DRAFT 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 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 typically 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) Both the choice of an appropriate test method and the use of this Test Guideline (TG) should be considered in the context of the OECD Guidance Document on an Integrated Approach on Testing and Assessment for Serious Eye Damage and Eye Irritation. (39)

3. This Test Guideline describes an *in vitro* procedure for identification of chemicals (substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. The Vitrigel-Eye Irritancy Test (EIT) method is an in vitro eye irritation test method that can distinguish chemicals that induce serious eye damage or eye irritation from those that do not induce either, as defined by the UN GHS. (1)

4. 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 of a plurality of test methods have been proposed for

use in place of the Draize test. (3) Test Guidelines adopted by the OECD include No. 437: 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, FL, and STE test methods are considered useful in a top-down approach to distinguishing Category 1 from No Category chemicals per the UN GHS, but only the RhCE test method is considered useful in a bottom-up approach to distinguishing Category 1 and Category 2 chemicals from No Category chemicals.

5. The Vitrigel-EIT method is intended to distinguish chemicals that induce either serious eye damage (Category 1) or eye irritation (Category 2) from those that do not require classification for eye irritation or serious eye damage (No Category) without further testing. (9, 10, 11) It is not, however, intended to distinguish Category 1 chemicals from Category 2 chemicals, and to do so would require the addition of another tier to the test strategy.

6. 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 human corneal epithelium (HCE) models used in the Vitrigel-EIT method. Time-dependent changes in TEER values following exposure of the HCE model to a test chemical is an important mode of action (MOA) leading to damage of a corneal epithelium and eye irritation. The Vitrigel-EIT method involves analysis of time-dependent changes in TEER values using three indexes, and eye irritation potential of the test chemical is predicted according to the score of each index according to predetermined criteria.

7. 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.

8. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. This TG is based on a protocol developed by Yamaguchi and Takezawa, (12) 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).

(13) 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 demonstrated reproducibility and predictive capacity suitable for use in predicting eye irritation potential. (14)

10. The results of the validation study showed within-laboratory reproducibility to be 80–100% at all three laboratories and an excellent between-laboratory reproducibility of 92%. The predictive capacity was evaluated based on in-house data for 114 test chemicals. (13) The test chemicals were selected to ensure that a diverse range of substances were represented in terms of eye-irritant level per UN GHS categories, physical state, and chemical class. The 114 test chemicals comprised 89 liquids and 25 solids, including 62 eye irritant chemicals (i.e., UN-GHS Category 1, Category 2A, or Category 2B) and 52 non-irritant chemicals (i.e., UN-GHS No Category). Of these 114 test chemicals, 75 were predicted to be irritants and 39 were predicted to be non-irritants. Furthermore, results for 85 of the 114 test chemicals matched their UN GHS category. In contrast, 8 of the 62 of test chemicals classified as irritants by in vivo data were identified as non-irritants, a false-negative rate of 13%. Additionally, 21 of the 52 test chemicals classified as non-irritants under UN GHS were identified as irritants, a false-positive rate of 40%. Thus, the Vitrigel-EIT method achieved a sensitivity of 87%, a specificity of 60%, and an accuracy of 75%.

11. 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. The TEER values of the HCE models after exposure to these five acidic test chemicals that yielded false-negatives increased from their initial values. Interestingly, 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. (15, 16) On the other hand, two of the five non-acidic false-negative chemicals were water-insoluble solids that were easily separated from the culture medium at room temperature. Based on the above, two restrictions to the applicability domain were stipulated:

- All chemicals that have a pH level of 5 or less in solution are excluded from the

applicability domain.

- All solids that have both a logP value of 2.5 or more and a density of either less than 0.95 g/cm^3 or over 1.10 g/cm^3 are excluded from the applicability domain.

Under this applicability domain, 12 of the original 114 test chemicals were excluded, which improved sensitivity from 87 to 98%, specificity from 60 to 61%, and accuracy from 75 to 79%.

12. Any chemical 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).

PRINCIPLE OF THE TEST

13. The Vitrigel-EIT test method is an in vitro assay using the HCE models fabricated in a collagen vitrigel membrane 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.

14. 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. (17) 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 the HCE models as an endpoint to assess the extent of damage to the HCE model during a three-minute exposure to the test chemical.

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 1in 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 stipulate 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. (13) 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 (12). The following paragraphs describe the main components and procedures of the Vitrigel-EIT method.

Culture of HCE-T cells

17. An SV40-immortalized HCE cell strain (HCE-T cells, RCB no. 2280) is obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells 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.

Preparation of collagen vitrigel membrane chambers

18. A collagen xerogel membrane chamber (ad-MED Vitrigel™) is purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The collagen xerogel membrane chamber is set in the well of a 12-well plate and immersed in culture medium by pouring 1.5 mL outside and 0.5 mL inside the chamber in the well for 10 min to convert the xerogel into vitrigel immediately before use.

Fabrication of a human corneal epithelium model

19. The culture medium outside the chamber in the well of a 12-well plate is replaced with 1.5 mL of a fresh medium. The medium inside the chamber is removed and 0.5 mL of a cell suspension in a culture medium at a density of 1.2×10^5 cells/mL is poured onto the CVM in the chamber and cultured for 2 days at 37°C. Subsequently, the cells are cultured for 4 days at the air–liquid interface to fabricate the HCE model after removing the inside medium and changing the outside medium to a fresh medium. The medium outside the chamber is changed on the third day of culture at the air–liquid interface.

The quality of the HCE models is checked as follows. First, 500 μL of a 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 human corneal epithelium 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 is measured. Only HCE models with a TEER value between 140 $\Omega \cdot cm^2$ and 220 $\Omega \cdot cm^2$ are acceptable for the following chemical exposure test performed on the same day.

Measurement of TEER value in a human corneal epithelium model

20. An ad-MED TEER Recorder, an apparatus for measuring TEER value using the CVM chamber is purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The electrical resistance value of the 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 the HCE model is calculated as follows:

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

The pre-operation check of the ad-MED TEER Recorder is performed as follows. The individual CVM-free chamber (ad-MED Vitrigel without a CVM) is set for the 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 ad-MED TEER Recorder. The TEER measurement is functioning normally when the measured TEER values satisfy the following conditions.

(TEER value of 0.45% NaCl aqueous solution) − (TEER value of 0.9% NaCl aqueous solution) $\geq 60\ \Omega \cdot cm^2$

Preparation of Control Substances

21. The Vitrigel-EIT method uses saline as a negative control, benzalkonium-chloride as a positive control, and ethanol as a reference control. 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

22. A test chemical solution is prepared in the culture medium at a concentration of 2.5% (weight/volume), and each test chemical solution should be suitable for measuring TEER values without undue influence from the electrical resistance of the test chemical itself. 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 as 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, and the solubility of the test chemical is checked. Move on to the next step only if 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 level of each 2.5% test chemical solution is measured using universal pH test paper from ADVANTEC (Tokyo, Japan).

Application of the Test Chemicals and Control Substances

23. The HCE models that pass the quality check are suitable for use in a chemical exposure experiment. The medium inside the chamber is replaced with 500 μL of a test chemical solution, and R_{model} values are measured at intervals of 10 seconds for a period of 3 min after exposure to the test chemical solution. Three runs are made for each test chemical and the new HCE model is used in each test.
    To ensure reproducibility, it is essential that measurements begin between 2 to 5 s 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 s after adding the test chemical solution. Also, the HCE model has already been exposed to the test chemical for over 5 s.
    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 confirmed by measuring the actual temperature of culture medium outside the HCE model.

Calculating eye irritancy of test chemicals

24. 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 human corneal epithelium 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 ($100 - P_2$). Time lag ($t_1$) is defined as the maximum time at which a profile is maintained at $0 \geq dP/dT > -0.03\%$/second. The starting time of plateau level (t_2) is defined as the initial time at which the profile is maintained at $0 \geq dP\ (P_3 - P_2)/dT\ (t_3 - t_2) > -0.03\%$/s after the profile is maintained at $dP/dT \leq -0.03\%$/second for a particular period. The time (t_3) is represented in the equation ($t_3 = t_2 + 30$ seconds) because the plateau level is evaluated by the profile for 30 seconds. P_1, P_2, and P_3 are the percentages against the initial TEER value at t_1, t_2, and t_3 after exposure to the test chemical. Subsequently, the eye irritation potential of a test chemical is predicted to be either irritant or non-irritant in accordance with the criteria for each index shown in Table 3.

Table 3. Eye irritancy criteria.

Criteria	Prediction
Time lag ≤ 180 or Intensity ≥ 0.05 or Plateau level > 5.0	Irritant (I)
Time lag > 180 and Intensity < 0.05 and Plateau level ≤ 5.0	Non-irritant (NI)

Acceptance Criteria

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

a) Negative control: The plateau level is 5% or less of the TEER value at 0 seconds.

b) Positive control: The plateau level is 40% or more of the TEER value at 0 seconds.

c) Reference control: The plateau level is 10% or more of the TEER value at 0 seconds.

DATA AND REPORTING

Data

25. TEER values for each individual HCE model measured during each repetition as well as the scores of each index and the final prediction by the Vitrigel-EIT method should be reported.

Test Report

The test report should include the following information:

Test Chemical and Control Substances

- Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers
- Physical state, volatility, pH, LogP, molecular weight, density, 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
- Duration of each step for preparation of the 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
- Duration of exposure to test chemical solution for each HCE model
- Number of repetitions and replicates used (if different than the ones recommended)
- 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
- Demonstration of proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or demonstration of reproducible performance of the test method over time

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 min for each independent repetition, scores of three indexes, and the final judgment of eye irritancy of each test chemical
- Description of other effects observed

Discussion of the Results

Conclusions

LITERATURE

1) United Nations UN (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1. Available at: [http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html].

2) OECD. (2012). OECD Guideline for The Testing Chemicals Acute Eye Irritation/Corrosion No. 405. Organisation for Economic Cooperation and Development, Paris.

3) Scott L, et al. (2010). A proposed Eye Irritation Testing Strategy to Reduce and Replace in vivo Studies Using Bottom-Up and Top-Down Approaches. Toxicol. In Vitro 24, 1-9.

4) OECD. (2013). Guidelines for Testing of Chemicals No. 437. Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage. Organisation for Economic Cooperation and Development, Paris.

5) OECD. (2013). Guideline for Testing of Chemicals No. 438, Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification. Organisation for Economic Cooperation and Development, Paris.

6) OECD (2012). Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guideline for Testing of Chemicals No. 460. Organisation for Economic Co-operation and Development, Paris.

7) OECD (2015). 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. OECD Guideline for Testing of Chemicals No. 491. Organisation for Economic Co-operation and Development, Paris.

8) OECD (2015). Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. OECD Guideline for Testing of Chemicals No. 492. Organisation for Economic Co-operation and Development, Paris.

9) Takezawa T, et al. (2011). Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals. Toxicol in Vitro 25, 1237–1241.

10) Yamaguchi H, et al. (2013). Vitrigel-eye irritancy test method using HCE-T cells. Toxicol Sci 135, 347–355.

11) Yamaguchi H, et al. (2016). Predictive performance of the Vitrigel-eye irritancy test method using 118 chemicals. J Appl Toxicol 36, 1025-1037.

12) Standard Protocol for the Vitrigel-EIT method, Version 1.80e. (April 02, 2015).

13) 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

14) Peer review report

15) Farré R, et al. (2008). Short exposure of oesophageal mucosa to bile acids, both in acidic and weakly acidic conditions, can impair mucosal integrity and provoke dilated intercellular spaces. Gut 57, 1366-1374.

16) Oshima, et al. (2012). Acid modulates the squamous epithelial barrier function by modulating the localization of claudins in the superficial layers. Lab. Invest 92, 22-31.

17) Mikkelson TJ, et al. (1973). Altered Bioavailability of Drugs in the Eye Due to Drug-Protein Interaction. J. Pharm. Sci.1648-1653.

18) OECD (2005), Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Environmental, Health and Safety Publications, Series on Testing and Assessment (No. 34.). Organisation for Economic Cooperation and Development, Paris.

19) Takezawa T, et al. (2004). Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. Cell Transplant 13, 463–473.

20) Takezawa T, et al. (2007). Reconstruction of hard connective tissue utilizing a pressed silk sheet and type-I collagen as the scaffold for fibroblasts. Tissue Engineering 13, 1357–1366.

21) Takezawa T, et al. (2007). A protein-permeable scaffold of a collagen vitrigel membrane useful for reconstructing crosstalk models between two different cell types. Cells Tissues Organs 185, 237–241.

22) Takezawa T, et al. (2007). Collagen vitrigel membrane useful for paracrine assays in vitro and drug delivery systems in vivo," Journal of Biotechnology 131, 76–83.

23) Takezawa T, et al. (2008). A novel culture model of rabbit corneal epithelium utilizing a handy scaffold of collagen vitrigel membrane and its cryopreservation," Alternatives to Animal Testing and Experimentation 13, 176.

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. (18)

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.

Collagen vitrigel membrane: A membrane composed of high density collagen fibrils equivalent to 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 the various studies utilizing it as a cell culture scaffold advances so well. (19-23)

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.

LogP: Logarithm of the octanol-water partitioning coefficient

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 determine 100%

tissue viability.

Not Classified: Chemicals that are not classified for Eye irritation (UN GHS Category 2, 2A, or 2B) or Serious eye damage (UN GHS Category 1). Interchangeable with "UN GHS No Category."

Performance standards: Standards, based on a validated test method which was considered scientifically valid, 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. (16)

Positive control: A replicate 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.

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. (17)

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. (23)

Sensitivity: The proportion of all positive/active chemicals 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. (17)

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with "irreversible effects on the eye"

and with UN GHS Category 1. (1)

Specificity: The proportion of all negative/inactive chemicals 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. (23)

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 it 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.

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).

Transepithelial electrical resistance: electrical resistance of an epithelium or an epithelial cell layer(s). it is known as a suitable method 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: See “Serious eye damage”.

UN GHS Category 2: See “Eye irritation”.

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

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 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 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 1: Recommended chemicals for demonstrating technical proficiency with the Vitrigel-Eye Irritancy Test Method

		Result in VRM[1]								
	Chemical Name	CASRN	Organic Functional Group	Physical State	Time lag (s)	Intensity (%/s)	Plateau level (%)	Prediction[2]	AD1 (Acidic)	AD2 (insoluble)
	In vivo category 1[3]									
1	3-（2-Aminoethylaminno）propyltrimethoxysilane	1760-24-3	Silicon compound	Liquid	0	0.41	73	1/2A/2B	No	No
2	Imidazole	288-32-4	Hetrocyclics	Solid	80	0.33	33	1/2A/2B	No	No
	In vivo category 2A[3]									

3	gamma-Butyrolactone	96-48-0	Heterocyclic compounds, Ketones	Liquid	10	0.23	42	1/2A/2B	No	No
4	Dibenzyl phosphate	1623-08-1	Organophosphorus compound	Solid	0	0.39	59	1/2A/2B	Yes	No
	In vivo category 2B[3]									
5	2-Methyl-1-pentanol	105-30-6	Alcohols	Liquid	0	0.26	48	1/2A/2B	No	No
6	Camphene	79-92-5	Hydrocarbons	Solid	>180	-0.03	0	No category[4]	No	No
	In vivo no category[3]									
7	*iso*-Octyl acrylate	29590-42-9	Acrylates	Liquid	>180	-0.01	0	No category	No	No
8	*iso*-Octylthioglycolate	25103-09-7	Thiocompound, Ester	Liquid	>180	-0.02	0	No category	No	No
9	2,4-Pentanediol	625-69-4	Alcohols	Liquid	130	0.12	8	1/2A/2B	No	No
10	Gluconolactone	90-80-2	Lactone	Solid	0	0.31	9	1/2A/2B	Yes	No

Abbreviations: CASRN, Chemical Abstracts Service Registry Number; UN GHS, United Nations Globally Harmonized System of Classification and Labelling of Chemicals; VRM, Validated Reference Method; AD1, Applicability domain 1, i.e., Exclude all test chemicals that have a pH level of 5 or less in solution; AD2, Applicability domain 2., i.e., Exclude all solids that have both a logP value of 2.5 or more and a density of either less than 0.95 g/cm^3 or over 1.10 g/cm^3.

[1] Based on results obtained with validation Study of the Vitrigel-EIT method) (13).

[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. Discordant results obtained in one of three laboratories.