Section 4
Health effects

Test Guideline No. 494

Vitrigel®-Eye Irritancy Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Vitrigel®-Eye Irritancy Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage

INTRODUCTION

1. Serious eye damage refers to 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, 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, which are fully reversible, occurring after exposure of the eye to a substance or mixture. 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 (TG) 405, which was adopted in 1981 and revised in several occasions (2). The choice of the most appropriate test method and the use of this TG should be seen in the context of the OECD Guidance Document (GD) 263 on Integrated Approaches to Testing and Assessment (IATA) for Serious Eye Damage and Eye irritation (3). However, this method does not address the toxicity and reversibility aspects of ocular toxicity. Therefore, consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (3) when selecting the combination of tests to use for classification purposes.

3. The Vitrigel®-Eye Irritancy Test (EIT) method is an *in vitro* test method that allows the identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Category) as defined by the UN GHS (1) without further testing (4, 5, 6), and therefore is performed in a bottom-up approach, as suggested by Scott et al (7). However, the Vitrigel®-EIT method is not intended to identify nor differentiate between UN GHS Category 1 and UN GHS Category 2. This differentiation will need to be addressed by another tier of a testing strategy (3).

4. This TG is based on a protocol developed by Yamaguchi and Takezawa (8), which was subjected to a validation study by a validation management team (VMT) organized by the Japanese Centre for the Validation of Alternative Methods (JaCVAM) in cooperation with the International Collaboration on Alternative Test Methods (ICATM) (9). The validation study was carried-out by three participating Japanese laboratories. The validation report was evaluated by an independent peer-review panel composed of

international experts (10). Further, the OECD Expert Group concluded that the Vitrigel®-EIT method is valid for use as an initial step in a bottom-up approach for identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Cat. chemicals).

5. This TG describes a procedure for assessing the eye irritation 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 existing *in vitro* test methods, the viability of cells in culture *in vitro* or the corneal opacity of isolated eyeballs *ex vivo* have been utilized as an endpoint. It is known that chemicals that are irritating to the eye first destroy tear film and epithelial barrier function of the eye, subsequently induce epithelial cell death, and finally produce stromal degeneration and endothelial cell death, resulting in corneal opacity (11, 12). Therefore, the change of the epithelial barrier function is a relevant endpoint for detecting eye irritation (13, 14). In the Vitrigel®-EIT method, time-dependent changes in the Transepithelial Electrical Resistance (TEER) values are indicative of damage to the barrier function of the corneal epithelium following exposure to a test chemical; this situation is similar to the observed damage of the rabbit cornea following exposure to a test chemical, which is an important mode of action leading to damage of the corneal epithelium and eye irritation (4, 13).

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 chemicals. The term “test chemical preparation” is used to describe the mixture of the test chemical with a culture medium (see paragraph 27).

7. Definitions are provided in ANNEX 1 - .

PRINCIPLE OF THE TEST

8. The Vitrigel®-EIT method is an *in vitro* assay using hCE models fabricated in a collagen vitrigel® membrane (CVM) chamber (5). The eye irritation potential of the test chemical is predicted by analyzing time-dependent changes in TEER values using the score of three indexes (see the prediction model in Table 2).

9. The Vitrigel®-EIT method makes use of the destructive activity of the test chemical against the barrier function of hCE models as an endpoint to assess the extent of damage to the hCE model. The test chemical is dissolved or suspended in the culture medium before exposure of the hCE model to prevent a delay of the reaction due to slow dissolution. In a previous study it was observed that the TEER values of the hCE models decreased immediately after exposing the test chemical preparations and became constant within 3 minutes (4). Therefore, the exposure period of the hCE model to the test chemical preparation was limited to 3 minutes.

INITIAL CONSIDERATIONS AND LIMITATIONS

10. The Vitrigel®-EIT can be applied to test chemicals absorbing light in the same range as formazan dye, and test chemicals able to directly reduce the tetrazolium dye (see paragraph 16). However, test chemical preparations of both solids and liquids showing acidity (pH ≤ 5) and rapid phase separation are not in the applicability domain of the test method as explained below. When the absolute difference of the absorbance values of the 2.5% weight/volume (w/v) test chemical preparation at 0 and 3 minutes is greater than 0.1, the chemical should not be tested. See paragraph 28 for further details.

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 the developer's in-house data for 93 chemicals (9, 15, 16, 17). The Vitrigel®-EIT method achieved a sensitivity of 83% (50/60), a specificity of 70% (23/33), and an accuracy of 78% (73/93). The assay, as described in this paragraph, would not be the method of choice because of its limited predictivity (i.e., low specificity).

12. Analysis of the false-negative reactions showed that five of the 10 false-negative chemicals were acidic, and the 2.5% w/v preparations used for exposure had a pH level lower than five. Typically, the TEER values of the hCE model after exposure to UN GHS No Category chemicals changed little from their initial TEER values. The TEER values of the hCE models increased after exposure to the five acidic test chemicals that yielded false-negatives as previously reported (18, 19). Also, water-insoluble solids that readily separate from the culture medium may yield false-negative results. It should be noted that these chemicals lead to variable and extreme exposure conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (2). The Vitrigel®-EIT method can be applied not only to liquids but also to solids by performing a pre-test to exclude solid test chemical preparations showing pH ≤ 5 and/or rapid phase separation during the testing time of three minutes (see paragraph 28).

13. Following these considerations, 158 test chemicals comprising 94 liquids and 64 solids were tested. Among these 158 tested chemicals, 22 solids were excluded on the basis of the pre-test: 7 showed a pH≤ 5 (2 of which also showed rapid phase separation), and 15 showed rapid phase-separation only; 29 liquids were excluded: 5 showed acidity (2 of which also showed rapid phase- separation) and 24 showed rapid phase-separation only as well. Accounting for these exclusions, the sensitivity, specificity and accuracy of the assay for the 107 test chemicals remaining in-domain is 96% (51/53), 67% (36/54) and 81% (87/107), respectively. Table 1 shows the predictive capacity for the test liquids and solids separately.

Table 1. Predictive Capacity

Liquids	Solids
Sensitivity: 100% (34/34) Specificity: 71% (22/31) Accuracy: 86% (56/65)	Sensitivity: 89% (17/19) Specificity: 61% (14/23) Accuracy: 74% (31/42)

The two false negative chemicals were GHS Cat. 2B (mild eye irritants).

14. The test method shows a high percentage of false positive results. The false positive rates obtained with the Vitrigel®-EIT method are not critical in the context of this TG since all test chemicals that result in "No Stand-alone Prediction Can Be Made" will require further information and/or testing, depending on regulatory requirements, according to the OECD GD 263 (3). The appropriate regulatory authorities should be consulted before using the test method under classification schemes other than UN GHS.

15. A limitation of this TG is that it does not allow discrimination between eye irritation/reversible effects on the eye (UN GHS Category 2) and serious eye damage/irreversible effects on the eye (UN GHS Category 1), nor between eye irritants (optional Category 2A) and mild eye irritants (optional Category 2B), as defined by UN GHS (1). For these purposes, further testing with other *in vitro* test methods is required

(3).

16. This TG is technically applicable to mono-constituent substances, multi-constituent substances, substances of unknown or variable composition, complex reaction products or biological materials (UVCBs). Mixtures, gases and aerosols, however, have not been assessed in the validation study. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable or water reactive), or test chemicals not clearly within the applicability domain described in this TG , upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Test chemicals absorbing light in the same range as formazan dye and test chemicals able to directly reduce the tetrazolium dye MTT can also be tested using the Vitrigel®-EIT method.

17. Any test chemical satisfying the criteria of the pre-test described above (i.e., test chemical preparations showing pH > 5 and keeping dissolution or homogeneous dispersion for at least three minutes) in a 2.5% w/v concentration in culture medium can be tested with the Vitrigel®-EIT method. 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, and/or c) heating to a maximum temperature of 70°C (See PROCEDURE).

DEMONSTRATION OF PROFICIENCY

18. Prior to routine use of the Vitrigel®-EIT method described in this TG, laboratories should demonstrate technical proficiency by correctly classifying the 10 substances recommended in Table 32 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 TG 405 on *in vivo* rabbit eye tests (2) and the UN GHS classification system (1). Other selection criteria stipulated that the proficiency substances should be commercially available, with high-quality *in vivo* reference data and high-quality *in vitro* data from the Vitrigel®-EIT method available (9, 10). In situations where a listed substance is unavailable or cannot be used for other justifiable reason, it should be substituted with another proficiency substance for which adequate *in vivo* and *in vitro* reference data are available.

PROCEDURE

19. The following paragraphs describe the main components and procedures of the Vitrigel®-EIT method, being referred as the Validated Reference Method (VRM)(8). Testing should be performed in accordance with Good In Vitro Method Practices (20). 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

20. Immortalized hCE cells[1] are maintained in a culture medium comprising a 1:1

[1] HCE-T cells, RCB no. 2280 obtained from RIKEN BioResource Research Center, Tsukuba, Japan. MTA with RIKEN BRC are required to perform this Test Guideline.

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□ in air. The cells should be free of contamination by bacteria, viruses, mycoplasma, and fungi except for the application of test chemical preparations to hCE models.

Preparation of CVM chambers

21. A collagen xerogel membrane chamber (i.e. ad-MED Vitrigel®, as used for the VRM[2]) 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 and waiting for 10 minutes to allow the xerogel to convert into vitrigel® immediately before use. If alternative chambers are used, hCE models prepared in the chamber should show the appropriate TEER values.

Fabrication of a hCE model

Culture procedure

22. The culture medium outside the chamber in the well of the 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 1.2×10^5 cells/mL is poured onto the CVM in the chamber and cultured for two 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 four more days at the air-liquid interface to obtain the hCE model. On the third day of culture at the air-liquid interface, the medium outside the chamber is changed.

Quality check

23. 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. 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. For the VRM, hCE models with a TEER value between 140 $\Omega \cdot cm^2$ and 220 $\Omega \cdot cm^2$ are acceptable for the testing.

Measurement of TEER value in a hCE model

24. 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

[2] ad-MED Vitrigel® (Kanto Chemical Co., Inc., Tokyo, Japan.)

0.1–3 kΩ. Photographic images of the TEER measuring system for the VRM[3] are shown in Annex 3. If an alternative apparatus is used, it should be demonstrated that it produces the same results. The electrode unit is 23 mm in diameter and 35 mm in height. 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 is fixed, 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 is also fixed. The electrical resistance value of the hCE model cultured in the CVM chamber (Rmodel) and that of a blank empty CVM chamber (Rblank) are measured. The TEER value of a hCE model is calculated as follows:

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

25. 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. For the VRM, pre-operation check of the TEER Measuring System is performed as follows. The individual CVM-free chamber (ad-MED Vitrigel® without a CVM) is set in two wells of a 12-well plate, and subsequently one well is filled with 3.0 mL of 0.90% NaCl aqueous solution and another well is filled with 0.45% NaCl aqueous solution at 25±5°C. Then, the TEER values in both wells are measured using the TEER Measuring System. The TEER measurement is functioning normally when the measured TEER values satisfy the following conditions.

$$\text{(TEER value of 0.45\% NaCl aqueous solution)}$$
$$- \text{(TEER value of 0.90\% NaCl aqueous solution)} \geq 60\ \Omega \cdot \text{cm}^2$$

Preparation of Control Substances

26. The Vitrigel®-EIT method uses saline as a negative control, benzalkonium chloride as a positive control, and ethanol which induces a response in the medium range 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% w/v 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. In addition, 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.

[3] e.g., TEER Measuring System (Kanto Chemical Co., Inc., Tokyo, Japan.)

Preparation of Test Chemicals

27. A test chemical solution or suspension is prepared in the culture medium at a concentration of 2.5% w/v, as the electrical resistance value of 2.5% w/v preparation usually has little or no effect on the electrical resistance of the culture medium irrespective of the test chemical 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, one of the following techniques is used, 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 preparation 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 taken only once the test chemical preparation is well dissolved or homogeneously dispersed. For test chemicals that prove to be insoluble or immiscible using the above techniques, the test chemical preparation is prepared as a homogeneous suspension by vortexing the test chemical in the medium for up to one minute immediately before use.

28. The pH of each 2.5% w/v test chemical preparation is measured using a universal pH test paper covering a range from pH 1 to 11 or a pH meter. If pH of a 2.5% w/v preparation is ≤ 5 the chemical should not be tested. Also, absorbance at 660 nm of the 2.5% w/v test chemical preparation is measured by a UV-VIS spectrophotometer at 0 and 3 minutes after the preparation. However, a different wavelength has to be used in case the absorption of the test chemical at this wavelength disturbs the measurement. In the case where the absolute difference measured is above 0.1, the chemical should not be tested.

Application of the Test Chemicals and Control Substances

29. The 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 two hours after drawing it from a CO_2 incubator. The medium inside the chamber is replaced with 500 µL of test chemical preparation, and Rmodel values are measured at intervals of 10 seconds for a period of three minutes after exposure to the test chemical preparation. At least three hCE models should be used for each control substance solution and each test chemical preparation in each run. The eye irritation potential of the test chemical is predicted using the result of one run.

30. To ensure reproducibility, it is essential that measurements begin between two to five seconds after adding the test chemical preparation. A minimum of a two-second wait before beginning measurements is necessary, because the liquid around the electrode is often unstable for up to two seconds after adding the test chemical preparation. However, it should begin before 5 seconds after adding the test chemical preparation, as the TEER value of the hCE model changes in the presence of the test chemical for over five seconds.

31. The temperature of the hCE models and the test chemical preparations 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

32. 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 (t1), intensity (− [P2 − P1] / [t2 − t1]), and plateau level (100 − P2). Annex 4 provides a graph showing an analysis of a TEER profile after exposure of an hCE model to a test chemical. The 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 2. In this case, no further testing with another test method is considered necessary. If the scores of the indexes are Time lag ≤ 180 seconds or Intensity ≥ 0.05 %/seconds or Plateau level > 5.0 %, then no stand-alone prediction can be made from this result in isolation, as shown in Table 2. This is because in case of a true positive, the method cannot resolve between UN GHS Categories 1 and 2. Furthermore, the Vitrigel®-EIT method shows a high percentage of false positive results (see paragraph 14). In both cases, further information and or testing will be required for classification purposes according to GD 263 (3).

Table 2. Prediction Models according to UN GHS classification*

Criteria	Prediction
Time lag ≤ 180 seconds or Intensity ≥0.05 %/seconds or Plateau level >5.0 %	No Stand-alone Prediction Can Be Made[1]
Time lag > 180 seconds and Intensity <0.05 %/seconds and Plateau level ≤ 5.0 %	No Category[2]

Notes:
[1]No Stand-alone Prediction Can Be Made corresponds to chemicals that require further information for classification purposes according to the IATA guidance document (3).
[2]No Category corresponds to chemicals that do not require classification for serious eye damage or eye irritation according to UN GHS
* Consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (3), when deriving a classification.

Acceptance Criteria

33. Test run is judged to be acceptable when the following four 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 ≤ 15%.

The range of historical results for the positive control in the validation study is from 65% to 90%.

DATA AND REPORTING

Data

34. 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

35. 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 possible
- Physical state, pH, volatility, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent possible
- Purity, chemical identity of impurities as appropriate and practically feasible, etc.
- Treatment prior to testing, if applicable (e.g., warming)
- Storage conditions and stability to the extent possible

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 measuring system
- 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 preparation, and absorbance values at 660 nm values at 0 and 3 minutes of the test chemical preparation)
- Temperature of the hCE models and test chemical preparation at the start of exposure test

- Lot number of a hCE model
- Time of removal of the hCE model from CO_2 incubator, and exposure of the hCE model to the test chemical
- 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)
- Number of runs and number of hCE models used within each run (if different than 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 preexposure TEER values and time dependent TEER values after exposing test chemicals for three minutes for each hCE model used, scores of three indexes, and the *in vitro* prediction of the test chemical.
- Description of other effects observed

Discussion of the Results

Conclusions

LITERATURE

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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 (21).

Applicability domain: A description of the physicochemical or other properties of the chemicals for which a test method is applicable for use (21).

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 (22-26). The CVM chamber is prepared from a commercially available collagen xerogel membrane chamber.

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 chemicals falsely identified by a test method as negative. It is one indicator of test method performance (21).

False positive rate: The proportion of all negative (non-active) chemicals that are falsely identified as positive. It is one indicator of test method performance (21).

Hazard: The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level (21).

hCE: human corneal epithelium.

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).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 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 (i) essential test method components; (ii) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) 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 (21).

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 (21).

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 (21).

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 (21).

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 (21).

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 stepwise sequential animal testing procedure is performed until an unequivocal classification can be made (21).

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.

Vitrigel: The ad-MED Vitrigel® collagen xerogel membrane chamber was used for the VRM. The definition of vitrigel is a gel in a stable state produced by rehydration after the vitrification of a traditional hydrogel (22, 23, 24, 25, 26). The literal element for “VITRIGEL” is a trademark of National Agriculture and Food Research Organization (NARO) and registered in Japan, USA, EU and China.

VRM: Validated Reference Method.

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 10 chemicals recommended in Table 3. The Vitrigel®-Eye Irritancy Test Method outcomes provided represent examples of the results observed during its validation study (9, 10). 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)

(iv) cover a broad range of the chemical classes and organic functional groups, representative of those used in the validation study, (10)

(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 (27).

Table 3. Recommended chemicals for demonstrating technical proficiency with the Vitrigel®-Eye Irritancy Test Method

1) CASRN and physicochemical properties

	Chemical Name	CASRN	Organic Functional Group	Physical State	pH
***In vivo* UN GHS Category 1**[1]					
1	3-(2-Aminoethylamino) propyl]trimethoxysilane	1760-24-3	Silicon compound	Liquid	10
2	Tetraethylene glycol diacrylate	17831-71-9	Acrylate, Ester	Liquid	7
3	Sodium salicylate	54-21-7	Organic salts	Solid	7
***In vivo* UN GHS Category 2A**[1]					
4	Cyclopentanol	96-41-3	Alcohols	Liquid	7
5	Methyl cyanoacetate	105-34-0	Esters, Nitrile compounds	Solid	7
***In vivo* UN GHS Category 2B**[1]					
6	Ethyl-2-methylacetoacetate	609-14-3	Esters	Liquid	7
7	Ammonium nitrate	6484-52-2	Inorganic salts	Solid	8
***In vivo* UN GHS No Category**[1]					
8	*iso*-Octylthioglycolate	25103-09-7	Thiocompound, Ester	Liquid	7
9	Tetrabromobisphenol A	79-94-7	Aryl; Aryl halide Phenol	Solid	7
10	4,4'-Sulfonylbisbenzenamide	80-08-0	Dianilines, Sulfone	Solid	7

2) Test results

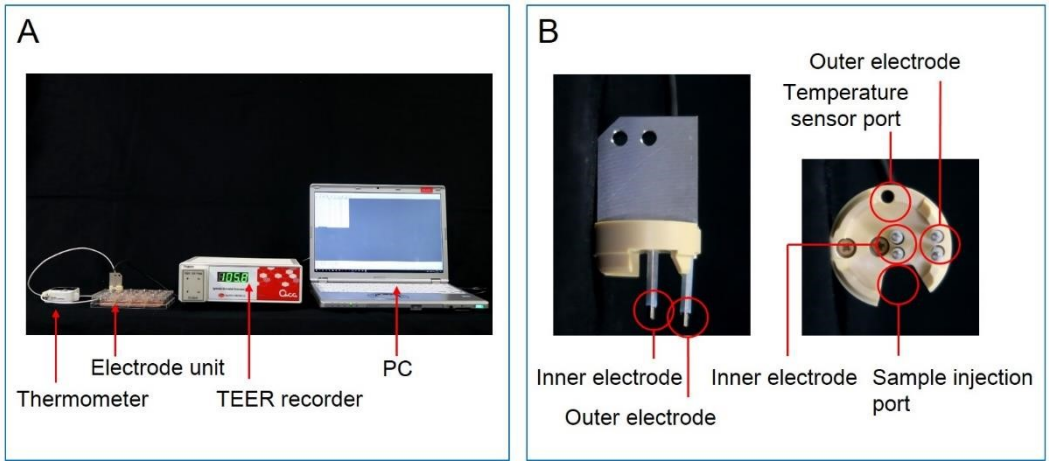
	Chemical Name	Time lag (seconds)[2]			Intensity (%/seconds)[2]			Plateau level (%)[2]			Prediction
		Mean± SD	Min.	Max	Mean± SD	Min.	Max	Mean± SD	Min.	Max	
***In vivo* UN GHS Category 1[1]**											
1	3-(2-Aminoethylamino) propyltrimethoxysilane	0 ± 0	0	0	0.36 ± 0.05	0.33	0.41	64 ± 8	60	73	No Stand-alone Prediction Can Be Made
2	Tetraethylene glycol diacrylate	7 ± 12	0	20	0.23 ± 0.02	0.20	0.24	40 ± 5	35	43	No Stand-alone Prediction Can Be Made
3	Sodium salicylate	0 ± 0	0	0	0.48 ± 0.11	0.35	0.54	41 ± 3	38	43	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS Category 2A[1]**											
4	Cyclopentanol	0 ± 0	0	0	0.29 ± 0.01	0.28	0.30	52 ± 2	51	55	No Stand-alone Prediction Can Be Made
5	Methyl cyanoacetate	10 ± 10	0	20	0.10 ± 0.06	0.06	0.17	18 ± 11	11	30	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS Category 2B[1]**											
6	Ethyl-2-methylacetoacetate	3 ± 6	0	10	0.22 ± 0.03	0.19	0.25	41 ± 6	34	46	No Stand-alone Prediction Can Be Made
7	Ammonium nitrate	0 ± 0	0	0	0.69 ± 0.12	0.58	0.82	29 ± 17	18	49	No Stand-alone Prediction Can Be Made
***In vivo* UN GHS No Category[1]**											
8	*iso*-Octylthioglycolate	>180	>180	>180	-0.01 ± 0.01	-0.02	0.00	1 ± 1	0	2	No Category
9	Tetrabromobisphenol A	>180	>180	>180	-0.02 ± 0.01	-0.02	-0.01	0 ± 0	0	0	No Category
10	4,4'-Sulfonylbis benzenamide	>180	>180	>180	-0.02 ± 0.01	-0.03	-0.01	0 ± 0	0	0	No Category

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 from the *in vivo* rabbit eye test (OECD TG 405) (2) and using the UN GHS. (1)

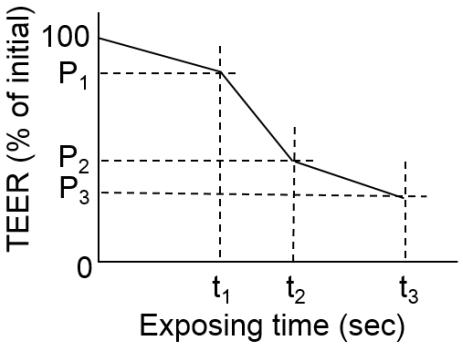
[2] Based on results obtained with validation Study of the Vitrigel®-EIT method (9,10). Data for chemical No. 5, 7, 9 and 10 are based on results obtained with the developer's in-house data. Each score was calculated from the data of three runs. Three hCE models were used for each run.

ANNEX 3 - PHOTOGRAPHIC IMAGES OF THE TEER MEASURING SYSTEM



The TEER measuring system (A) and the electrode unit (B).

ANNEX 4 - GRAPH SHOWING AN ANALYSIS OF A TEER PROFILE AFTER EXPOSURE OF A MODEL TO A TEST CHEMICAL



Time lag (second) = t_1

Intensity (% / second)= $-(P_2 - P_1)/(t_2 - t_1)$

Plateau level (%)= $100 - P_2$

Notes:

t1 (second); The maximum time at which a profile is maintained at 0 ≥ dP/dT > -0.03%/second.
t2 (second); The initial time at which the profile is maintained at 0 ≥ dP (P3 - P2)/dT (t3 - t2) > −0.03%/second after the profile is maintained at dP/dT ≤ -0.03%/second.
t3 (second); t2 + 30 seconds because the plateau level is evaluated by the profile for 30 seconds.
P1 (%); The percentage of TEER value at t1 against the TEER value at 0 second.
P2 (%); The percentage of TEER value at t2 against the TEER value at 0 second.
P3 (%); The percentage of TEER value at t3 against the TEER value at 0 second.
dP/dT; The derivative of P with respect to t.