INTRODUCTION

1. *Serious eye damage* refers to the production of tissue damage in the eye or serious physical decay of vision that follows application of a test chemical to the anterior surface of the eye and which is not fully reversible within 21 days of application, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). Also according to UN GHS, *eye irritation* refers to the production of changes in the eye that follow the application of a test chemical to the anterior surface of the eye and which are fully reversible within 21 days of application. Test chemicals that induce serious eye damage are classified as UN GHS Category 1, and those that induce eye irritation are classified as UN GHS Category 2, which includes subcategories 2A or 2B. Test chemicals that are neither Category 1 nor Category 2 do not require classification for eye irritation or serious eye damage and are referred to as UN GHS No Category.

2. The assessment of serious eye damage and eye irritation has historically involved the use of laboratory animals as described in OECD Test Guideline 405, which was adopted in 1981 and revised in 1987, 2002, 2012, and 2017 (2). 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 (IATA) for Serious Eye Damage and Eye irritation (3).

3. Although much effort has been made to develop alternatives to animal testing, no single ex vivo or in vitro test is capable of fully replacing in vivo testing. Therefore, bottom-up and top-down approaches that combine multiple test methods have been proposed for use in place of the Draize test (3). Test Guidelines adopted by the OECD include No. 437: the Bovine Corneal Opacity Permeability (BCOP) test method (4), No. 438: Isolated Chicken Eye (ICE) test method (5), No. 460: Fluorescein Leakage (FL) test method (6), No. 491: Short Time Exposure (STE) test method (7), and No. 492: Reconstructed human Cornea-like Epithelium (RhCE) test method (8). The BCOP, ICE, and STE test methods are considered useful both in a top-down and bottom-up approach, to identify without further testing (i) chemicals inducing serious eye damage (UN GHS Category 1) and (ii) chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category).
GHS No Category). On the other hand, the FL test method is considered useful in a top-down approach to identify chemicals inducing serious eye damage (UN GHS Category 1) without further testing, and the RhCE test method is considered useful in a bottom-up approach to identify chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category) without further testing.

4. The Vitrigel-Eye Irritancy Test (EIT) method is an in vitro eye irritation test method that can identify chemicals that do not induce serious eye damage or eye irritation (GHS No Cat) from those that do induce serious eye damage (GHS Cat 1) or eye irritation (GHS Cat 2) (9, 10, 11), as defined by the UN GHS (1) without further testing. Here, the Vitrigel-EIT method is performed within a testing strategy such as the Bottom-Up/Top-Down approach suggested by Scott et al. e.g., as an initial step in a Bottom-Up approach or as one of the last steps in a Top-Down approach. However, the Vitrigel-EIT method is not intended to differentiate between UN GHS Category 1 and UN GHS Category 2. This differentiation will need to be addressed by another tier of a test strategy (3).

5. The purpose of this TG is to describe a procedure for assessing the eye hazard potential of a test chemical based on its ability to induce damage to the barrier function of the human corneal epithelium (hCE) models used in the Vitrigel-EIT method. In traditional test methods, the viability of cells in culture in vitro or the corneal opacity of isolated eyeballs ex vivo was utilized as an endpoint. Meanwhile, it is known that eye hazardous chemicals first destroy tear film and epithelial barrier function, and subsequently induce epithelial cell death, and finally produce stromal degeneration and endothelial cell death, resulting the corneal opacity. Therefore, the change of the epithelial barrier function is suitable for the endpoint for estimating moderate eye irritation. Time-dependent changes in the Transepithelial Electrical Resistance (TEER) values indicating the barrier function of epithelium following exposure of not only the hCE model but also rabbit cornea to a test chemical is an important mode of action (MoA) leading to damage of the corneal epithelium and eye irritation (9, 12). The change in TEER occurs when the barrier of corneal epithelial cell layers has been damaged by exposing the hCE models with eye irritant chemicals. The Vitrigel-EIT method involves analysis of time-dependent changes in TEER values using three indexes. Using predetermined criteria, the score of each index is employed to predict the irritation potential of the test chemical.

6. The term “test chemical” is used in this TG to refer to the chemicals being tested and is not
a reference to the applicability of the Vitrigel-EIT method to the testing of substances.

7. Definitions are provided in Annex 1.

PRINCIPLE OF THE TEST

8. The Vitrigel-EIT test method is an in vitro assay using hCE models fabricated in a collagen vitrigel membrane (CVM) chamber (10). Eye irritation potential of the test chemical is predicted by analyzing the relative changes over time in TEER measured during a three-minute period following exposure to a test chemical.

9. It has been reported that 80% of a solution applied to the eye of a rabbit is excreted through the conjunctival sac within three to four minutes, and more than 80% of a solution applied to the human eye is excreted within one to two minutes (13). The Vitrigel-EIT test method attempts to approximate these exposure times and makes use of the destructive activity of the chemicals against the barrier function of hCE models as an endpoint to assess the extent of damage to the hCE model during a three-minute exposure to the test chemical.

INITIAL CONSIDERATIONS AND LIMITATIONS

10. This TG is based on a protocol developed by Yamaguchi and Takezawa (14), which was the subject of a validation study by a validation management team (VMT) organized in cooperation with the International Collaboration on Alternative Test Methods (ICATM) (15). The validation study was performed with the participation of three Japanese laboratories. The validation report was evaluated by an independent peer review panel of international experts, which concluded that the Vitrigel-EIT method is valid for use as an initial test in a bottom-up testing strategy approach for identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Cat. chemicals), when used within the limited applicability domain of test chemicals having pH > 5.0 (based on 2.5% solution), and excluding solids having both a log P ≥2.5 and a density of < 0.95 g/cm³ or > 1.10 g/cm³ (16).

11. The results of the validation study showed within-laboratory reproducibility to be
80–100% at all three laboratories and a between-laboratory reproducibility of 92%. The predictive capacity was evaluated based on validation and developer’s in-house data for 93 test chemicals (15). The test chemicals were selected to ensure that a diverse range of substances were represented in terms of eye-irritancy potential per UN GHS categories, physical state, and chemical class. The majority of these chemicals represented mono-constituent substances, but 9 multi-constituent substances (polymers) were also included in the study. The 93 test chemicals comprised 56 liquids and 37 solids, including 60 classified chemicals for ocular hazards and 33 non-classified chemicals. In detail, 28 chemicals were predicted to be Category 1, 4 chemical was predicted to be Category 2, 16 chemicals were predicted to be Category 2A, 12 chemicals were predicted to be Category 2B, and 33 chemicals were predicted to be No Category under the UN GHS classification. Furthermore, results for 73 of the 93 test chemicals matched their in vivo UN GHS category. In contrast, 10 of the 60 test chemicals classified as irritants by in vivo data were identified as non-classified in vitro, resulting in a false-negative rate of 17%. Additionally, 10 of the 33 test chemicals classified as non-classified under UN GHS were identified as requiring classification in vitro, resulting in a false-positive rate of 30%. Thus, the Vitrigel-EIT method achieved a sensitivity of 83%, a specificity of 70%, and an accuracy of 78%.

Analysis of the false-negative reactions shows that five of the ten false-negative chemicals were acidic, and the 2.5% solutions used for exposure had a pH level lower than 5. Typically, the TEER values of the hCE model after exposure to UN-GHS No Category chemicals almost no changed from their initial TEER values. Also, the TEER values of the hCE model after exposure to UN-GHS Category 1/2A/2B chemicals decreased from their initial TEER values. The TEER values of the hCE models after exposure to these five acidic test chemicals that yielded false-negatives increased from their initial values. It was reported that isolated rabbit esophageal mucosal epithelium and normal human bronchial epithelial cell layers in culture displayed increased TEER values when exposed to weak acidic solutions (17, 18). Two of the five non-acidic false-negative chemical were water-insoluble solids that were easily separated from the culture medium at room temperature in visual observation. Here, LogP of 2.5 or more and a density of either less than 0.95 g/cm³ or over 1.10 g/cm³ mean low solubility and high separability in aqueous solution, respectively. Based on the above, two restrictions to the applicability domain were stipulated:

- All chemicals that have a pH of 5 or less in solution are excluded from the applicability domain.
- All solids that have an estimated or measured LogP of 2.5 or more, and also a density of 
either less than 0.95 g/cm$^3$ or over 1.10 g/cm$^3$ are excluded from the applicability 
domain.

LogP value can be measured by the shake-flask method (19), high-performance liquid 
chromatography method (20) or be predicted by in silico method such as EPI Suite (21).
Under this applicability domain, 17 of the original 93 test chemicals were excluded. Here, 
the 17 test chemicals were 11 acidic chemicals including 5 false-negatives, 5 true-positives 
and 1 true-negative and 6 insoluble chemicals including 2 false-negatives and 4 
true-positives, suggesting 9 true-positives were excluded. Consequently, sensitivity, 
specificity, and accuracy were improved from 83 to 93%, from 70 to 69%, and from 78 to 
83%, respectively.

13. In the analysis of false-positive reactions, Zonula occludens-I(ZO-1) and Mucin 1(MUC1)
expressions in hCE models were maintained after exposing chemicals predicted as 
No-Category and disappeared after exposing the false-positives by the Vitrigel-EIT 
method (11). These data demonstrated that such false-positives induced the unhealthy 
conditions for the hCE models, suggesting that the chemicals have an eye irritant potential. 
The false positive rates obtained with the Vitrigel-EIT method are not critical in the 
context of this Test Guideline since all test chemicals that predicted 1/2A/2B will require 
further testing depending on regulatory requirements, according to the OECD Guidance 
Document on an Integrated Approaches on Testing and Assessment for Serious Eye 
Damage and Eye irritation (3).

14. Any substance not excluded by the applicability domain can be tested with the 
Vitrigel-EIT method by dissolving it in a culture medium at a concentration of 2.5% 
(weight/volume). Test chemicals that do not dissolve readily can be tested after using one 
of the following techniques: a) mix mechanically using a vortex mixer, b) sonication, or c) 
heating to a maximum temperature of 70°C (See PROCEDURE). The Test Guideline is 
applicable to mono-constituent substances, multi-constituent substances, substances of 
unknown or variable composition, complex reaction products or biological materials 
(UVBC), and mixtures, and to solids, liquids, semi-solids and waxes. Also, highly volatile 
substances, test chemicals absorbing light in the same range as formazan dye and test 
chemicals able to directly reduce the tetrazolium dye can be applied to the test method. 
Consequently, the test method can be used with other methods complementary. Gases and 
aerosols have not been assessed in a validation study. Therefore, the current Test 
Guideline does not allow testing of gases and aerosols.
DEMONSTRATION OF PROFICIENCY

15. Prior to routine use of the Vitrigel-EIT method described in this test guideline, laboratories should demonstrate technical proficiency by correctly classifying the ten substances recommended in Table A1 in Annex 2. 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 stipulates 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 Vitrigel-EIT method should be available (15). In situations where a listed substance is unavailable or cannot be used for other justifiable reason, it should be substituted with another chemical substance for which adequate in vivo and in vitro reference data are available.

PROCEDURE

16. The protocol for the Vitrigel-EIT method was developed by Yamaguchi and Takezawa (14). The following paragraphs describe the main components and procedures of the Vitrigel-EIT method. Testing should be performed in accordance with the Good Laboratory Practice (23). Values specified in this protocol as integers are considered to be accurate to one additional significant digit. Thus, “37°C” indicates an acceptable range from 36.5°C to 37.4°C.

Culture of hCE cells

17. An SV40-immortalized hCE cells\(^1\) are maintained in a culture medium comprising a 1:1 mixture of Dulbecco’s modified Eagle medium and nutrient mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 μg/mL recombinant human insulin, 10 ng/mL recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells are grown at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The cells should be free of contamination by bacteria, viruses, mycoplasma, and fungi except for the application of test chemical solutions to hCE models.
**Preparation of CVM chambers**

18. A collagen xerogel membrane chamber is set in the well of a 12-well plate and immersed in the culture medium by pouring 1.5 mL outside and 0.5 mL inside the chamber in the well for 10 minutes to convert the xerogel into vitrigel immediately before use.

**Fabrication of a hCE model**

19. The culture medium outside the chamber in the well of a 12-well plate is replaced with 1.5 mL of the fresh medium. The medium inside the chamber is carefully removed by using a micropipette and 0.5 mL of the cell suspension in the culture medium at a density of $1.2 \times 10^5$ cells/mL is poured onto the CVM in the chamber and cultured for 2 days at 37°C. After carefully removing the inside medium by using a micropipette and changing the outside medium to fresh medium, the cells are cultured for 4 more days at the air–liquid interface to fabricate the hCE model. On the third day of culture at the air-liquid interface, the medium outside the chamber is changed.

The quality of hCE models is checked as follows. First, 500 μL of fresh culture medium is poured in the chamber of the hCE models and the temperature of the culture medium is adjusted to 28±2°C. Next, the longer electrode of a TEER Measuring System (Refer to the section “Measurement of TEER value in a hCE model”) is set into the culture media outside the chamber, and the shorter electrode is set into the culture media inside the chamber, after which the TEER value of each hCE model (pre-exposure TEER values) is measured. Only hCE models with a TEER value within adequate range are acceptable for the testing of chemicals conducted on the same day.

**Histomorphology**

20. The hCE model should show a hCE-like structure including about 6 layers of viable epithelial cells with a superficial layer of non-keratinized cells.

**Barrier function**

21. The hCE model should possess sufficient robustness equivalent to hCE in order to avoid rapid disruption after chemical exposure. The barrier function of each hCE model is checked by measuring its TEER value. Adequate ranges should be provided for any proposed similar or modified test method.

**Measurement of TEER value in a hCE model**

22. TEER values of the hCE model should be measured by using an electrical resistance meter with low-voltage and alternating current. General specifications of the instrument are an
alternating current of 50–1,000 Hz and a measuring range of at least 0.1–3 kΩ. Schematic illustrations of the TEER measuring system are shown in Annex 3. The inner electrode is positioned inside the chamber, and the outer electrode is positioned outside the chamber. The distance between the inner and outer electrode must be consistent, because this distance affects the electrical resistance value obtained. Also, during resistance measurement, the depth to which the electrodes are submerged in the medium or buffer solution inside and outside of the chamber must also be consistent. The electrical resistance value of a hCE model fabricated in a CVM chamber (R_{model}) and that of its blank, an empty CVM chamber (R_{blank}) are measured. The TEER value of a hCE model is calculated as follows:

$$\text{TEER value of a hCE model (}\Omega\cdot\text{cm}^2) = \{R_{\text{model}} (\Omega) - R_{\text{blank}} (\Omega)\} \times \text{effective surface area (cm}^2)$$

The sensitivity of the TEER Measuring System should be checked before testing, and adequate ranges should be provided. This can be achieved by measuring the electrical resistance of two or more solutions having different conductivities, thereby confirming that the differences of these conductivities are within the predetermined value.

**Preparation of Control Substances**

23. The Vitrigel-EIT method uses saline as a negative control, benzalkonium-chloride as a positive control, and ethanol as a reference control. The reference control is used to check the quality of the hCE models. Control substance solutions are prepared in the culture medium at a concentration of 2.5% (weight/volume) by adding 0.1–0.2 g of saline, benzalkonium chloride, or ethanol to a 15-ml tube, pouring an appropriate volume of the culture medium into the tube, and mixing until dispersed uniformly. As long as the proper concentration is maintained for each control solution, the actual quantity is unimportant.

**Preparation of Test Chemicals**

24. A test chemical solution is prepared in the culture medium at a concentration of 2.5% (weight/volume), because at 2.5% (w/v), the test chemical does not influence the electrical resistance of the culture medium irrespective of conductivity. The test chemical is manually mixed in the medium until dissolved or for a maximum of one minute. If the test chemical does not dissolve readily, use one of the following techniques, which are listed here in order of preference:

a) mix mechanically for a maximum of one minute using a vortex mixer,
b) sonication for a maximum of 20 minutes, or
c) heating to a maximum temperature of 70°C.

After mixing, the temperature of the test chemical solution is adjusted to 28±2°C using a hot plate, a water bath, or an air conditioner, and the solubility of the test chemical is checked by visual inspection. **The next step is only taken once** the test chemical solution is well dissolved or homogeneously dispersed. For test chemicals that prove to be insoluble or immiscible using the above techniques, a test chemical solution is prepared as a homogeneous suspension by vortexing the test chemical in the medium for up to 1 minute immediately before use. The pH of each 2.5% test chemical solution is measured using a universal pH test paper covered a range from pH 1 to 11 or a pH meter. If pH of a 2.5% solution is ≤ 5 the chemical should not be tested.

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

25. Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals, or for evaluating the relative ocular irritancy potential of a chemical within a specific range of irritant responses. hCE models that pass the quality check can be used for exposure to the test chemical. The hCE model should be subjected to the chemical exposure experiment within 2 hours after drawing it from a CO₂ incubator. The medium inside the chamber is replaced with 500 μL of test chemical solution, and R_{model} values are measured at intervals of 10 seconds for a period of 3 minutes after exposure to the test chemical solution. **At least three hCE models should be used for each control substance solution and each test chemical solution in each run.**

To ensure reproducibility, it is essential that measurements begin between 2 to 5 seconds after adding the test chemical solution. A minimum of a two-second wait before beginning measurements is necessary, because the liquid around the electrode is often unstable for up to 2 seconds after adding the test chemical solution. Also, the TEER value of the hCE model has already been changed by adding the test chemical for over 5 seconds.

The temperature of the hCE models and the test chemical solutions should be maintained at 28±2°C during the chemical exposure tests. This can be done using a hot plate, a water bath, or an air conditioner. The temperature of the hCE model can be checked by measuring the actual temperature of culture medium outside the hCE model.

**Prediction Model**

26. The TEER values of the hCE model after exposure to a test chemical is calculated using the formula given above in the section “Measurement of TEER value in a hCE model”. The mean TEER values for all three tests are analyzed by using the following three
indexes: time lag \((t_1)\), intensity \((- [P_2 - P_1] / [t_2 - t_1])\), and plateau level \((100 - P_2)\). Annex 4 provides a graph showing an analysis of a TEER profile after exposure of a model to a test chemical. Score of each index is calculated. The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the scores of the indexes are Time lag > 180 seconds and Intensity < 0.05 %/seconds and Plateau level ≤ 5.0 %, as shown in Table 1. In this case no further testing in other test methods is required. If the scores of the indexes are Time lag ≤ 180 seconds or Intensity ≥ 0.05 %/seconds or Plateau level > 5.0 %, no prediction can be made from this result in isolation, as shown in Table 1. This is because in case of a true positive, the method cannot resolve between UN GHS Categories 1 and 2 (see paragraph 4). Furthermore, the Vitrigel-EIT method shows a high percentage of false positive results (see paragraphs 10-13). In both cases, further information will be required for classification purposes according to the IATA guidance document (3).

Table 1. Prediction Model.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time lag ≤ 180 seconds or Intensity ≥ 0.05 %/seconds or Plateau level ≥ 5.0 %</td>
<td>No Prediction Can Be Made¹</td>
</tr>
<tr>
<td>Time lag &gt; 180 seconds and Intensity &lt; 0.05 %/seconds and Plateau level ≤ 5.0 %</td>
<td>No Category²</td>
</tr>
</tbody>
</table>

¹No Prediction Can Be Made corresponds to chemicals that require further information for classification purposes according to the IATA guidance document (3).

²No Category corresponds to chemicals that do not require classification for serious eye damage or eye irritation according to UN GHS.

Acceptance Criteria

27. Test run is judged to be acceptable when the following criteria are all satisfied:

a) Negative control: The plateau level is ≤ 5%.

b) Positive control: The plateau level is ≥ 40%.

c) Reference control: The plateau level is ≥ 10%.

d) The average standard deviation of the overall TEER profile for each test chemical is ≤
The range of historical results for the positive control in the validation study is from 65% to 90%.

DATA AND REPORTING

Data

28. TEER values obtained for each individual hCE model, the scores of each index, and the final prediction by the Vitrigel-EIT method should be reported.

Test Report

29. The test report should include the following information:

Test Chemical and Control Substances
- Mono-constituent substance: Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers
- Multi-constituent substance, UVCB and mixture: Characterization as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available
- Physical state, pH, LogP, density, volatility, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available
- Purity, chemical identity of impurities as appropriate and practically feasible, etc.
- Treatment prior to testing, if applicable (e.g., warming, grinding)
- Storage conditions and stability to the extent available

Test Method Conditions and Procedures
- Name and address of the sponsor, test facility and study director
- Description of the test method used
- Details of test procedure used
- Cell line used, its source, passage number and confluence of cells used for testing
- Supplier, catalog number and lot number of a reagent
- Time and date of sub-culturing hCE cells, duration of tripsinization, dilution ratio of the cells
- Duration of each step for preparation of hCE models
- Data of QC check for TEER recorder
- Record of test chemical preparation (e.g. weight of test chemical, volume of medium, mixing method and solubility of test chemical, pH of the test chemical solution)
- Temperature of the hCE models and test chemical solution at the start of exposure test
- Lot number of a hCE model
- Time of pulling out a hCE model from CO₂ incubator, exposing a test chemical to a hCE model
- Time of starting TEER measurement by a TEER Measuring System
- Test chemical concentrations used (if different than the ones recommended)
- Duration of exposure to the test chemical (if different than the one recommended)
- Description of any modifications of the test procedure
- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Results
- For each test chemical and control substance, tabulation should be given for pre-exposure TEER values and time dependent TEER values after exposing test chemicals for 3 minutes for each independent repetition, scores of three indexes, and in vitro prediction of the test chemical.
- Description of other effects observed

Discussion of the Results
Conclusions

FOOT NOTE
1 e.g., HCE-T cells, RCB no. 2280 obtained from RIKEN BioResource Center, Tsukuba, Japan. MTA with RIKEN BRC is required.
2 e.g., ad-MED Vitrigel™ (Kanto Chemical Co., Inc., Tokyo, Japan.)
3 e.g., ad-MED TEER Recorder (Kanto Chemical Co., Inc., Tokyo, Japan.)

LITERATURE


15) VITRIGEL-EIT Validation Management Team (2017). Validation Study of the Vitrigel-EIT method as an alternative to in vivo eye irritation testing Study Report, Version 2.0

16) Vitrigel-Eye Irritation Test (EIT) method Report of the Peer Review Panel


**ANNEX 1**

**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 (24).

**Applicability domain:** A description of the physicochemical or other properties of the substances for which a test method is applicable for use (24).

**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)(3).

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

**Collagen vitrigel membrane (CVM):** A membrane composed of high density collagen fibrils modelling the connective tissues in vivo and is easily handled with tweezers. Also, it possesses excellent transparency and permeability of protein with high molecular weight and consequently provides an ideal cell culture scaffold (25-29).

**Effective surface area:** The bottom surface area of the CVM chamber.

**Eye irritation:** The production of changes in the eye, which are fully reversible, occurring after the exposure of the eye to a substance or mixture (1).

**False negative rate:** The proportion of all positive substances falsely identified by a test method as negative. It is one indicator of test method performance (24).

**False positive rate:** The proportion of all negative (non-active) substances that are falsely identified as positive. It is one indicator of test method performance (24).

**Hazard:** The potential for an adverse health or ecological effect. The adverse effect is
manifested only if there is an exposure of sufficient level (24).

**hCE**: human corneal epithelium.

**LogP**: Logarithm of the octanol-water partitioning coefficient.

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

**MoA**: mode of action.

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

**Mucin 1 (MUC1)**: A cell membrane spanning mucin families and expressed in the superficial layer of the corneal epithelium, which plays a protective role against the adherence of pathogens.

**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.

**Negative control**: A sample containing all components of a test system and treated with a substance known not to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples and is used to check the durability of the hCE models.

**Performance standards**: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (1) essential test method components; (2) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (3) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should
demonstrate when evaluated using the minimum list of reference chemicals (24).

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Reference control: A sample containing all components of a test system and treated with a substance known to induce a middle class response in the system. This sample is processed with test chemical-treated samples and other control samples and is used to check the quality of the hCE models.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (24).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (24).

Run: A run consists of one or more test chemicals tested concurrently with a negative control, a positive control and a reference control.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (24).

Serious eye damage: The production of tissue damage in the eye, or serious physical decay of vision, which is not fully reversible, occurring after exposure of the eye to a substance or mixture (1).

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (24).
**Substance**: Chemical elements and their compounds in the natural state or obtained by any production process, inducing any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

**Test chemical**: The term "test chemical" is used to refer to what is being tested.

**Tiered testing strategy**: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight of evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made (24).

**Top-Down Approach**: step-wise approach used for a test chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome) (3).

**Transepithelial electrical resistance (TEER)**: The electrical resistance of an epithelium or epithelial cell layers. It is considered a suitable means (index) for evaluating the integrity of the tight junction of corneal epithelium.

**United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS)**: A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

**UN GHS Category 1**: Serious eye damage/irreversible effects on the eye (1).

**UN GHS Category 2**: Eye irritation/reversible effects on the eye (1).

**UN GHS Category 2A**: Irritating to eyes (1).
UN GHS Category 2B: Mildly irritating to eyes (1).

UN GHS No Category: Chemicals that are not classified as UN GHS Category 1 or 2 (2A or 2B) (1).

UVCB: Substances of unknown or variable composition, complex reaction products or biological materials.

Zonula occludens-1 (ZO-1): A tight junction-related protein, associated with the principal barrier that separates the eye from the outside environment.
ANNEX 2

PROFICIENCY CHEMICALS FOR THE VITRIGEL-EYE IRRITANCY TEST METHOD

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the chemicals recommended in Table 1. The Vitrigel-Eye Irritancy Test Method outcomes provided represent examples of the results observed during its validation study (13). The selection includes, insofar as possible, chemicals that (i) cover the full range of in vivo serious eye damage/eye irritation responses based on the UN GHS classification system (i.e., Categories 1, 2A, 2B or No Category), (ii) are based on high quality results obtained in the reference in vivo rabbit eye test (OECD TG 405), (2) (iii) cover different physical states, (iv) cover a broad range of the chemical classes and organic functional groups, representative of those used in the validation study, (13) (v) cover the range of in vitro responses based on high quality Vitrigel-EIT data, (vi) produced correct and reproducible predictions in the VRM, (vii) are commercially available, and (viii) are not prohibitively expensive either to acquire or dispose of.

In situations where a listed chemical is unavailable or cannot be used for other justifiable reason, it should be substituted with another chemical that fulfills the criteria described above, e.g. from the chemicals used in the validation of the Vitrigel-Eye Irritancy Test Method or listed as a reference chemical within the Performance Standards. (OECD, 20XX)
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CASRN</th>
<th>Organic Functional Group</th>
<th>Physical State</th>
<th>Time lag (seconds)$^1$</th>
<th>Intensity (%/seconds)$^3$</th>
<th>Plateau level (%)$^3$</th>
<th>Prediction</th>
<th>pH</th>
<th>LogP</th>
<th>Density (g/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-[[2-(2-Aminoethylamino)]propytrimethoxysilane</td>
<td>1760-24-3</td>
<td>Silicon compound</td>
<td>Liquid</td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0.36 ± 0.04</td>
<td>0.33</td>
<td>0.41</td>
</tr>
<tr>
<td>2</td>
<td>Imidazole</td>
<td>288-32-4</td>
<td>Heterocyclics</td>
<td>Solid</td>
<td>87 ± 6</td>
<td>80</td>
<td>90</td>
<td>0.26 ± 0.04</td>
<td>0.24</td>
<td>0.31</td>
</tr>
<tr>
<td>In vivo UN GHS Category 2A³</td>
<td>Heterocyclic compounds, Ketones</td>
<td>Liquid</td>
<td>3 ± 6</td>
<td>0</td>
<td>10</td>
<td>0.22 ± 0.01</td>
<td>0.21</td>
<td>0.23</td>
<td>40 ± 3</td>
<td>37</td>
</tr>
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</tr>
<tr>
<td>Dibenzyl phosphate 1623-08-1</td>
<td>Organophosphorus compound</td>
<td>Solid</td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0.41 ± 0.10</td>
<td>0.32</td>
<td>0.51</td>
<td>62 ± 8</td>
<td>57</td>
</tr>
<tr>
<td>In vivo UN GHS Category 2B³</td>
<td>Alcohol</td>
<td>Liquid</td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0.32 ± 0.09</td>
<td>0.26</td>
<td>0.42</td>
<td>57 ± 15</td>
<td>48</td>
</tr>
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</tr>
<tr>
<td>6</td>
<td>Camphene</td>
<td>79-92-5</td>
<td>Hydrocarbons</td>
<td>Solid</td>
<td>$&gt;180$</td>
<td>160</td>
<td>$&gt;180$</td>
<td>0.01 ± 0.06</td>
<td>-0.03</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>In vivo UN GHS No Category¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>iso-Octyl acrylate</td>
<td>29590-42-9</td>
<td>Acrylates</td>
<td>Liquid</td>
<td>$&gt;180$</td>
<td>$&gt;180$</td>
<td>$&gt;180$</td>
<td>0.01 ± 0.01</td>
<td>-0.02</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>No Category</td>
<td>7</td>
<td>4.</td>
<td>0.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>iso-Octylthio glycolate</td>
<td>25103-09-7</td>
<td>Thiocompound, Ester</td>
<td>Liquid</td>
<td>$&gt;180$</td>
<td>$&gt;180$</td>
<td>$&gt;180$</td>
<td>0.01 ± 0.01</td>
<td>-0.02</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>No Category</td>
<td>7</td>
<td>4.</td>
<td>0.</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2,4-Pentanediol</td>
<td>625-69-4</td>
<td>Alcohols</td>
<td>Liquid</td>
<td>87 ± 67</td>
<td>10</td>
<td>130</td>
<td>0.10 ± 0.02</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>No Prediction Can Be Made</td>
<td>8</td>
<td>0.</td>
<td>35</td>
<td>0.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Gluconolactone</td>
<td>90-80-2</td>
<td>Lactone</td>
<td>Solid</td>
<td>0 ± 0</td>
<td>0</td>
<td>0</td>
<td>0.30 ± 0.04</td>
<td>0.26</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>No Prediction Can Be Made</td>
<td>6</td>
<td>-2.</td>
<td>48</td>
<td>1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CASRN, Chemical Abstracts Service Registry Number; UN GHS, United Nations Globally Harmonized System of Classification and Labelling of Chemicals; VRM, Validated Reference
Method.

1 Based on results obtained with validation Study of the Vitrigel-EIT method (13). Each score was calculated from the data of three runs.

2 When discordant results were obtained within and/or between laboratories in the validation study, the prediction of the VRM indicated in the table is based on the mode of all predictions. (See footnote 4.) False-positive and false-negative predictions from VRM are underlined.

3 Based on results from the in vivo rabbit eye test (OECD TG 405) (2) and using the UN GHS. (1, 2)

4 The VRM prediction is based on the mode of all predictions obtained in the validation study. One of the three laboratory predicted the chemical as No Prediction Can Be Made, other two laboratories predicted it as No Category.
ANNEX 3

SCHEMATIC ILLUSTRATIONS AND PHOTOGRAPHIC IMAGES OF THE TEER RECORDER

Figure A shows the electrode unit, Figure B the electrode unit applied to the culture media via HCE model, and Figure C the TEER recorder system.
ANNEX 4

GRAPH SHOWING AN ANALYSIS OF A TEER PROFILE AFTER EXPOSING OF A MODEL TO A TEST CHEMICALS

\[ t_1 \text{ (second)}; \text{The maximum time at which a profile is maintained at } 0 \geq \frac{dP}{dT} > -0.03\%/\text{second.} \]

\[ t_2 \text{ (second)}; \text{The initial time at which the profile is maintained at } 0 \geq \frac{dP}{dT} = \frac{(P_2 - P_1)}{(t_2 - t_1)} > -0.03\%/\text{second after the profile is maintained at } \frac{dP}{dT} \leq -0.03\%/\text{second.} \]

\[ t_3 \text{ (second)}; t_2 + 30 \text{ seconds because the plateau level is evaluated by the profile for 30 seconds.} \]

\[ P_1 \text{ (%)}; \text{The percentage of TEER value at } t_1 \text{ against the TEER value at 0 second.} \]

\[ P_2 \text{ (%)}; \text{The percentage of TEER value at } t_2 \text{ against the TEER value at 0 second.} \]

\[ P_3 \text{ (%)}; \text{The percentage of TEER value at } t_3 \text{ against the TEER value at 0 second.} \]

\[ \frac{dP}{dT}; \text{The derivative of P with respect to t.} \]