standard deviation from the data (n=144).

Protocol for SIRC-CVS cytotoxicity test
Version 3.8

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Contents

1 Purpose

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

2 The principle of SIRC cytotoxicity test

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

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

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

3 Materials

3.1 Cell line

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

3.2 Equipment

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

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

3.3 Instruments

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

3.4 Culture medium and reagents

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

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

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

3.5 Medium

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

3.6 Crystal violet solution

A 0.4% crystal violet solution is prepared using methanol.

3.7 Test chemicals

3.7.1 Determining solubility or suspensibility of test chemicals in the Medium

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

10,000 µg/mL. If not, the test substance is considered to be outside the applicability domain of the test. These judgments can all be performed by visually confirming the absence or presence of precipitate in the solution.

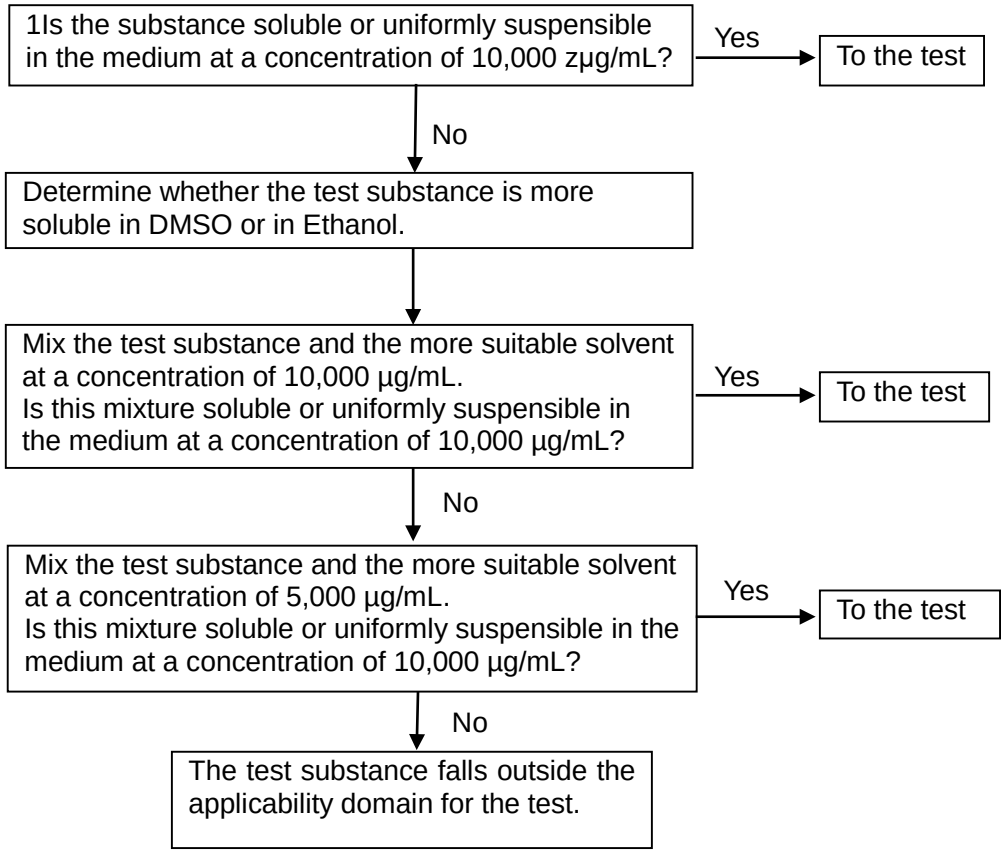

Figure 1: Determining solubility or suspensibility of test chemicals in the Medium

3.7.2 Preparing test chemicals

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

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

3.7.3 Preparing test chemical dilution series

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

3.8 Reference substances

3.8.1 Positive control

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

3.8.2 Relative control

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

3.8.3 Negative control

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

4 Test procedure

4.1 Passaging SIRC cells

Cell culture

1. Culture SIRC cells in MEM supplemented with 10% FBS and 1% P/S/F (the Medium) at 37°C in a humidified incubator at 5% CO2 in air. The concentrations of the antibiotics are 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of Amphotericin B.
2. Remove the Medium from the culture flask, then rinse the SIRC cells twice with 10 mL of modified PBS to remove the serum, which is a trypsin inhibiter.
3. Remove the modified PBS, then add and ensure that all the cells in the culture flask are exposed to 1.5 to 2.0 mL of 0.25% trypsin solution.
4. Remove the 0.25% trypsin solution, then incubate the cells as is for two or three minutes at 37°C. Detach the cells from the inside surface of the flask by tapping. Collect the cells in an appropriate volume of MEM (10% FBS). Count the cells and prepare a cell suspension at a density of 6 to 8 × 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 Appendix 1.). The IC_{50} of triethanolamine should be between 1,000 and 2,500 μg/mL when tested using the standard protocol (See Appendix 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 Appendix 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 appendix 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 appendix 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 appendix 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 appendix 1 and the related sentences were deleted.

In reference of appendix 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)

Appendix 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.

Appendix 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).