9-3 Positive control

Table 6 shows the absorbance values for the positive control. All data for the positive control met the acceptance criteria.

Table 6. Viability of the positive control

Laboratory	Average OD/ triplicate tissues	Average, SD at all OD
Lab a	**2.29**	**2.65±0.68**
	3.62	
	2.59	
	2.10	
Lab b	**4.98**	**3.87±0.84**
	3.22	
	4.50	
	3.02	
	3.62	
Lab c	**2.87**	**2.69±0.49**
	3.37	
	2.64	
	2.55	
	2.02	

9-4 Viability of chemicals

Table 7 shows the mean viability of testing chemicals at each tissue. Two data points at Lab a, eight data points at Lab b, and four data points at Lab c showed a SD > 18% and did not meet the acceptance criteria. Instead of generating insufficient data, each laboratory re-tested up to two additional runs. At Lab b, No. 15 resulted in a single invalid run, thereby invalidating an entire run sequence of three runs. In addition, the VMT did not accept all data from the fourth or fifth runs. The original data are shown in Appendix 5.

All study acceptance criteria were met as shown below.

1. All 20 Reference Chemicals had at least one complete run sequence at each laboratory.
2. In each of three participating laboratories, at least 95% of the run sequences were complete (One invalid run sequence was allowed in Lab b).
3. 99.4% of all possible run sequences from the three laboratories were complete (for 20 chemicals tested in three laboratories, a total of one invalid run sequence is allowed).

These experiments confirmed the feasibility of the LabCyte EPI-MODEL24 SIT test method.

Table 7. Mean viability of chemicals at each laboratory

Chem.	Lab	Exp. 1	2	3	4	5
01	a	12.4	11.3	19.0		
	b	16.5	10.7	10.6		
	c	9.0	9.8	9.8		
02	a	91.7	81.5	69.6	80.1	
	b	60.9	57.5	65.5	69.5	
	c	90.5	77.4	102.0	93.0	88.7
03	a	108.0	113.0	105.0		
	b	96.5	96.7	90.2		
	c	89.4	90.8	106.0	98.9	96.0
04	a	19.1	43.4	65.1	59.3	
	b	66.6	70.6	48.1	66.2	
	c	90.1	93.0	93.2		
05	a	89.6	77.0	67.6		
	b	75.9	57.5	74.8	77.1	
	c	68.5	86.6	66.4	67.2	74.4
06	a	16.2	15.9	17.0		
	b	17.3	13.5	11.4		
	c	15.5	16.1	12.0		
07	a	110.0	110.0	104.0		
	b	98.8	93.1	76.3		
	c	91.2	102.0	108.0		
08	a	109.0	122.0	111.0		
	b	93.1	106.0	86.6		
	c	95.5	106.0	119.0		
09	a	105.0	111.0	102.0		
	b	98.0	95.7	83.5		
	c	99.6	100.0	113.0		
10	a	15.7	20.3	16.0		
	b	11.5	15.9	11.4		
	c	17.3	14.1	14.9		
11	a	14.2	16.5	9.4		
	b	12.4	17.3	16.2		
	c	22.1	15.1	14.1		
12	a	8.9	15.9	10.0		
	b	11.0	7.8	9.0		
	c	6.0	7.4	5.7		
13	a	48.0	16.2	16.1	15.5	
	b	39.5	6.6	49.6	17.2	19.0
	c	17.5	17.0	16.2		
14	a	2.1	4.3	4.1		
	b	4.9	5.2	9.1		
	c	2.8	3.4	3.2		
15	a	19.9	95.9	83.5		
	b	39.1	28.0	52.7	17.5	18.5
	c	81.1	83.2	86.3		
16	a	0.9	1.7	1.6		
	b	4.6	2.0	3.3		
	c	0.9	3.1	11.6		
17	a	6.9	46.6	1.0		
	b	10.6	21.0	11.6		
	c	6.3	5.0	6.6		
18	a	6.7	4.5	3.6		
	b	9.8	10.9	11.0		
	c	1.3	1.8	2.2		
19	a	9.4	10.3	10.4		
	b	9.5	7.0	9.5		
	c	11.9	10.2	10.9		
20	a	8.7	12.0	7.8		
	b	9.1	7.9	37.6	17.4	
	c	7.6	7.0	6.8		

Red block
VRM SD > 18%, Not acccepted data

Light block
Not acceped for 4th da

9-5. Classification of three independent viabilities at each laboratory

The classifications from individual viabilities and the median of three independent viabilities are shown in Table 8. Lab a misclassified two data points (No. 4 and 15 at the first test), Lab b misclassified two data points (No. 15 at the first and second tests), and Lab c missed no classifications. As previously discussed, the third data point of the test with No. 15 at Lab b induced a single invalid run, thereby invalidating the entire run sequence of three runs. Therefore, the VMT judged "not detected" in the classification of No.15.

Table 8. Classification using three independent viabilities
P: Positive, N: Negative, F: Final detemination by median, ND: Not detected

No	UN GHS *in vivo* Cat.	Lab a				Lab b				Lab c			
		1	2	3	F	1	2	3	F	1	2	3	F
1	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
2	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
3	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
4	No Cat.	P	N	N	N	N	N	N	N	N	N	N	N
5	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
6	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
7	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
8	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
9	No Cat.	N	N	N	N	N	N	N	N	N	N	N	N
10	No Cat.	P	P	P	P	P	P	P	P	P	P	P	P
11	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
12	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
13	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
14	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
15	Cat.2	P	N	N	N	P	P	ND	ND	N	N	N	N
16	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
17	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
18	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
19	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P
20	Cat.2	P	P	P	P	P	P	P	P	P	P	P	P

10. Discussion

10-1. Reliability

Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show a concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals at each laboratory. As shown in Table 8 above, Lab a missed two classifications (No. 4 and 15) and the rate of within-laboratory reproducibility was 90.0% (18/20). Lab b missed one data point (No. 15) and the rate of reproducibility was 95.0% (19/20). Lab c missed no classifications and had a reproducibility rate of 100%. Therefore, results of all laboratories were sufficient, having a reproducibility rate equal to or higher than (≥) 90%.

Between-laboratory reproducibility

For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals between three laboratories was evaluated. As shown in Table 8, all laboratories missed four classifications and the rate of between-laboratory reproducibility was 95.0% (19/20). Therefore, all laboratories had a sufficient between-laboratory reproducibility that was equal to or higher than (≥) 80%.

Table 9. 2x2 tables

Lab a & c		*In vivo* classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	1	7	8
	Total	10	10	20

Sensitivity (%)	90.0
Specificity (%)	70.0
Accuracy (%)	80.0

Lab b		*In vivo* classification		
		Irritant	Non-Irritant	Total
In vitro prediction	Irritant	9	3	12
	Non-irritant	0	7	7
	Total	9	10	19

Sensitivity (%)	100.0
Specificity (%)	70.0
Accuracy (%)	84.2

10-2. Predictivity

The accuracy (sensitivity, specificity, and overall accuracy) of the LabCyte EPI-MODEL24 SIT skin irritation test was evaluated by cell viabilities (MTT) as an indicator, and the UN-GHS classifications are shown in Table 9. The sensitivity, specificity, and accuracy of this prediction model at each laboratory were 90–100%, 70%, and 80–84.2%, respectively. Some deviations from the OECD Performance standard (sensitivity of 80%, specificity of 70%, and accuracy of 75%;

shown in Table 3) were specific adaptations for the Japanese model.

11. Conclusions

Based on the reference list in the OECD Performance Standards, a catch-up validation of the LabCyte EPI-MODEL24 SIT by three labs was performed. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of accuracy (80–84.2% overall accuracy, 90–100% overall sensitivity, and 70% overall specificity) on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

12. Acknowledgement

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Short Time Exposure (STE) Test

2nd Phase Validation Study Report

(Version 1.1)

STE Test 2nd Phase Validation Management Team

STE Test 2nd Phase Validation Management Team

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Original terms or meanings of abbreviations

DMSO: dimethyl sulfoxide
ECVAM: European Center for the Validation of Alternative Methods
GHS: Globally Harmonised System
GLP: Good Laboratory Practice
ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods
JSAAE: Japanese Society for Alternative to Animal Experiments
JaCVAM: Japanese Center for the Validation of Alternative Methods
MSDS: Material Safety Data Sheet
MTT: methylthiazolydiphenyl-tetrazolium bromide
OECD: Organisation for Economic Co-Operation and Development
QU: Quality Control
SLS: Sodium lauryl sulfate
STE: Short Time Exposure
UN: United Nation
VMT: Validation Management Team

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Appendix:

Attached document 1: The STE test 1st phase validation study report

Attached document 2: Short Time Exposure (STE) Test Protocol (version 1.7E)

Attached document 3: Validation Study Plan for Alternative STE Test for Eye Irritation

Attached document 4: The report of selection of test substances for STE test

Summary

Introduction

The Short Time Exposure (STE) test is an easy-to-use *in vitro* eye irritation test using the cell viability of SIRC (rabbit corneal cell line) cells as an end point following one 5 min treatment. The Validation Management Team (VMT) was organized by JSAAE (Japanese Society for Alternative to Animal Experiments) and conducted a validation study with five laboratories to assess transferability, inter-laboratory reproducibility, and predictive capacity of the STE test from 2008-2009. These data showed good transferability of the STE test. Assignment of 25 blinded substances to the STE irritation classifications, "Non-Irritant" or "Irritant" (STE classification) showed good intra-and inter-laboratory agreement and high predictivity compared with irritation classification based on GHS category (United Nation: UN GHS Category 1 and UN GHS No Category: GHS classification) Furthermore, STE rank (the eye irritation property rank classifications based on the score at each 5% and 0.05% concentration) was compared with GHS categories (i.e., UN GHS Category 1, UN GHS Categories 2A and 2B, and UN GHS No Category). In this 2nd phase validation study, new VMT organized by JaCVAM (Japanese Center for the Validation of Alternative Methods) and we re-evaluated the predictive capacity of the STE test using an additional 40 blinded substances with three laboratories. After that, we evaluated the predictive capacity of GHS category on the STE test using 63 blinded substances with the results in the 1st phase validation study.

Materials and Methods

This study was conducted based on the same test protocol of the STE test 1st phase validation study. Using 40 blinded substances, three experiments for each substance were evaluated using substances at 5% and 0.05% concentrations in either saline, 5% DMSO in saline or mineral oil as a solvent. The STE classification based on cell viability in 5% substance solutions was compared with GHS classification. In addition, the STE rank was compared with GHS categories.

Results and Discussion

The results showed that the STE test was not only easy to acquire and implement among three laboratories, but it also had a high intra- and inter-laboratory reproducibility, and had a high predictive ability of the STE classification for predicting the GHS classification of various substances. However, a predictive ability for predicting the STE rank was not good compared with that of GHS categories. Therefore, the STE test can assess not only the severe/corrosive ocular irritant (correspond to UN GHS Category 1) but also the mild or moderate ocular irritants (correspond to UN GHS Category 2). The predictive ability for predicting the STE rank was insufficient for identification of UN GHS categories (Category 1, Category 2, and No Categoriy).

From these results, the STE test is recommended as an initial step within a Bottom-Up approach to identify substances that do not require classification for eye irritation (UN GHS No Category) as well as a step within a Top-Down approach to identify severe, moderate or mild irritants and substances that do not require classification for eye irritation (UN GHS No Category) from other toxicity classes, specially for limited types of substances. On the other hand, it is not considered adequately valid for the identification of mild or moderate

irritants (ie.UN GHS Categories 2A and 2B) and severe irritants (UN GHS Category 1).

Preface

The present report describes the results of the 2nd phase validation study of Short Time Exposure (STE) Test conducted by the STE test validation Management Team (VMT).

The STE test, developed by Kao Corporation, is a short time exposure cytotoxicity test that uses SIRC cells to predict eye irritation. It solves the problems associated with conventional cytotoxicity tests, and it is very simple to use and provides rapid results. In collaborative research conducted in three laboratories—namely, those of Kao Corporation, Kanebo Cosmetics Inc., and Lion Corporation—similar test results were obtained for positive and negative controls, indicating that the STE test has excellent "transferability." In addition, in an evaluation of 51 substances, high reproducibility and a strong predictive ability were found in each of the three laboratories (inter-laboratory accuracy was 98.0% or higher; Takahashi et al., 2008b). However, the laboratories were not used coded and blinded test substances in the collaboration research.

In the previous validation study conducted by the JSAAE (Japanese Society for Alternative to Animal Experiments), the transferability was initially evaluated using three standard substances while inter-laboratory reproducibility and predictive ability (i.e., agreement with the irritation category of Globally Harmonised System : GHS) were then evaluated with five laboratories using 25 blinded test substances from 2008-2009.

In this 2nd phase validation study, we evaluated the predictive capacity of the STE test with three laboratories using an additional 40 blinded substances and 25 substances in the 1st phase validation study.

This report contains all the results of these evaluations and the data support the usefulness of the STE test as an alternative test method for eye irritation.

1. Background

1.1 Eye irritation

Eye irritation is a reaction caused by the direct contact of a test substance with the eye, inducing symptoms such as clouding of the cornea, inflammation of the iris, and redness/edema/secretion of the conjunctiva. It is important to assess eye irritation, especially in products used on the face (such as cosmetics) or hair or household products, any of which can accidentally enter the eye.

1.2 Test method using rabbits (Draize test)

The Draize test (Draize et al., 1944) using rabbits has been widely used to evaluate eye irritation. In the Draize test, 0.1 ml or 0.1 g of a test substance is instilled into the palpebra of a rabbit; reactions in the cornea, iris, and conjunctiva are then macroscopically judged over time on the basis of a set of evaluation criteria. In evaluating the cornea, a maximum of 80 points are assigned on the basis of degree and area of opacity; for the iris, a maximum of 10 points are assigned on the basis of degree of congestion, swelling, and bleeding; and for the conjunctiva, a maximum of 20 points are assigned on the basis of redness, edema, and secretion. Thus, the total score is a maximum of 110 points. More weight is placed on changes in the cornea—as reflected in the higher number of points assigned there—given the significance of corneal injury. In this test method, recovery from a reaction can be evaluated through successive judgments. Degree of irritation is evaluated on the basis of judgments made, and the five-step evaluation using the Maximum Average Score (MAS) obtained during the observation period (Kay and Calandra 1962) is used as the judgment standard. The eye irritation tests described in the Organisation for Economic Co-Operation and Development (OECD) test guidelines (OECD number 405, 2002).

1.3 Globally Harmonized System (GHS)

The Globally Harmonized System (GHS) of Classification and Labeling of Chemicals is a system by which, according to globally standardized rules, a chemical is classified as to its type and degree of hazard and labeling so that the information can be understood easily when conveyed in a material safety data sheet (MSDS) for that chemical (UN: United Nations 2003). This standard was published by the United Nations in 2003 and was to be implemented internationally as of 2008 (from the home page of the Ministry of the Environment, http://www.env.go.jp/chemi/ghs/).

Mainly on the basis of Draize test results using rabbits, GHS eye irritation is classified as irreversible eye effects (UN GHS Category 1), reversible eye effects (UN GHS Categories 2A and 2B), and not-classified (UN GHS No Category).

In the present report, GHS Categories 1 and 2 are combined, and termed as irritants. Furthermore, all chemicals were classified into GHS Category 1 and No as defined by GHS classification, and it analyzed. In addition, similar to the approach of Goethem et al. (2006), three categories of eye irritation (UN GHS Category 1/Category 2A and B; no Category) were made as defined by GHS categories and the analysis was performed.

1.4 STE test

The STE test was developed by the Kao Corporation (Takahashi et al., 2008). It is a cytotoxicity test in which rabbit cornea-derived SIRC cells are exposed to a substance evaluated at a constant concentration for 5 min, with mean cell viability as the endpoint. Mean cell viability is determined by the incorporation of methylthiazolyldiphenyl-tetrazolium bromide (MTT: tetrazolium salt substance). In the STE test, two test concentrations of test substance, 5% and 0.05%, are used for evaluating the irritation potential. The irritation score can differ depending on whether cell viability is greater than 70%.

Physiological saline is used as a test solvent to evaluate water-soluble substances, while physiological saline containing 5% dimethyl sulfoxide (DMSO) or mineral oil is used for water-insoluble substances. The test protocol used in the present study is shown in attached document 1, "Short Time Exposure (STE) Test Protocol (version 1.7E)."

1.5 pre-validation study

The STE test is a cytotoxicity test that precludes the problems associated with conventional cytotoxicity tests, and it can be performed easily and rapidly. In a collaborative study conducted in three laboratories—namely, those of the Kao Corporation, Kanebo Cosmetics Inc., and Lion Corporation—transferability of the STE test was found to be excellent, since similar test results were obtained for negative and positive controls among the three laboratories. In addition, high reproducibility and predictive ability for Draize test results were found among the three laboratories following the evaluation of 51 substances (i.e., inter-laboratory accuracy was 98.0% or greater; Takahashi et al., 2008b). However, since the laboratories involved were not blinded to the identity of the test substances.

1.6 The 1st Phase validation study

This validation of the STE test blinded the identification of the blinded 25 test substances. The specific objectives of the study are to establish:

1) "Transferability," i.e., the extent to which a laboratory can adapt and easily implement the STE test;
2) "Inter-laboratory reproducibility," i.e., the extent to which results agree among a number of laboratories; and
3) "Predictive ability," i.e., the extent to which results agree with GHS categories, which are based on the results of the Draize test.

2. Goal statement

- The ultimate goal of the test strategy will be to replace the regulatory Draize eye irritation test according OECD (Organisation for Economic Co-operation and Development) Test Guideline 405 (OECD, 2002).
- The primary goal of the two validation studies is an evaluation of the ability of the *in vitro* tests to reliably discriminate ocular irritant from non-irritant chemicals, as defined according to the OECD and

UN proposal for GHS for the classification and labeling of eye irritation (UN GHS Category 1/Category 2A and B; no Category).

3. Methods

3.1 Organization and Roles

The STE test Validation Management Team (VMT) organized as shown in Figure 3.1. The correlated groups were comprised of the chemical management group, data analysis group, record management group under VMT. These groups managed the operations of the validation study.

The VMT, which played a central role overseeing the conduct of the validation study, includes:

Goal statement, project plan including objective, study protocol / amendments, outcome of QC (Quality Control) audits, test substances, data management procedures, timeline/ study progression, data collect and analysis, study interpretation and conclusions and reports and publication.

The final decision on which laboratories participate in the validation study was the responsibility of the VMT.

3.2 Sub-groups

1) Chemical management group

The group, which played a conduct of the validation management team, includes:

Definition of selection criteria, chemical selection, liaises with suppliers, final check of substances provided, acquisition, coding and distribution. The ECVAM and ICCVAM liaisons suggested selecting substances.

2) Data analysis group

The group, which played a conduct of the validation study, includes:

Approve spreadsheets, Collect data and Analysis data

3) Record management group

The group, which played a conduct of the validation management team, includes:

Check the protocol and the document sheets,

3.3. Participating laboratories

The laboratories participating in the study are to be defined as shown in **Fig.** 3.1.

The following three laboratories participated in the validation study for the evaluation of the STE test:

Laboratory 1 — Kanebo Cosmetics Inc., Quality Management Department

Laboratory 2 — POLA Chemical Industries Inc., Quality Research Department

Laboratory 3 — Lion Corporation, Human & Environmental Safety Evaluation Center

A lead laboratory was also identified as Kao Corporation. This laboratory was not participated and supported technically in the validation study.

Each laboratory also was responsible for complying with Good Laboratory Practice (GLP) -like principles and specifying QC aspects.

STE test – Validation Management Team

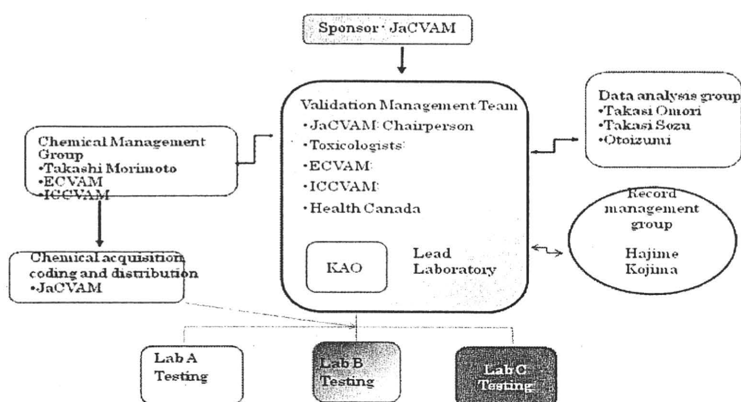

Figure 3.1. STE test validation execution organization

3.4. Overview of STE test

An overview of the STE test method is shown in Figure 3.2. In addition, the protocol of the present test (version 1.7E) is attached as attached document 1, and procedures are described in greater detail below and protocol.

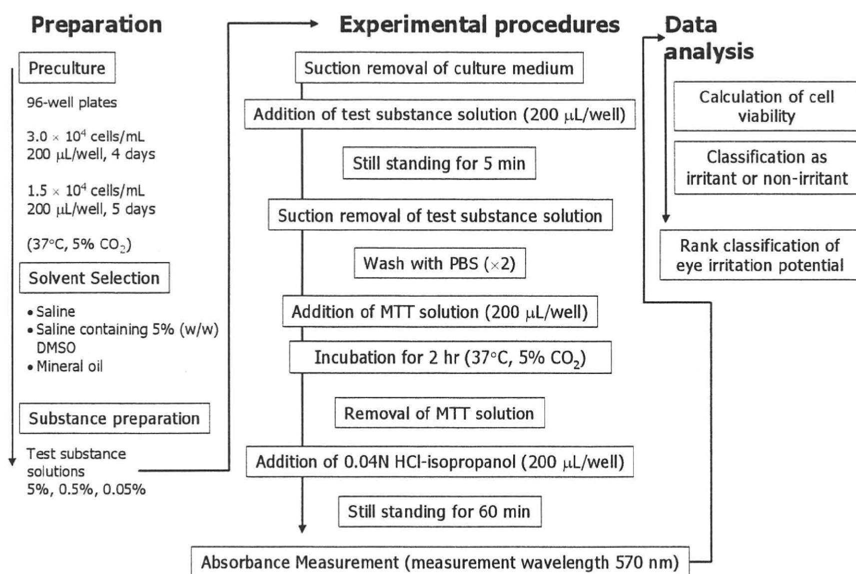

Figure 3.2. STE test procedure

1) Cells

* SIRC cells were purchased from ATCC (cat. #: CCL60; lot #: 3981569). Cells were used between three weeks and three months after initiation of culture or up to passage No. 25.

* SIRC cells were cultured in a culture flask (37°C, 5% CO_2) in Eagle MEM media containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50–100 units/mL of penicillin, and 50–100 μg/mL streptomycin. Confluent cells were dispersed in the culture flask to single cells by using trypsin-EDTA solution; they were then transferred into culture flask or seeded onto 96-well plates.

2) Pre-culture

* The cell suspension was prepared at 3.0×10^4 cells/mL or 1.5×10^4 cells/mL with medium; it was then pre-cultured (37°C, 5% CO_2) for 4 d when seeding at 3.0×10^4 cells/mL onto 96-well plates (200 μl/well) or for 5 d when seeding at 1.5×10^4 cells/mL.

3) Solvent selection

The work flow from solvent selection to substance preparation is depicted in Figure 3.3.

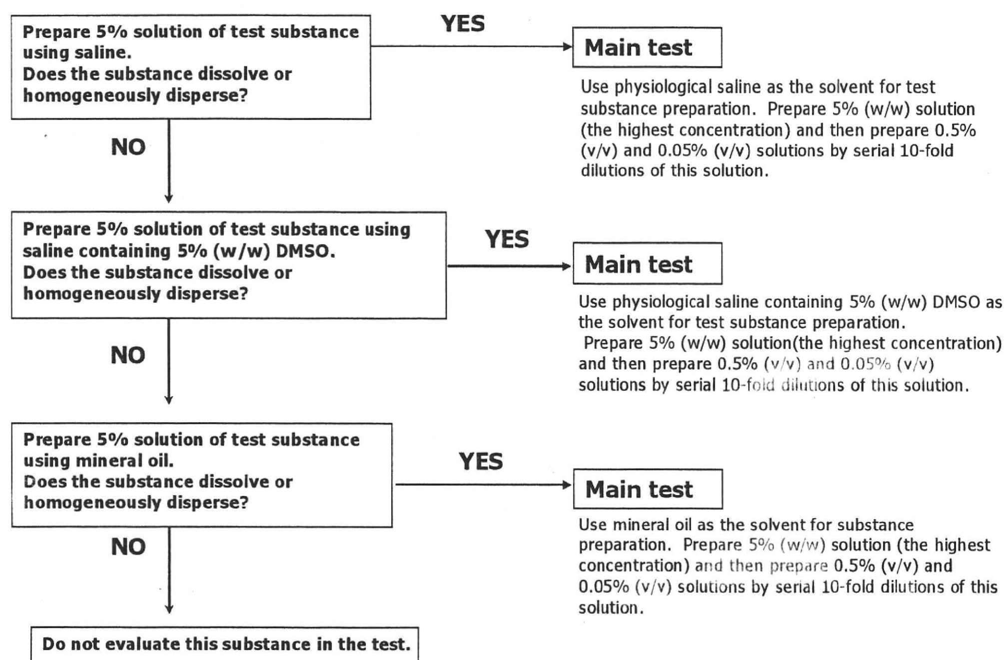

Figure 3.3. Solvent selection and substance preparation

* First, a 5% (w/w) solution of a test substance was prepared using saline as solvent and the dissolution pattern of the substance was observed. If the substance dissolved or homogeneously dispersed [Note 1), 2)], saline was chosen as the solvent for this substance.

* When the substance did not dissolve or homogeneously disperse in saline, saline containing 5% (w/w) dimethyl sulfoxide (DMSO) was tried as the solvent. If the substance dissolved or homogeneously dispersed, then saline containing 5% DMSO was chosen as the solvent for this substance.

* When the substance did not dissolve or homogeneously disperse in saline containing 5% (w/w) DMSO, mineral oil was tried as the solvent. If the substance dissolved or homogeneously dispersed, the 5% (w/w) solution of the substance in mineral oil mineral oil was used as the solvent for this substance. If the substance did not dissolve or homogeneously disperse in mineral oil, the substance was not evaluated in this test.

Note 1) A test substance was considered homogeneously dispersed in a fluid, and this condition was maintained for 5 min or longer.

Note 2) Dissolution was aided by vortexing, sonication, or warming, as appropriate.

4) Preparation of test substances

Three concentrations—5%, 0.5%, and 0.05%—were prepared in order to evaluate the ease of technical transferability. In the present evaluation of the blinded test substances, these three concentrations were prepared, and the first and third concentrations were used in the evaluation. The following were the steps used in preparing substances for testing.

* Test substance was diluted using the solvent selected in 3.5., 3).

* Test substance was weighed in a screw-capped tube and a 5% (w/w) solution was prepared using the selected solvent. The highest concentration of 5% (w/w) was diluted 10-fold to prepare a 0.5% (v/v) solution; the 0.5% solution was further diluted 10-fold to prepare a 0.05% (v/v) solution.

* A 0.01% physiological saline solution of SLS was used as the positive control. Here, a 1% (w/w) physiological saline solution of SLS was first prepared, and then diluted 10-fold to prepare a 0.1% (v/v) solution. This solution was further diluted 10-fold to prepare a 0.01% (v/v) physiological saline solution of SLS.

* The solvent selected in 3.5., 3) was used as the solvent control.

5) Experimental protocol

* Confluent SRC cells were transferred to a 96-well plate after pre-culturing.

* A 1-mL disposal syringe was filled with 0.6 mL of the prepared test substance (for 0.2 mL × three wells).

* The medium from each well was removed with a suction tube while tilting the plate. (This is easier if a Pasteur pipette is attached to the end of the suction tube. Care must be taken not to touch the bottom of the well with the Pasteur pipette.)

* The test substances were then added to the wells. With a stopwatch, add test substances to the respective wells at a rate of three wells per 7- to 10-second intervals. Figure 3.4 describes the process used to expose cells to the Standard substances and the 40 blinded substances.

* After 5 min, the test substances were removed from the wells by suction at a rate of three wells per 7- to 10-s intervals.

* Two hundred (200) μL of PBS were slowly added to the wells from which the test substances had been removed and then the PBS was removed by suction. This procedure was repeated twice to wash inside the wells. (The washing procedure can be efficiently performed with an eight-channel pipetter or the like.)

* PBS washing solution was carefully removed by suction so that none remained in the wells