

# *OECD GUIDELINE FOR THE TESTING OF CHEMICALS*

## **SHORT TIME EXPOSURE IN VITRO TEST METHOD FOR IDENTIFYING I) CHEMICALS INDUCING SERIOUS EYE DAMAGE AND II) CHEMICALS NOT REQUIRING CLASSIFICATION FOR EYE IRRITATION OR SERIOUS EYE DAMAGE**

### **INTRODUCTION**

1. The Short Time Exposure (STE) test method is an in vitro method that can be used under certain circumstances and with specific limitations for hazard classification and labeling of chemicals (substances and mixtures) that induce serious eye damage as well as those that do not require classification for either serious eye damage or eye irritation, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (1).

2. For many years, the eye hazard potential of chemicals has been evaluated primarily using an in vivo rabbit eye test (TG 405). It is generally accepted that, in the foreseeable future, no single in vitro alternative test will be able to fully replace the in vivo rabbit eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of alternative test methods used in a (tiered) testing strategy may well be able to fully replace the rabbit eye test (2). The top-down approach is designed for the testing of chemicals that can be expected, based on existing information, to have a high irritancy potential or induce serious eye damage. Conversely, the bottom-up approach is designed for the testing of chemicals that can be expected, based on existing information, not to cause sufficient eye irritation to require a classification. While the STE test method is not considered to be a complete replacement for the in vivo rabbit eye test, it is suitable for use as part of a tiered testing strategy for regulatory classification and labeling, such as the top-down/bottom-up approach, to identify without further testing (i) chemicals inducing serious eye damage (UN GHS Category 1) and (ii) chemicals (excluding all solid chemicals other than surfactants) that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (1) (2). However, a chemical that is neither predicted to cause serious eye damage (UN GHS Category 1) nor UN GHS No Category (does not induce either serious eye damage or eye irritation) by the STE test method would require additional testing to establish a definitive classification. Furthermore, the appropriate regulatory authorities should be consulted before using the STE in a bottom-up approach under classification schemes other than the UN GHS. The choice of the most appropriate test method and the use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (14).

3. The purpose of this test guideline (TG) is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in the Short Time Exposure Test method. The cytotoxic effect of chemicals on corneal epithelial cells is an important mode of action (MOA) leading to corneal epithelium damage and eye irritation. Cell viability in the STE test method is assessed by the quantitative measurement, after extraction from cells, of blue formazan salt produced by the living cells by enzymatic conversion of the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), also known as Thiazolyl Blue Tetrazolium Bromide (3). The obtained cell viability is compared to the solvent control (relative viability) and used to estimate the potential eye hazard of the test chemical. A test chemical is classified as UN GHS Category 1 when both the 5% and 0.05% concentrations result in a cell viability smaller than or equal to ( $\leq$ ) 70%. Conversely, a chemical is predicted as UN GHS No Category when both 5% and 0.05% concentrations result in a cell viability higher than ( $>$ ) 70%.

4. The term “test chemical” is used in this Test Guideline to refer to what is tested and is not related to the applicability of the STE test method to the testing of substances and/or mixtures. Definitions are provided in Annex I.

## INITIAL CONSIDERATIONS AND LIMITATIONS

5. This Test Guideline is based on a protocol developed by Kao Corporation (4), which was the subject of two different validation studies: one by the Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE) (5) and another by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6). A peer review was conducted by NICEATM/ICCVAM based on the validation study reports and background review documents on the test method (7).

6. When used to identify chemicals (substances and mixtures) inducing serious eye damage (UN GHS Category 1 (1), data obtained with the STE test method on 125 chemicals (including both substances and mixtures), showed an overall accuracy of 83% (104/125), a false positive rate of 1% (1/86), and a false negative rate of 51% (20/39) as compared to the in vivo rabbit eye test (7). The false negative rate obtained is not critical in the present context, since all test chemicals that induce a cell viability of  $\leq$  70% at a 5% concentration and  $>$  70% at 0.05% concentration would be subsequently tested with other adequately validated in vitro test methods or, as a last option, in the in vivo rabbit eye test, depending on regulatory requirements and in accordance with the sequential testing strategy and weight-of-evidence approaches currently recommended (1) (8). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. The STE test method showed no other specific shortcomings when used to identify test chemicals as UN GHS Category 1. Investigators could consider using this test method on test chemicals, whereby cell viability  $\leq$  70% at both 5% and 0.05% concentration should be accepted as indicative of a response inducing serious eye damage that should be classified as UN GHS Category 1 without further testing.

7. When used to identify chemicals (substances and mixtures) not requiring classification for eye irritation and serious eye damage (i.e. UN GHS No Category), data

obtained with the STE test method on 130 chemicals (including both substances and mixtures), showed an overall accuracy of 85% (110/130), a false negative rate of 12% (9/73), and a false positive rate of 19% (11/57) as compared to the *in vivo* rabbit eye test (7). If highly volatile substances (i.e. vapour pressure > 6kPa) and solid substances other than surfactants are excluded from the dataset, the overall accuracy improves to 90% (92/102), the false negative rate to 2% (1/54), and the false positive to 19% (9/48) (7). Further work demonstrated that highly volatile substances can be correctly tested when using mineral oil instead of saline as a solvent (15). The accuracy of the STE test for highly volatile substances (i.e. vapour pressure > 6kPa) was then 95% (19/20), the false negative rate was 0% (0/7), and the false positive rate was 8% (1/13). As a consequence, the potential shortcoming of the STE test method when used to identify test chemicals not requiring classification for eye irritation and serious eye damage (UN GHS No Category) is a high false negative rate for solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants. Such chemicals are excluded from the applicability domain of the STE test method (7).

8. In addition to the chemicals mentioned in paragraphs 6 and 7, the STE test method generated dataset also contains in-house data on 40 mixtures, which when compared to the *in vivo* Draize eye test, showed an accuracy of 88% (35/40), a false positive rate of 50% (5/10), and a false negative rate of 0% (0/30) for predicting mixtures that do not require classification under the UN GHS classification system (9). The STE test method can therefore be applied to identify mixtures as UN GHS No Category in a bottom-up approach with the exception of solid mixtures other than those composed only of surfactants as an extension of its limitation to solid substances. Furthermore, mixtures containing substances with vapour pressure higher than 6kPa that do not dissolve in mineral oil, or that do not form stable suspensions for at least 5 minutes, should be evaluated with care to avoid potential under-predictions, and should be justified on a case-by-case basis.

9. The STE test method cannot be used for the identification of test chemicals as UN GHS Category 2, Category 2A (eye irritation) or UN GHS Category 2B (mild eye irritation), due to the considerable number of UN GHS Category 1 chemicals under-predicted as UN GHS Category 2, 2A, or 2B and UN GHS No Category chemicals over-predicted as UN GHS Category 2, 2A, or 2B (7). For this purpose, further testing with another suitable method may be required.

10. The STE test method is suitable for test chemicals that are dissolved or uniformly suspended for at least 5 minutes in physiological saline, 5% dimethyl sulfoxide (DMSO) in saline, or mineral oil. The STE test method is not suitable for test chemicals that are insoluble or cannot be uniformly suspended for at least 5 minutes in physiological saline, 5% DMSO in saline, or mineral oil. The use of mineral oil in the STE test method is possible because of the short-time exposure. Therefore, the STE test method is suitable for predicting the eye hazard potential of water-insoluble test chemicals (e.g., long-chain fatty alcohols or ketones) provided that they are miscible in at least one of the three above proposed solvents (4).

## PRINCIPLE OF THE TEST

11. The STE test method is a cytotoxicity-based *in vitro* assay that is performed on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells, cultured on a 96-well polycarbonate microplate (4). After five-minute exposure to a test chemical, the

cytotoxicity is quantitatively measured as the relative viability of SIRC cells using the MTT assay (4). Decreased cell viability is used to predict potential adverse effects leading to ocular damage.

12. It has been reported that 80% of a solution dropped into the eye of a rabbit is excreted through the conjunctival sac within three to four minutes, while greater than 80% of a solution dropped into the human eye is excreted within one to two minutes (10). The STE test method attempts to approximate these exposure times and makes use of cytotoxicity as an endpoint to assess the extent of damage to SIRC cells following a five-minute exposure to the test chemical.

## DEMONSTRATION OF PROFICIENCY

13. Prior to routine use of the STE test method described in this test guideline, laboratories should demonstrate technical proficiency by correctly classifying the eleven substances recommended in Table 1. These substances were selected to represent the full range of responses for serious eye damage or eye irritation based on results of in vivo rabbit eye tests (TG 405) and the UN GHS classification system (1). Other selection criteria included that the substances should be commercially available, that high-quality in vivo reference data should be available, and that high quality in vitro data from the STE test method should be available (3). In situations where a listed substance is unavailable or where justifiable, another substance for which adequate in vivo and in vitro reference data are available could be used provided that the same criteria as described here are used.

**Table 1: List of Proficiency Substances**

| Substance                            | CASRN      | Chemical class <sup>1</sup>                                | Physical state | <i>In Vivo UN GHS Cat.</i> <sup>2</sup> | Solvent in STE test | STE UN GHS Cat.           |
|--------------------------------------|------------|--|----------------|---|---------------------|---------------------------|
| Benzalkonium chloride (10%, aqueous) | 8001-54-5  | Onium compound   | Liquid         | Category 1                              | Saline              | Category 1                |
| Triton X-100 (100%)                  | 9002-93-1  | Ether  | Liquid         | Category 1                              | Saline              | Category 1                |
| Acid Red 92                          | 18472-87-2 | Heterocyclic compound; Bromine compound; Chlorine compound | Solid          | Category 1                              | Saline              | Category 1                |
| Sodium hydroxide                     | 1310-73-2  | Alkali; Inorganic chemical                                 | Solid          | Category 1 <sup>3</sup>                 | Saline              | Category 1                |
| Butyrolactone                        | 96-48-0    | Lactone; Heterocyclic compound                             | Liquid         | Category 2A                             | Saline              | No prediction can be made |
| 1-Octanol                            | 111-87-5   | Alcohol  | Liquid         | Category 2A/B <sup>4</sup>              | Mineral Oil         | No prediction can be made |
| Cyclopentanol                        | 96-41-3    | Alcohol; Hydrocarbon, cyclic                               | Liquid         | Category 2A/B <sup>5</sup>              | Saline              | No prediction can be made |

|                               |          |                          |        |             |             |             |
|-------------------------------|----------|--------------------------|--------|-------------|-------------|-------------|
| 2-Ethoxyethyl acetate         | 111-15-9 | Alcohol; Ether           | Liquid | No Category | Saline      | No Category |
| Dodecane                      | 112-40-3 | Hydrocarbon, acyclic     | Liquid | No Category | Mineral Oil | No Category |
| Methyl isobutyl ketone        | 108-10-1 | Ketone                   | Liquid | No Category | Mineral Oil | No Category |
| n,n-Dimethylguanidine sulfate | 598-65-2 | Amidine; Sulfur compound | Solid  | No Category | Saline      | No Category |

1 Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

2 <sup>1</sup> Chemical classes were assigned using information obtained from previous NICEATM  
3 publications and if not available, using the National Library of Medicine's Medical Subject  
4 Headings (MeSH®) (via ChemIDplus® [National Library of Medicine], available at  
5 <http://chem.sis.nlm.nih.gov/chemidplus/>) and structure determinations made by  
6 NICEATM.

7 <sup>2</sup> Based on results from the in vivo rabbit eye test (OECD TG 405) and using the UN GHS  
8 (1).

9 <sup>3</sup> Classification as Cat.1 is based on skin corrosive potential of 100% sodium hydroxide  
10 (listed as a proficiency chemical with skin corrosive potential in OECD TG 435) and the  
11 criterion for UN GHS category 1 (1).

12 <sup>4</sup> Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for  
13 distinguishing between these two categories, i.e., 2 out of 6 vs 4 out of 6 animals with  
14 effects at day 7 necessary to generate a Category 2A classification. The in vivo dataset  
15 included 2 studies with 3 animals each. In one study two out of three animals showed effects  
16 at day 7 warranting a Cat. 2A classification (11), whereas in the second study all endpoints  
17 in all three animals recovered to a score of zero by day 7 warranting a Cat. 2B classification  
18 (12).

19 <sup>5</sup> Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for  
20 distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with  
21 effects at day 7 necessary to generate a Category 2A classification. The in vivo study  
22 included 3 animals. All endpoints apart from corneal opacity and conjunctivae redness in  
23 one animal recovered to a score of zero by day 7 or earlier. The one animal that did not  
24 fully recover by day 7 had a corneal opacity score of 1 and a conjunctivae redness of 1 (at  
25 day 7) that fully recovered at day 14 (11).

## 26 PROCEDURE

### 27 *Preparation of the Cellular Monolayer*

28 14. The rabbit cornea cell line, SIRC should be used for performing the STE test  
29 method. It is recommended that SIRC cells are obtained from a well-qualified cell bank,  
30 such as American Type Culture Collection CCL60.

31 15. SIRC cells are cultured at 37°C under 5% CO<sub>2</sub> and humidified atmosphere in a  
32 culture flask containing a culture medium comprising Eagle's minimum essential medium  
33 (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50–100  
34 units/mL penicillin and 50–100 µg/mL streptomycin. Cells that have become confluent in

1 the culture flask should be separated using trypsin-ethylenediaminetetraacetic acid  
2 solution, with or without the use of a cell scraper. Cells are propagated (e.g. 2 to 3 passages)  
3 in a culture flask before being employed for routine testing, and should undergo no more  
4 than 25 passages from thawing.

5 16. Cells ready to be used for the STE test are then prepared at the appropriate density  
6 and seeded into 96-well plates. The recommended cell seeding density is  $6.0 \times 10^3$  cells  
7 per well when cells are used four days after seeding, or  $3.0 \times 10^3$  cells per well when cells  
8 are used five days after seeding, at a culture volume of 200  $\mu\text{L}$ . Cells used for the STE test  
9 that are seeded in a culture medium at the appropriate density will reach a confluence of  
10 more than 80% at the time of testing, i.e., four or five days after seeding.

### 11 *Application of the Test Chemicals and Control Substances*

12 17. The first choice of solvent for dissolving or suspending test chemicals is  
13 physiological saline. If the test chemical demonstrates low solubility or cannot be dissolved  
14 or suspended uniformly for at least five minutes in saline, 5% DMSO (CAS#67-68-5) in  
15 saline is used as a second choice solvent. For test chemicals that cannot be dissolved or  
16 suspended uniformly for at least five minutes in either saline or 5% DMSO in saline,  
17 mineral oil (CAS#8042-47-5) is used as a third choice solvent. For highly volatile test  
18 chemicals (i.e. vapor pressure over 6 kPa) mineral oil can be used as a solvent, provided  
19 the test chemical dissolves or forms a stable suspension for at least five minutes in mineral  
20 oil.

21 18. Test chemicals are dissolved or suspended uniformly in the selected solvent at 5%  
22 (w/w) concentration and further diluted by serial 10-fold dilution to 0.5% and 0.05%  
23 concentration. Each test chemical is to be tested at both 5% and 0.05% concentrations.  
24 Cells cultured in the 96-well plate are exposed to 200  $\mu\text{L}$ /well of either a 5% or a 0.05%  
25 concentration of the test chemical solution (or suspension), for five minutes at room  
26 temperature. Test chemicals (mono-constituent substances or multi-constituent substances  
27 or mixtures) are considered as neat substances and diluted or suspended according to the  
28 method, regardless of their purity.

29 19. The culture medium described in paragraph 15 is used as a medium control in each  
30 plate of each repetition. Furthermore, cells are to be exposed also to solvent control samples  
31 in each plate of each repetition. The solvents listed in paragraph 17 have been confirmed  
32 to have no adverse effects on the viability of SIRC cells.

33 20. In the STE test method, 0.01% Sodium lauryl sulfate (SLS) in saline is to be used  
34 as a positive control in each plate of each repetition. In order to calculate cell viability of  
35 the positive control, each plate of each repetition has to also include a saline solvent control.

36 21. A blank is necessary to determine compensation for optical density and should be  
37 performed on wells containing culture medium or phosphate buffered saline, but no  
38 calcium and magnesium (PBS-) or cells.

39 22. Each sample (test chemical at 5% and 0.05%, medium control, solvent control, and  
40 positive control) should be tested in triplicate in each repetition by exposing the cells to  
41 200  $\mu\text{L}$  of the appropriate test or control chemical for five minutes at room temperature.

42 23. Benchmark substances are useful for evaluating the ocular irritancy potential of  
43 unknown chemicals of a specific chemical or product class, or for evaluating the relative  
44 irritancy potential of an ocular irritant within a specific range of irritant responses.

### *Cell Viability Measurement*

24. After exposure, cells are washed twice with 200 µL of PBS and 200 µL of MTT solution (0.5 mg MTT/mL of culture medium) is added. After a two-hour reaction time in an incubator (37°C, 5% CO<sub>2</sub>), the MTT solution is decanted, MTT formazan is extracted by adding 200 µL of 0.04 N hydrochloric acid-isopropanol for 60 minutes in the dark at room temperature, and the absorbance of the MTT formazan solution is measured at 570 nm with a plate reader. Interference of test chemicals with the MTT assay (by colorants or direct MTT reducers) only occurs if significant amount of test chemical is retained in the test system following rinsing after exposure. While this is often the case for the 3D reconstructed human cornea or Reconstructed human epidermis, it is less likely to occur in the 2D cell cultures used for the STE test method. However, because residual material from colorants or direct MTT reducers could interfere with the measurement of optical density, STE users should evaluate such results with caution. If the test results in a Category 1 prediction, then no further actions to address potential interference are needed. Where possible, data should be generated to determine whether such interference is occurring (e.g., conducting an experiment to compare MTT assay OD measurements from test article-treated wells containing SIRC cells in comparison to test article-treated wells containing no cells). If MTT interference is expected to affect the results, alternative cytotoxicity assays (e.g. neutral red) can be used as long as it can be shown to provide similar results as MTT assay, e.g. by testing the proficiency substances in Table 1, and if historical data are available to derive comparable run acceptance criteria (see paragraph 29).

### *Interpretation of Results and Prediction Model*

25. The optical density (OD) values obtained for each test chemical are then used to calculate cell viability relative to the solvent control, which is set at 100%. The relative cell viability is expressed as a percentage and obtained by dividing the OD of test chemical by the OD of the solvent control after subtracting the OD of blank from both values.

$$\text{Cell viability (\%)} = \frac{(\text{OD}_{570} \text{ of test chemical}) - (\text{OD}_{570} \text{ of blank})}{(\text{OD}_{570} \text{ of solvent control}) - (\text{OD}_{570} \text{ of blank})} \times 100$$

Similarly, the relative cell viability of each solvent control is expressed as a percentage and obtained by dividing the OD of each solvent control by the OD of the medium control after subtracting the OD of blank from both values.

26. Three independent repetitions, each containing three replicate wells (i.e., n=9), should be performed. The arithmetic mean of the three wells for each test chemical and solvent control in each independent repetition is used to calculate the arithmetic mean of relative cell viability. The final arithmetic mean of the cell viability is calculated from the three independent repetitions.

27. The cell viability cut-off values for identifying test chemicals inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter.

Table 2: Prediction model of the STE test method

| Cell viability |          | UN GHS Classification                  | Applicability   |
|----------------|----------|--|---|
| At 5%          | At 0.05% |  |   |
| > 70%          | > 70%    | No Category                            | Substances and mixtures <sup>1</sup> , with the exception of solid chemicals (substances and mixtures) other than surfactants and mixtures composed only of surfactants |
| ≤ 70%          | > 70%    | No prediction can be made <sup>3</sup> | Not applicable  |
| ≤ 70%          | ≤ 70%    | Category 1                             | Substances and mixtures <sup>2</sup>  |

<sup>1</sup> Mixtures containing test chemicals with vapour pressure higher than 6kPa and that do not either dissolve in mineral oil or form a stable suspension should be tested and evaluated with care to avoid potential under-predictions; results should be justified on a case-by-case basis.

<sup>2</sup> Based on results obtained mainly with mono-constituent substances, although a limited amount of data also exist on the testing of mixtures. The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. Before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

<sup>3</sup> No prediction can be made from this result in isolation. The result of the STE test should be considered in the context of an IATA (14) for classification purposes.

### Acceptance Criteria

28. Test results are judged to be acceptable when the following criteria are all satisfied:

- a) Optical density of the medium control (exposed to culture medium) should be 0.3 or higher after subtraction of blank optical density.
- b) Viability of the solvent control should be 80% or higher relative to the medium control. If multiple solvent controls are used in each repetition, each of those controls should show cell viability greater than 80% to qualify the test chemicals tested with those solvents.
- c) The cell viability obtained with the positive control (0.01% SLS) should be within two standard deviations of the historical mean. The upper and lower acceptance boundaries for the positive control should be frequently updated i.e., every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e., less than once a month). Where a laboratory does not complete a sufficient number of experiments to establish a statistically robust positive control distribution, it is acceptable that the upper and lower acceptance boundaries established by the method developer are used, i.e.,



1 between 21.1% and 62.3% according to its laboratory historical data, while an  
2 internal distribution is built during the first routine tests.

3 If any of the above criteria a), b) or c) are not met, an additional repetition should  
4 be performed.

5 d) Standard deviation of the final cell viability derived from three independent  
6 repetitions should be less than 15% for both 5% and 0.05% concentrations of the  
7 test chemical. If the standard deviation is greater than or equal to 15%, the results  
8 should not be used and three more repetitions should be performed.

## 9 DATA AND REPORTING

### 10 *Data*

11 29. Data for each individual well (e.g., cell viability values) of each repetition as well  
12 as overall mean, standard deviation (SD), and classification are to be reported.

### 13 *Test Report*

14 30. The test report should include the following information:

#### 15 *Test Chemical and Control Substances*

- 16 • Mono-constituent substance : chemical identification, such as IUPAC or CAS  
17 name(s), CAS registry number(s), SMILES or InChI code, structural formula,  
18 and/or other identifiers;
- 19 • Multi-constituent substance, UVCB and mixture: Characterization as far as  
20 possible by e.g., chemical identity (see above), purity, quantitative occurrence and  
21 relevant physicochemical properties (see above) of the constituents, to the extent  
22 available;
- 23 • Physical state, volatility, pH, LogP, molecular weight, chemical class, and  
24 additional relevant physicochemical properties relevant to the conduct of the study,  
25 to the extent available;
- 26 • Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- 27 • Treatment prior to testing, if applicable (e.g., warming, grinding);
- 28 • Storage conditions and stability to the extent available, including solubility for at  
29 least five minutes in a selected solvent (e.g. dissolution or stable suspension).

#### 30 *Test Method Conditions and Procedures*

- 31 • Name and address of the sponsor, test facility and study director;
- 32 • Description of the test method used;
- 33 • Cell line used, its source, passage number and confluence of cells used for testing;
- 34 • Details of test procedure used;
- 35 • Number of repetitions and replicates used;
- 36 • Test chemical concentrations used (if different than the ones recommended);

- 1 • Justification for choice of solvent for each test chemical;
- 2 • Duration of exposure to the test chemical (if different than the one recommended);
- 3 • Description of any modifications of the test procedure;
- 4 • Description of evaluation and decision criteria used;
- 5 • Reference to historical positive control mean and Standard Deviation (SD):
- 6 • Demonstration of proficiency of the laboratory in performing the test method (e.g.
- 7 by testing of proficiency substances) or demonstration of reproducible performance
- 8 of the test method over time.

### 9 ***Results***

- 10 • For each test chemical and control substance, and each tested concentration,
- 11 tabulation should be given for the individual OD values per replicate well, the
- 12 arithmetic mean OD values for each independent repetition, the % cell viability for
- 13 each independent repetition, and the final arithmetic mean % cell viability and SD
- 14 over the three repetitions;
- 15 • Results for the medium, solvent and positive control demonstrating suitable study
- 16 acceptance criteria;
- 17 • Description of other effects observed, including retainment of significant amounts
- 18 of test chemical following rinsing after exposure;
- 19 • The overall derived classification with reference to the prediction model/decision
- 20 criteria used.

### 21 ***Discussion of the Results***

### 22 ***Conclusions***

23

## 1 LITERATURE

- 2 1) United Nations UN (2013). Globally Harmonized System of Classification and  
3 Labelling of Chemicals (GHS). Fifth revised edition. New York & Geneva: United Nations  
4 Publications. ISBN: 978-92-1-117006-1. Available at:  
5 [[http://www.unece.org/trans/danger/publi/ghs/ghs\\_rev05/05files\\_e.html](http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html)].
- 6 2) Scott L, et al. (2010). A proposed Eye Irritation Testing Strategy to Reduce and  
7 Replace in vivo Studies Using Bottom-Up and Top-Down Approaches. *Toxicol. In Vitro*  
8 24, 1-9.
- 9 3) Mosmann T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival:  
10 Application to 7 Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* 65, 55-63.
- 11 4) Takahashi Y, et al. (2008). Development of the Short Time Exposure (STE) Test:  
12 an In Vitro Eye Irritation Test Using SIRC Cells. *Toxicol. In Vitro* 22,760-770.
- 13 5) Sakaguchi H, et al. (2011). Validation Study of the Short Time Exposure (STE)  
14 Test to Assess the Eye Irritation Potential of Chemicals. *Toxicol. In Vitro* 25,796-809.
- 15 6) Kojima H, et al. (2013). Second-Phase Validation of Short Time Exposure Tests  
16 for Assessment of Eye Irritation Potency of Chemicals. *Toxicol. In Vitro* 27, 1855-1869.
- 17 7) ICCVAM (2013). Short Time Exposure (STE) Test Method Summary Review  
18 Document, NIH. Available at:  
19 [[http://www.ntp.niehs.nih.gov/iccvam/docs/ocutox\\_docs/STE-SRD-NICEATM-508.pdf](http://www.ntp.niehs.nih.gov/iccvam/docs/ocutox_docs/STE-SRD-NICEATM-508.pdf)].
- 20 8) OECD (2012). Test Guideline for Testing of Chemicals (No.405): Acute Eye  
21 Irritation/Corrosion.Organisation for Economic Cooperation and Development, Paris.
- 22 9) Saito K, et al. (2015). Predictive Performance of the Short Time Exposure Test for  
23 Identifying Eye Irritation Potential of Chemical Mixtures. *Toxicol. In Vitro* 29, 617–620
- 24 10) Mikkelson TJ, Chrai SS and Robinson JR. (1973). Altered Bioavailability of Drugs  
25 in the Eye Due to Drug-Protein Interaction. *J. Pharm. Sci.*1648-1653.
- 26 11) ECETOC. (1998). Eye Irritation Reference Chemicals Data Bank. Technical  
27 Report (No. 48. (2)), Brussels, Belgium.
- 28 12) Gautheron P, et al. (1992). Bovine Corneal Opacity and Permeability Test: an In  
29 Vitro Assay of Ocular Irritancy. *Fundam Appl Toxicol.* 18, 442–449.
- 30 13) OECD (2005), Guidance Document on the Validation and International Acceptance  
31 of New or Updated Test Methods for Hazard Assessment. Environmental, Health and  
32 Safety Publications, Series on Testing and Assessment (No. 34.). Organisation for  
33 Economic Cooperation and Development, Paris.
- 34 14) OECD (2018). Guidance Document on an Integrated Approaches on Testing and  
35 Assessment for Serious Eye Damage and Eye irritation. Series on Testing and Assessment  
36 No.263. ENV Publications, Organisation for Economic Cooperation and Development,  
37 Paris.
- 38 15) Abo T, et al. (2018). Expansion of the applicability domain for highly volatile  
39 substances on the Short Time Exposure test method and the predictive performance in  
40 assessing eye irritation potential. *J. Toxicol. Sci.*, 43, 407-422

## ANNEX I

## DEFINITIONS

**Accuracy:** The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (13).

**Benchmark substance:** A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

**Bottom-Up Approach:** A step-wise approach used for a test chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome)

**Chemical:** means a substance or mixture.

**Eye irritation:** Production of change in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with “reversible effects on the eye” and with UN GHS Category 2 (1)

**False negative rate:** The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance.

**False positive rate:** The proportion of all negative chemicals that are falsely identified by a test method as positive. It is one indicator of test method performance.

**Hazard:** Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

**Medium control:** An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

**Mixture:** A mixture or a solution composed of two or more substances in which they do not react (1).

**Mono-constituent substance:** A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

**MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

**Multi-constituent substance:** A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration  $\geq 10\%$  (w/w) and  $< 80\%$  (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

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- 2 **OD:** Optical Density.
- 3 **Positive control:** A replicate containing all components of a test system and treated with a  
4 substance known to induce a positive response. To ensure that variability in the positive  
5 control response across time can be assessed, the magnitude of the positive response should  
6 not be excessive.
- 7 **Relevance:** Description of relationship of the test to the effect of interest and whether it is  
8 meaningful and useful for a particular purpose. It is the extent to which the test correctly  
9 measures or predicts the biological effect of interest. Relevance incorporates consideration  
10 of the accuracy (concordance) of a test method (10).
- 11 **Reliability:** Measures of the extent that a test method can be performed reproducibly within  
12 and between laboratories over time, when performed using the same protocol. It is assessed  
13 by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability  
14 (13).
- 15 **Sensitivity:** The proportion of all positive/active chemicals that are correctly classified by  
16 the test. It is a measure of accuracy for a test method that produces categorical results, and  
17 is an important consideration in assessing the relevance of a test method (10).
- 18 **Serious eye damage:** Production of tissue damage in the eye, or serious physical decay of  
19 vision, following application of a test chemical to the anterior surface of the eye, which is  
20 not fully reversible within 21 days of application. Interchangeable with “irreversible effects  
21 on the eye” and with UN GHS Category 1 (1).
- 22 **Solvent/vehicle control:** An untreated sample containing all components of a test system,  
23 including the solvent or vehicle that is processed with the test chemical-treated and other  
24 control samples to establish the baseline response for the samples treated with the test  
25 chemical dissolved in the same solvent or vehicle. When tested with a concurrent medium  
26 control, this sample also demonstrates whether the solvent or vehicle interacts with the test  
27 system.
- 28 **Specificity:** The proportion of all negative/inactive chemicals that are correctly classified  
29 by the test. It is a measure of accuracy for a test method that produces categorical results  
30 and is an important consideration in assessing the relevance of a test method (13).
- 31 **Substance:** Chemical elements and their compounds in the natural state or obtained by any  
32 production process, inducing any additive necessary to preserve the stability of the product  
33 and any impurities deriving from the process used, but excluding any solvent which may  
34 be separated without affecting the stability of the substance or changing its composition (1).
- 35 **Surfactant:** Also called surface-active agent, this is a chemical such as a detergent, that  
36 can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is  
37 also known as a wetting agent.
- 38 **Test chemical:** The term "test chemical" is used to refer to what is being tested.
- 39 **Tiered testing strategy:** A stepwise testing strategy where all existing information on a  
40 test chemical is reviewed, in a specified order, using a weight of evidence process at each  
41 tier to determine if sufficient information is available for a hazard classification decision,  
42 prior to progression to the next tier. If the irritancy potential of a test chemical can be  
43 assigned based on the existing information, no additional testing is required. If the irritancy  
44 potential of a test chemical cannot be assigned based on the existing information, a step-

- 1 wise sequential animal testing procedure is performed until an unequivocal classification  
2 can be made.
- 3 **Top-Down Approach:** step-wise approach used for a test chemical suspected of causing  
4 serious eye damage, which starts with the determination of chemicals inducing serious eye  
5 damage (positive outcome) from other chemicals (negative outcome).
- 6 **United Nations Globally Harmonized System of Classification and Labelling of  
7 Chemicals (UN GHS):** A system proposing the classification of chemicals (substances and  
8 mixtures) according to standardized types and levels of physical, health and environmental  
9 hazards, and addressing corresponding communication elements, such as pictograms,  
10 signal words, hazard statements, precautionary statements and safety data sheets, so that to  
11 convey information on their adverse effects with a view to protect people (including  
12 employers, workers, transporters, consumers and emergency responders) and the  
13 environment (1).
- 14 **UN GHS Category 1:** See “Serious eye damage”.
- 15 **UN GHS Category 2:** See “Eye irritation”.
- 16 **UN GHS No Category:** Chemicals that are not classified as UN GHS Category 1 or 2 (2A  
17 or 2B).
- 18 **UVCB:** substances of unknown or variable composition, complex reaction products or  
19 biological materials.
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