Version 1.9E, May 29th, 2012; Prepared by Yuko Nukada, Kao Corporation

Short time exposure (STE) test protocol

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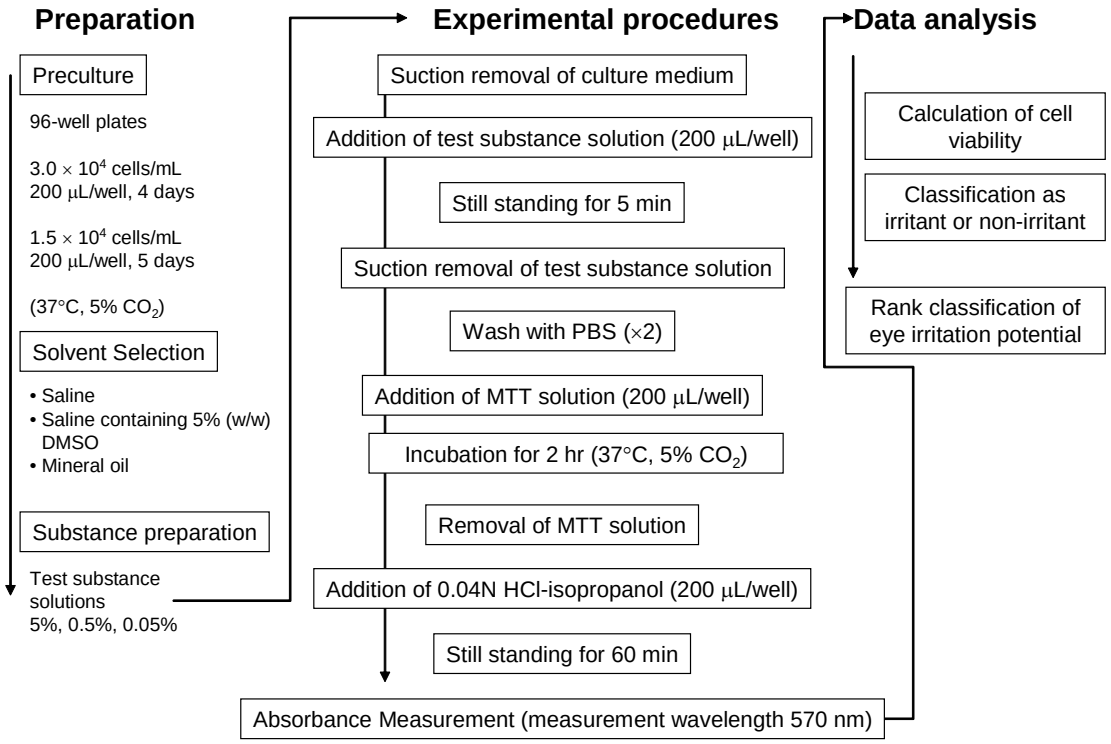
Test methods

Outline of the STE test procedures is described in Fig. 1.

Fig. 1 STE test methods



MTT: Methyl Thiazolyl Diphenyl-Tetrazolium Bromide

1. Cells

- Use SIRC cells purchased from ATCC (catalog No. CCL60, recommended lot No. 3981569). The cells should be used between 3 weeks and 3 months after the start of cultivation or within 25 passages.
- The SIRC cells purchased from other cell bank can be used. In this case, the reactivity for 0.01% SLS should be checked before use. If the cell viability of 0.01% SLS is within a range of 21.1 to 62.3 % (within a range of mean cell viability 41.7 % ± 10.3 × 2 standard deviations) stably, it can be used for assay as same as the SIRC cells from ATCC (lot No. 3981569).
- The reactivity of the SIRC cells should be also checked by three chemicals if the

new lots of SIRC cells. The three chemicals are SLS, Calcium thioglycolate and Tween 80. If the STE rank of these chemicals corresponds with the following (SLS is rank 3, Calcium thioglycolate is rank 2 and Tween 80 is rank 1), it can be used for assay as same as the SIRC cells from ATCC (lot No. 3981569).

- Culture SIRC cells at 37°C under 5% CO_2 and humidifying in a culture flask containing Eagle's MEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50-100 unit/mL penicillin and 50-100 μg/mL streptomycin. Dissociate the cells that have become confluent in the culture flask using trypsin-EDTA solution with or without the use of a cell scraper and subculture in a culture flask or inoculate into 96-well plates.

2. Culture media and materials

2-1. Preparation of culture medium

- Add the following materials to 500 mL of E-MEM:

  -FBS 56 mL

  -L-glutamine 6 mL

  -Penicillin-streptomycin 6 mL

2-2. Precautions in preparing culture medium

- If white turbidity is seen in L-glutamine solution, dissolve it in a constant-temperature bath.
- Prior to the preparation of penicillin-streptomycin solution, lightly shake the container bottle to mix the solution.
- Use disposable pipettes only once throughout the preparation procedure (use two 25 mL pipettes for FBS).

2-3. STE test-related agent and materials

Item	Maker	Item code	Note
SIRC cells	ATCC	CCL-60	Lot.3981569
Eagle's MEM medium	Sigma-Aldrich	M2279-500ML	
Fetal bovine serum	GIBCO	10099-141 or 10091-148	
L-glutamine 200 mM	GIBCO	25030-081	
Penicillin-streptomycin	GIBCO	15140-122	0.85% NaCl Penicillin 10,000U Streptomycin 10mg
MTT	Sigma-Aldrich	M-2128	
Saline for injection	Otsuka Pharmaceutical	081517	Japanese Pharmacopoeia, 20mL, 50 tubes
Dimethyl sulfoxide	SIGMA-Aldrich	154938	

(DMSO)			
Mineral oil	SIGMA	M5310	Mineral oil, Embryo tested; sterile-filtered
PBS tablet	Takara Bio	T900	
Trypsin-EDTA	Sigma-Aldrich	T-3924	Porcine trypsin 0.5 g EDTA-4 Na 0.2 g in HBSS 1 L
2-propanol	Kanto Chemical	32435-01	
1 mol/L hydrochloric acid (1N)	Kanto Chemical	18591-08	
Sumilon cell culture flask (middle size)	Sumilon	MS-21250	Tissue Culture Treated Polystyrene Sterile
96-well microplate (flat bottom)	Corning	3997	Tissue Culture Treated Polystyrene Sterile
Tissue culture dish	Corning	430165	
Sumilon serum tube	Sumitomo Bakelite	MS-4601X	
Cell scraper	Sumilon	MS-93170	
1 mL Terumo syringe	Terumo	SS-01T	
20 mL Terumo syringe	Terumo	SS-20ESZ	
Microfilter Millex-HA filter unit	Millipore	SLHA033SS	MF-Millipore (cellulose mixed ester)
Falcon tube	Becton Dickinson (Falcon)	352070	
15 mL tube	Asahi Techno Glass (IWAKI)	2314-015 or 2324-015	
Sumilon pipette	Sumilon	MS-66050	
Disposable pipette	Sumilon	MS-66250	
Eppendorf combi-tip	Eppendorf	30069455	

plus (Sterilized)			
SCC screw tube bottle (white) No. 2	As One	7-2110-04	
5 mL polypropylene Round-Bottom Tube	Becton Dickinson (Falcon)	352063	

Note: The recommended makers, item code, and lot were listed. But the materials not listed can be also used, regardless of makers, if the reliability of materials was confirmed.

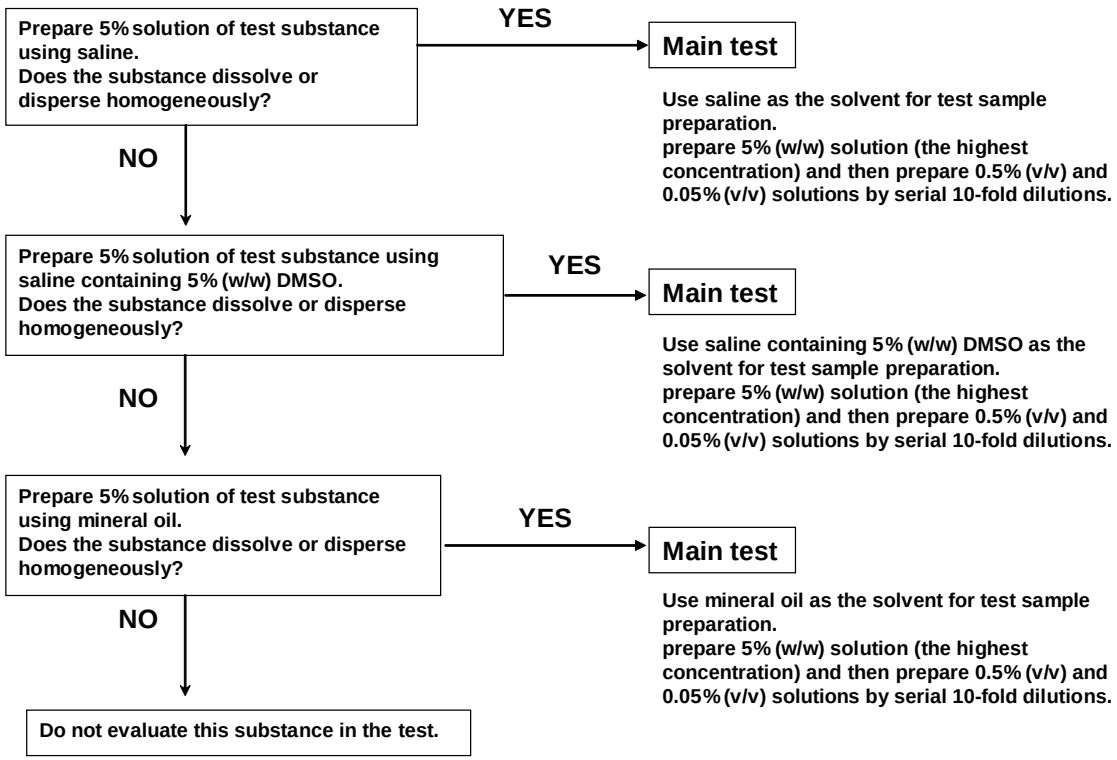
3. Preculture

- Prepare cell suspensions of 3.0×10^4 cells/mL or 1.5×10^4 cells/mL in culture medium. Preculture the cells for 4 days at 37°C under 5% CO_2 and humidifying if 200 μL each of the 3.0×10^4 cells/mL suspension is to be added to each well of the 96-well plate; for 5 days if the 1.5×10^4 cells/mL suspension is to be used for this procedure.

4. Selection of solvents for test substance preparation

A flowchart for the procedures from selection of solvents to preparation of substance is shown in Fig. 2.

Fig. 2 Solvent selection and test sample preparation



- First, prepare 5% (w/w) solution of a test substance using saline as solvent and observe the dissolution pattern of the substance. If the substance dissolves or homogeneously disperses [Note 1), 2)], choose saline as the solvent for this substance.
- When the substance does not dissolve or homogeneously disperse in saline, try saline containing 5% (w/w) DMSO. If the substance dissolves or homogeneously disperses, choose saline containing 5% DMSO as the solvent for this substance.
- When the substance does not dissolve or homogeneously disperse in saline containing 5% (w/w) DMSO, prepare this substance using mineral oil as solvent. Prepare 5% (w/w) solution of the substance using mineral oil as solvent and observe the dissolution pattern of the substance. If the substance dissolves or homogeneously disperses, choose mineral oil as the solvent for this substance. If the substance does not dissolve or homogeneously disperse in mineral oil, do not evaluate this substance in this test.

Note 1): A condition in which a substance is dispersed homogeneously and remains dispersed for at least 5 minutes

Note 2): Vortex, sonication or proper warm up can increase the solubility.

5. Preparation of test substance solutions

For assessment of the transferability, test substance is evaluated with 3 concentrations of 5%, 0.5% and 0.05%. For the main test in which blinded test substance is evaluated, 3 concentrations of test substance of 5%, 0.5% and 0.05% are prepared but two concentrations (5% and 0.05%) are tested.

- Prepare sample solutions of the test substances using the solvents selected as described in Section 4.
- Weigh the test substance in a screw tube and prepare 5% (w/w) solution using the selected solvent. Then, prepare 0.5% (v/v) and 0.05% (v/v) solutions by serial 10-fold dilutions of the original 5% (w/w) solution. These preparations are conducted for every three independent tests just before each test
- Use SDS 0.01% (v/v) saline solution as the positive control. Prepare SDS 1% (w/w) saline solution, and then prepare SDS 0.1% (v/v) and SDS 0.01% (v/v) saline solutions by serial 10-fold dilutions starting from the original SDS 1% (w/w) solution.
- Use the solvents used to prepare sample solutions as the solvent controls.

6. Experimental procedures

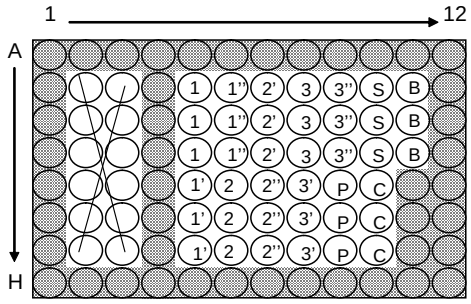
- Prior to initiating treatment conditions, familiarize yourself with the treatment diagram described in Figure 3.
- Preculture the cells to confluency in a 96-well plate.
- Take 0.6 mL (for 0.2 mL × 3 wells) of the prepared test sample using a 1 mL disposable syringe.

- Tilt the plate and remove culture medium from each well by suction using a suction tube (This can be done more easily by connecting a Pasteur pipette to tip of the suction tube. Be careful not to touch the well bottom with tip of the Pasteur pipette).
- Add test samples into wells. Start stopwatch when the first sample is added and then add test samples into their designated wells at a rate of 3 wells every 7-10 seconds (see Fig. 3).
- Exactly five minutes later, sequentially remove the test samples from all wells by suction at a rate of 3 wells every 7-10 seconds.
- Carefully add 200 μL each of PBS into the wells from which the test samples have been removed, and then remove it by suction. Repeat this manipulation twice to wash the inner surfaces of the wells (use of 8-channel pipettor, etc. will facilitate this wash).
- Thoroughly remove PBS by suction so that not a trace of it will remain in any of the wells.
- Add 200 μL of 0.5 mg/mL MTT (CAS No. 298-93-1) dissolved in culture medium [Note 3)] to each well and incubate in an incubator (37°C, 5% CO_2) for 2 hours.

Note 3): In the case of 21 mg MTT, add 42 mL of MEM culture medium, shake well and let the mixture stand for 20 minutes. Shake the mixture well, place it in a 20 mL syringe and filtrate through a 0.45 μm microfilter.
Protect MTT from light after weighing until immediately before use.

Fig. 3 Exposure and Evaluation of Treated Cultures

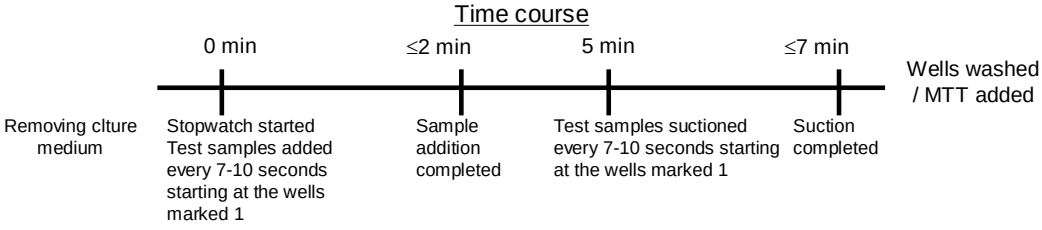
Evaluation of standard substances



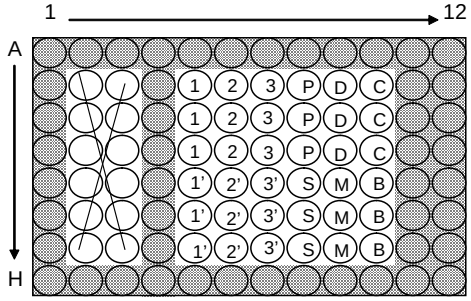
Diagonal lines: PBS to prevent drying (200 μL/well)
1-3': Test samples (3 compounds, 3 doses)

P: Positive control (0.01% SDS)
S: Saline
C: Procedural control (culture medium with cells)
B: Blank (culture medium without cells)

1. Remove culture medium from all wells (except wells for blank).
2. Add test samples starting at the wells marked 1.
   Start stopwatch and then add test sample solutions into designated wells at a rate of 3 wells every 7-10 seconds.
3. Five minutes later, sequentially suction off the test sample solutions at a rate of 3 wells every 7-10 seconds starting at the wells marked 1.
4. Wash the wells and add MTT.

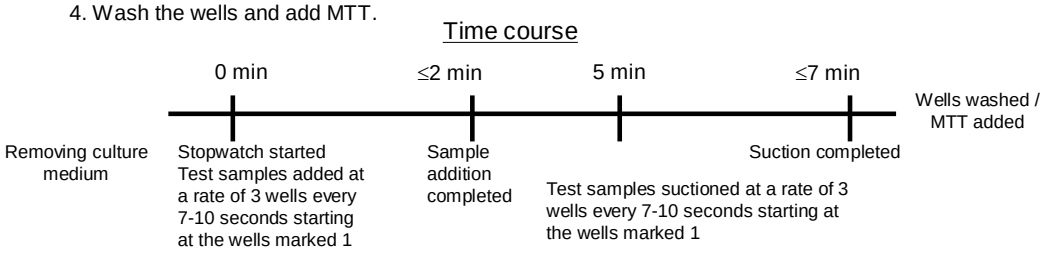


Evaluation of test substances



Diagonal lines: PBS to prevent drying (200 μL/well)
1-3': Test samples (3 compounds, 2 doses)
P: Positive control (0.01% SDS)
S: Saline
D: Saline containing 5% DMSO
M: Mineral oil
C: Procedural control (culture medium with cells)
B: Blank (culture medium without cells)

1. Remove culture medium from all wells (except wells for blank).
2. Add test samples starting at the wells marked 1.
   Start stopwatch and then add test sample solutions into designated wells at a rate of 3 wells every 7-10 seconds.
3. Five minutes later, sequentially suction off the test sample solutions at a rate of 3 wells every 7-10 seconds starting at the wells marked 1.
4. Wash the wells and add MTT.



- Take out the plate from incubator and discard the liquid in the wells. Remove the remaining drops completely by lightly tapping the plate onto a paper towel.
- Add 200 μL of 0.04N HCl-isopropanol (isopropanol 96 mL + 1N HCl 4 mL) to each well and let the plate stand for 60 minutes.
- Make the extracts in the wells uniform, set the plate onto a plate reader and measure absorbance at 570 nm wavelength.

Data analysis

7. Calculation of cell viability

- For each test sample, calculate cell viability (%) as the ratio of the optical density of test sample to the optical density of solvent control.

$$\text{Cell viability} = \frac{\text{optical density of test sample} (\text{optical density of test sample} - \text{optical density of blank})}{\text{optical density of solvent control} (\text{optical density of solvent control} - \text{optical density of blank})} \times 100$$

- In each assay, use 3 wells for each sample concentration and use the average of 3 optical density measurements to calculate cell viability.

- The cell viability of test sample concentration is treated as “zero”, when the average of 3 well optical densities is less than optical density of solvent control and the cell viability shows minus.

- Perform 3 independent assays[*1] for each sample concentration and use the average of 3 assays as the final cell viability.

Remark

*1: Each test sample concentration is evaluated with 3 wells on a plate. In case the

following 1) to 3) criteria are satisfied on the plate, the average cell viability of 3 wells is treated as an acceptable assay independently.

Criteria for acceptable test

1) Optical density of the culture medium procedural control (C in Fig. 3) at least 0.3 after subtraction of blank optical density
2) Cell viability for the solvent control at least 80% relative to that for the culture medium procedural control
3) Cell viability for the positive control (0.01% SDS) within a range of 21.1 to 62.3 % (within a range of mean cell viability 41.7 % $\pm$ 10.3 $\times$ 2 standard deviations)
4) Standard deviation of the final cell viability derived from 3 independent assays less than 15 % for all test sample concentrations

Approach in case the acceptance criteria are not satisfied

1) If any of the criteria 1) to 3) should be not satisfied, additional assay needs to be performed.
2) If the standard deviation of final cell viability is more than 15 %, another three independent assays are performed for that concentration and the standard deviation is re-calculated from the final viabilities derived from newly performed three assays. Old data is discarded.

8. Criteria for irritant and non-irritant classifications

- In the STE test, cell viability is the designated endpoint after a 5-minute exposure to test sample. Substances demonstrating higher than 70% cell viability are classified as non-irritant (NI) and those demonstrating a cell viability equal to or less than 70% are classified as irritant (I).

Classification	Cell viability
Non-irritant	> 70%
Irritant	≤ 70%

9. Rank classification of eye irritation potential in the STE test

- The procedure for rank classification of eye irritation potential in the STE test is shown in Fig. 4.
- With the 5% test sample solution, a score of 0 is given when cell viability is higher than 70% and score of 1 is given when cell viability is not higher than 70%. For 0.05% test sample solution, score of 1 is given when cell viability is higher than 70% and a score of 2 is given when cell viability is not higher than 70%. Next, the scores are added up for the 5% and 0.05% test sample solutions and final value is compared to the rank scale. The substance is classified according the following scale: Rank 1 = score of 1 = minimally irritant; rank 2 = score of 2 = moderately irritant; rank 3 = score of 3 = severely irritant.

Fig. 4 Rank classification of eye irritation potential in STE test

STE irritation score

Test sample solution 5%	Score	Test sample solution 0.05%	Score
If CV > 70%,	0	If CV > 70%,	1
If CV ≤ 70%,	1	If CV ≤ 70%,	2

CV: Cell viability

5% score + 0.05% score = STE rank

STE rank	Eye irritation potential
1	Minimally irritant
2	Moderately irritant
3	Severely irritant

10. Direct reduction of MTT by test substance

It should be noted that there are some chemicals which have a ability to directly reduce MTT. These abilities may cause the false negative results or under-prediction. To avoid these false results, it is necessary to know whether the test substance can reduce MTT directly.

- A MTT solution was prepared and 20 μL of 5% test substance was added to 200 μL of MTT solution.
- After incubation in the dark at 37°C for 2hours, the colors of mixed solution were checked visually.
- If the solution color turned blue or purple, the test substance has reduced the MTT; the absence of darkening indicates that the test substance did not directly reduce MTT.

- If the test substance reduced MTT directly, the false negative result may be obtained depending on the binding capacity of test substance to the tissue. In the case where these substances were assigned as non irritant in STE test, it would be assigned as “inconclusive” finally.

Amendment records

STE test protocol amendment

Date: May 27, 2010

Description of change

5. Experimental procedures and Fig.3

Delete all of the description about the record of exposure time.

Reason of amendment

At the 1st VMT meeting, the group agreed to not describe the procedure for the record of exposure time.in the protocol.

STE test protocol amendment

Date: May 27, 2010

Description of change

1. Cells, I7.

Replaced the word “sub-confluent” with the word “confluent”.

Reason of amendment

Incorrect word used.

STE test protocol amendment

Date: June 25, 2010

Description of change

6. Calculation of cell viability

The approach for when the acceptance criteria are not satisfied was added.

Reason of amendment

In the previous protocol, only criterion 4 was described. In order to make the protocol more complete, the approach for when the acceptance criteria are not satisfied was described in the updated protocol.

STE test protocol amendment

Date: July 9, 2010

Description of change

Fig.3, Evaluation of test substances

Replaced the numbering “1-2” with “1-3’.

Reason of amendment

Incorrect numbering order.

STE test protocol amendment

Date: July 9, 2010

Description of change

Document file name

Revised the version number from 1.1E to 1.6E.

Reason of amendment

Incorrect version was described.

STE test protocol amendment

Date: July 9, 2010

Description of changes

Additional information

Deleted the section “Additional Information” and re-numbered the description about culture media and materials as 2nd section.

Reason of amendment

In order to optimize the protocol.

STE test protocol amendment

Date: July 28, 2010

Description of changes

1. Cells, I7

The description of use of cell scraper was added.

Reason of amendment

In order to optimize the protocol

STE test protocol amendment

Date: July 28, 2010

Description of changes

2. Culture media and materials

2-3. STE test-related agent and materials

- Item name and code for 1mL Terumo syringe was added.
- Replaced the item code for Falcon tube “2070” with “352070”.
- Revised the maker’s name from Asahi Techno Glass to Asahi Techno Glass (IWAKI).
- Item code of replacement for 15 mL tube “2324-015” was added.
- Item “5mL polypropylene round-bottom tube” was added.

Reason of amendment

In order to optimize the protocol

STE test protocol amendment

Date: July 28, 2010

Description of changes

Fig.2

Deleted the words “of this solution” in text (at three parts).

Reason of amendment

In order to improve understanding of protocol.

STE test protocol amendment

Date: July 28, 2010

Description of changes

5. Preparation of test substance solutions, l6

Revised the section number from 3 to 4.

Reason of amendment

Incorrect number was described.

STE test protocol amendment

Date: July 28, 2010

Description of changes

Fig. 3

Evaluation of standard substances

Replaced the text that described the procedure (1-4) of “evaluation standard substances” with that of “evaluation of test substances”

Reason of amendment

In order to improve understanding of protocol.

STE test protocol amendment

Date: July 28, 2010

Description of changes

7. Calculation of cell viability, Remark, l2

Deleted the word “I” in the 2nd sentence.

Reason of amendment

In order to improve understanding of protocol. Non-essential word was deleted.

STE test protocol amendment

Date: July 28, 2010

Description of changes

Criteria for acceptable test 4), l2

Deleted the superscript “*2” at the end of sentence.

Reason of amendment

In order to improve understanding of protocol. Non-essential word was deleted.

STE test protocol amendment

Date: July 28, 2010

Description of changes

9. Rank classification of eye irritation potential in STE test

Inserted the word “the” before the words “STE test”.

Reason of amendment

In order to improve understanding of protocol.

Description of changes

2. Culture media and materials

2-3. STE test-related agent and materials

- Lot numbers of fetal bovine serum “Lot.1365536” and “Lot.665285” were deleted.
- Inserted the word “or” between the item code “10099-141” and “10091-148”.

Reason of amendment

In order to optimize the protocol

STE test protocol amendment

Date: April 20th, 2012

Description of changes

1. Cells

- Add the “recommended” just before the lot of SIRC cells.
- Add the following sentences;
  - The SIRC cells purchased from other cell bank can be used. In this case, the reactivity for 0.01% SLS should be checked before use. If the cell viability of 0.01% SLS is within a range of 21.1 to 62.3 % (within a range of mean cell viability 41.7 % $\pm$ 10.3 $\times$ 2 standard deviations) stably, it can be used for assay as same as the SIRC cells from ATCC (lot No. 3981569).
  - The reactivity of the SIRC cells should be also checked by three chemicals if the new lots of SIRC cells. The three chemicals are SLS, Calcium thioglycolate and Tween 80. If the STE rank of these chemicals corresponds with the following (SLS is rank 3, Calcium thioglycolate is rank 2 and Tween 80 is rank 1), it can be used for assay as same as the SIRC cells from ATCC (lot No. 3981569).

Reason of amendment

In order to improve the STE applicability

Description of changes

2-3. STE test-related agent and materials

- Removed the asterisks in the table
- Revised the footnote of table to following description;

Note: The recommended makers, item code, and lot were listed. But the materials not listed can be also used, regardless of makers, if the reliability of materials was confirmed.

Reason of amendment

In order to improve the STE applicability

Description of changes

5. Preparation of test substance solutions

- Added the sentence at the last of second point;

These preparations are conducted for every three independent tests just before each test.

Reason of amendment

In order to improve understanding of protocol.

STE test protocol amendment

Date: May 29th, 2012

Description of changes

10. Direct reduction of MTT by test substance

Added the procedure to know the ability direct reduction of MTT by test substances

Reason of amendment

In order to improve the reliability of STE data.