OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

Reconstructed Human Cornea-like Epithelium (RhCE) 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, following application of a test chemical to the anterior surface of the eye, 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 following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Test chemicals inducing serious eye damage are classified as UN GHS Category 1, while those inducing eye irritation are classified as UN GHS Category 2. Test chemicals not classified for eye irritation or serious eye damage are defined as those that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B) i.e., they are referred to as UN GHS No Category.

2. The assessment of serious eye damage/eye irritation has typically involved the use of laboratory animals (OECD Test Guideline 405 (TG 405); adopted in 1981 and revised in 1987, 2002 and 2012) (2). In relation to animal welfare concerns, TG 405 recommends the use of a sequential testing strategy for the determination of the serious eye damage/eye irritation potential of chemicals. This testing strategy is described in a Supplement to the Guideline and includes the use of validated and accepted *in vitro* test methods, thus decreasing or avoiding pain and suffering of animals.

3. This Test Guideline describes an *in vitro* procedure allowing the identification of chemicals (substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. It makes use of reconstructed human cornea-like epithelium (RhCE) which closely mimics the histological, morphological, biochemical and physiological properties of the human corneal epithelium. Three other *in vitro* test methods have been validated and adopted as OECD Test Guidelines 437 (3), 438 (4) and 460 (5) to address the human health endpoint serious eye damage/eye irritation.

4. The only *in vitro* test method currently covered by this Test Guideline is the EpiOcular™ Eye Irritation Test (EIT), which makes use of a commercially available RhCE model. The EpiOcular™ Eye Irritation Test (EIT) was validated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Cosmetics Europe between 2008 and 2013 (6)(7)(8)(9)(10). From this validation study and its independent peer review it was concluded that the EpiOcular™ EIT is able to correctly identify chemicals (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS (1), and the test method was recommended as scientifically valid for that purpose (11). The EpiOcular™ EIT is thus referred to as the Validated Reference Method (VRM) in the present Test Guideline.

5. It is currently generally accepted that, in the foreseeable future, no single *in vitro* test method will be able to replace the *in vivo* Draize eye test (12) to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. However, strategic combinations of several alternative test methods within ( tiered) testing strategies such as the Bottom-Up/Top-Down approach may be able to replace the Draize eye test (13). The Bottom-Up approach (13) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification,
while the Top-Down approach (13) is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT is recommended to identify chemicals that do not require classification for eye irritation or serious eye damage according to UN GHS (UN GHS No Category) (1) without further testing, 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 (13). However, the EpiOcular™ EIT is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation). This differentiation will need to be addressed by another tier of a test strategy (13). A chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT will thus require additional testing (in vitro and/or in vivo) to establish a definitive classification. The EpiOcular™ EIT is therefore not considered valid as a stand-alone replacement for the in vivo Draize rabbit eye test.

6. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in a RhCE model, as measured by the MTT assay (14) (see paragraph 21). The viability of the RhCE tissue following exposure to a test chemical is determined in comparison to tissues treated with the negative control substance (% viability), and is then used to predict the eye hazard potential of the test chemical.

7. Performance Standards (15) are available to facilitate the validation of new or modified in vitro RhCE-based test methods similar to EpiOcular™ EIT, in accordance with the principles of Guidance Document No. 34 (16), and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the Performance Standards, if these test methods have been reviewed and included in this Test Guideline by the OECD.

DEFINITIONS

8. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. This Test Guideline is based on a commercial RhCE-model reconstructed from primary human cells i.e., EpiOcular™ OCL-200, representing in vitro the target organ of the species of interest (17). It directly covers an important mechanistic step determining the overall in vivo serious eye damage/eye irritation response of a chemical upon ocular exposure, i.e., cell and tissue damage resulting in localised trauma.

10. A wide range of chemicals, covering a large variety of chemical types, chemical classes, molecular weights, LogPs, chemical structures, etc., has been tested in the validation study underlying this Test Guideline. The EpiOcular™ EIT validation database contained 112 chemicals in total, covering 95 different organic functional groups according to an OECD QSAR toolbox analysis (7)(8). The majority of these chemicals represented single-constituent substances, but several multi-constituent substances (including 3 homopolymers, 5 copolymers and 10 quasi polymers) were also included in the study. In terms of physical state and UN GHS Categories, the 112 tested chemicals distributed as follows: 13 Category 1 liquids, 14 Category 1 solids, 6 Category 2A liquids, 10 Category 2A solids, 7 Category 2B liquids, 7 Category 2B solids, 27 No Category liquids and 28 Category 1 solids (7)(8).

11. This Test Guideline is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed in a validation study. While it is conceivable that these can be tested using RhCE technology, the current Test Guideline does not allow testing of gases and
12. Test chemicals absorbing light in the same range as MTT formazan (intrinsically or after contact with water or isopropanol) and test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the tissue viability measurements and need the use of adapted controls for corrections (see paragraphs 36-40). For intensely coloured test chemicals strongly interfering with the MTT assay an alternative procedure to measure MTT formazan employing HPLC/UPLC-photometry, instead of the standard absorbance (Optical Density, OD) measurement, may be employed (18) (see paragraph 41 and 42).

13. Results generated in pre-validation (19) and full validation (7)(9)(10) studies have demonstrated that EpiOcular™ EIT is transferable to laboratories considered to be naive in the conduct of the assay and also to be reproducible within- and between laboratories. Based on these studies, the level of reproducibility in terms of concordance of predictions that can be expected from EpiOcular™ EIT is in the order of 95% within laboratories and 93% between laboratories.

14. The EpiOcular™ EIT can be used to identify chemicals that do not require classification for eye irritation or serious eye damage according to the UN GHS classification system (1). When used for this purpose, the EpiOcular™ EIT has an overall accuracy of 80% (82% based on 53 liquid chemicals and 78% based on 59 solid chemicals), sensitivity of 96% (98% based on 26 liquid chemicals and 94% based on 31 solid chemicals), false negative rate of 4% (2% based on 26 liquid chemicals and 6% based on 31 solid chemicals), specificity of 63% (65% based on 27 liquid chemicals and 61% based on 28 solid chemicals) and false positive rate of 37% (35% based on 27 liquid chemicals and 39% based on 28 solid chemicals), when compared to in vivo rabbit eye test data classified according to the UN GHS classification system (7). The false positive rate obtained (i.e., in vivo UN GHS No Category chemicals producing a mean percent tissue viability ≤ 60%, which are therefore predicted by EpiOcular™ EIT as requiring classification and labelling; see paragraph 44) is not critical in the context of this Test Guideline since all test chemicals that produce a tissue viability ≤ 60% will require further testing with other adequately valid in vitro test methods, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. This test method can be used for all types of chemicals, whereby a negative result (tissue viability > 60%) should be accepted for not classifying a chemical for eye irritation and serious eye damage (UN GHS No Category). The appropriate regulatory authorities should be consulted before using the EpiOcular™ EIT under classification schemes other than UN GHS.

15. A limitation of this Test Guideline is that it does not allow discrimination between eye irritation/reversible effects on the eye (Category 2) and serious eye damage/irreversible effects on the eye (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 suitable test methods may be required.

16. The validation study demonstrated that EpiOcular™ EIT is able to detect all types of ocular effects observed in vivo (i.e., corneal, iridal and conjunctival injuries). In this respect, it should be noted that effects on the iris are of lesser importance for classification of chemicals according to UN GHS, since iritis on its own rarely drives the UN GHS classification of chemicals in vivo (both Category 1 and Category 2) (1.8-3.1% of the chemicals). In fact, test chemical that cause classifiable effects to the iris also almost always cause classifiable corneal opacity (20).

17. Liquid test chemicals that are positive in EpiOcular™ EIT (i.e., that produce a tissue viability ≤ 60%) and have LogP > 2.5 should be carefully analysed as they may correspond to false positive predictions. For such test chemicals, additional testing should be considered using another in vitro test method able to identify chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category) rather than using an in vitro test method able to identify chemicals inducing serious eye damage (UN GHS Category 1) as is normally suggested in a Bottom-Up approach (13).
18. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the DPRA to the testing of substances and/or mixtures.

**PRINCIPLE OF THE TEST**

19. The test chemical is applied topically to a minimum of two three-dimensional RhCE tissues and tissue viability is measured following exposure and a post-treatment incubation period. The RhCE tissues are reconstructed from primary human cells, which have been cultured for several days to form a stratified, highly differentiated squamous epithelium morphologically similar to that found in the human cornea. The EpiOcular™ RhCE model consists of at least 3 viable layers of cells and a non-keratinized surface, showing a cornea-like structure analogous to that found in vivo.

20. Chemical-induced serious eye damage/eye irritation, manifested in vivo mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, is the result of a cascade of events beginning with penetration of the chemical through the cornea and production of damage to the cells. Cell damage can occur by several modes of action, including: cell membrane lysis (e.g., by surfactants, organic solvents); coagulation of macromolecules (particularly proteins) (e.g., by surfactants, organic solvents, alkalis and acids); saponification of lipids (e.g., by alkalis); and alkylation or other covalent interactions with macromolecules (e.g., by bleaches, peroxides and alkylators) (13)(21)(22). However, it has been shown that cytotoxicity plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical regardless of the physicochemical processes underlying tissue damage (23)(24). Moreover, the serious eye damage/eye irritation potential of a chemical is principally determined by the extent of initial injury (25), which correlates with the extent of cell death (23) and with the extent of the subsequent responses and eventual outcomes (26). Thus, slight irritants generally only affect the superficial corneal epithelium, the mild and moderate irritants damage principally the epithelium and superficial stroma and the severe irritants damage the epithelium, deep stroma and at times the corneal endothelium (24)(27). The measurement of viability of the EpiOcular™ RhCE tissue after topical exposure to a test chemical to discriminate chemicals not requiring classification for serious eye damage/eye irritation (UN GHS No Category) from those requiring classification and labelling (UN GHS Categories 1 and 2) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium.

21. RhCE tissue viability in EpiOcular™ EIT is measured by enzymatic conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS number 298-93-1], into a blue MTT formazan salt that is quantitatively measured after extraction from tissues (14). Chemicals not requiring classification and labelling according to UN GHS are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 60%, for UN GHS No Category).

**DEMONSTRATION OF PROFICIENCY**

22. Prior to routine use of EpiOcular™ EIT for regulatory purposes, laboratories should demonstrate technical proficiency by correctly predicting the fifteen proficiency chemicals listed in Table 1. These substances were selected to represent the range of responses for eye hazards based on results in the in vivo rabbit eye test (TG 405) (2)(12) and the UN GHS classification system (i.e., Categories 1, 2A, 2B, or No Category) (1). Other selection criteria were that substances were commercially available, high quality in vivo reference data were available, and high quality EpiOcular™ EIT in vitro data were available.

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1 In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CASRN</th>
<th>Organic Functional Group</th>
<th>Physical State</th>
<th>VRM viability (%)</th>
<th>VRM Prediction</th>
<th>MTT Reducer</th>
<th>Colour interf.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vivo Category 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylthioglycolate</td>
<td>2365-48-2</td>
<td>Carboxylic acid ester;</td>
<td>L</td>
<td>10.9±6.4</td>
<td>Cat 2 / Cat 1</td>
<td>Y (strong)</td>
<td>N</td>
</tr>
<tr>
<td>Tetraethylene glycol diacrylate</td>
<td>17831-71-9</td>
<td>Acrylate; Ether</td>
<td>L</td>
<td>34.9±15.3</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2,5-Dimethyl-2,5-hexanediol</td>
<td>110-03-2</td>
<td>Alcohol</td>
<td>S</td>
<td>2.3±0.2</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Sodium oxalate</td>
<td>62-76-0</td>
<td>Oxocarboxylic acid</td>
<td>S</td>
<td>29.0±1.2</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>In Vivo Category 2A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2,4,11,13-Tetraazatetradecane-</td>
<td>18472-51-0</td>
<td>Aromatic heterocyclic</td>
<td>L</td>
<td>4.0±1.1</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>Y (weak)</td>
</tr>
<tr>
<td>diimidamide, N,N''-bis(4-chlorophenyl)-</td>
<td></td>
<td>halide; Aryl halide;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3,12-diamo-, di-D-glucanate</td>
<td></td>
<td>Dihydroxyl group;</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(20%, aqueous)</td>
<td></td>
<td>Guanidine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1,5-Naphthalenediol</td>
<td>83-56-7</td>
<td>Fused carbocyclic</td>
<td>S</td>
<td>21.0±7.4</td>
<td>Cat 2 / Cat 1</td>
<td>Y (medium)</td>
<td>N</td>
</tr>
<tr>
<td>aromatic; Naphthalene; Phenol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>In Vivo Category 2B</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Diethyl toluamide</td>
<td>134-62-3</td>
<td>Benzamide</td>
<td>L</td>
<td>15.6±6.3</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2,2-Dimethyl-3-methylenebicylec</td>
<td>79-92-5</td>
<td>Alkane, branched with</td>
<td>S</td>
<td>4.7±1.5</td>
<td>Cat 2 / Cat 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>[2.2.1] heptane</td>
<td></td>
<td>tertiary carbon;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alkene; Bicycloheptane;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bridged-ring carbocycles;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cycloalkane</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>In Vivo No Category</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dipropyl disulphide</td>
<td>629-19-6</td>
<td>Disulfide</td>
<td>L</td>
<td>81.7±6.4</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>51-03-6</td>
<td>Alkoxy; Benzdioxole;</td>
<td>L</td>
<td>104.2±4.2</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1-Ethyl-3-methylimidazolium</td>
<td>342573-75-5</td>
<td>Alkoxy; Ammonium salt;</td>
<td>L</td>
<td>79.9±6.4</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>ethysulphate</td>
<td></td>
<td>Aryl; Imidazole;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol (PEG-40)</td>
<td>61788-85-0</td>
<td>Acylal; Alcohol;</td>
<td>Viscous</td>
<td>77.6±5.4</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>hydrogenated castor oil</td>
<td></td>
<td>Allyl; Ether</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium tetrafluoroborate</td>
<td>14075-53-7</td>
<td>Inorganic Salt</td>
<td>S</td>
<td>88.6±3.3</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
Table 1. Example of chemicals included in the validation study

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CASRN</th>
<th>Functional Group</th>
<th>Physical State</th>
<th>VRM viability (%)</th>
<th>VRM Prediction</th>
<th>MTT Reducer</th>
<th>Colour interf.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea</td>
<td>101-20-2</td>
<td>Aromatic heterocyclic halide; Aryl halide; Urea derivatives</td>
<td>S</td>
<td>106.7±5.3</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)</td>
<td>103597-45-1</td>
<td>Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl</td>
<td>S</td>
<td>102.7±13.4</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (1); VRM = Validated Reference Method, i.e., EpiOcular™ EIT; Colour interf. = colour interference with the MTT assay.

These chemicals were selected from the chemicals used in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (7)(8). The selection includes, to the extent possible, chemicals that: (i) cover the full range of in vivo serious eye damage/eye irritation responses; (ii) are representative of the chemical classes used in the validation study; (iii) have chemical structures that are well-defined; (iv) induce reproducible results in EpiOcular™ EIT; (v) are correctly predicted by EpiOcular™ EIT; (vi) cover the full range of in vitro responses (0 to 100% viability); (vii) are commercially available; and (viii) are not associated with prohibitive disposal costs.

2. Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (8).

3. Based on results from the in vivo rabbit eye test (OECD TG 405) (2)(12) and using the UN GHS (1).

4. Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The in vivo study included 3 animals. All endpoints apart from corneal opacity in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 (at day 7) that fully recovered at day 9.

23. As part of the proficiency testing, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhCE model producer. This is particularly important if tissues are shipped over long distance / time periods. Once a test method has been successfully established and proficiency in its use has been acquired and demonstrated, such verification will not be necessary on a routine basis. However, when using a test method routinely, it is recommended to continue to assess the barrier properties at regular intervals.

PROCEDURE

24. The only test method currently covered by this Test Guideline is the scientifically valid EpiOcular™ EIT (7)(11), referred to as the Validated Reference Method (VRM). The Standard Operating Procedure (SOP) for the EpiOcular™ EIT is available and should be employed when implementing and using the test method in a laboratory (28). The following paragraphs describe the main components and procedures of the EpiOcular™ EIT.

RHCE TEST METHOD COMPONENTS

General conditions

25. Relevant human-derived cells (e.g., human corneal epithelial cells or keratinocytes) should be used
to reconstruct the corneal epithelium-like three-dimensional tissue, which should be composed of progressively stratified but not cornified cells. The RhCE tissue is prepared in inserts with a porous synthetic membrane through which nutrients can pass to the cells. Multiple layers of viable, non-keratinized epithelial cells should be present in the reconstructed corneal epithelium. The RhCE tissue should have the epithelial surface in the direct contact with the air so as to allow for direct topical exposure of test chemicals in a fashion similar to how the corneal epithelium would be exposed in vivo. The RhCE tissue should form a functional barrier with sufficient robustness to resist rapid penetration of cytotoxic benchmark substances, e.g., Triton X-100. The barrier function should be demonstrated and may be assessed by determination of the exposure time required to reduce tissue viability by 50% (ET<sub>50</sub>) upon application of the benchmark substance at a specified, fixed concentration. The containment properties of the RhCE model should prevent the passage of test chemical around the edge of the viable tissue, which could lead to poor modelling of corneal exposure. The RhCE model should be free of contamination by bacteria, viruses, mycoplasma, and fungi.

**Functional conditions**

**Viability**

26. The assay used for determining the magnitude of tissue viability is the MTT assay (14). The vital dye MTT is reduced into a blue MTT formazan precipitate by the viable cells of the RhCE tissue, which is then extracted from the tissue using isopropanol (or a similar solvent). The OD of the extraction solvent alone should be sufficiently small, i.e., OD < 0.1. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-photometry procedure (18). The RhCE model users should ensure that each batch of the RhCE model used meets defined criteria for the negative control. An acceptability range (upper and lower limit) for the negative control OD values (in the test method conditions) are established by the RhCE model developer/supplier. Acceptability ranges for the VRM are given in Table 2. It should be documented that the tissues treated with the negative control substance are stable in culture (provide similar tissue viability measurements) for the duration of the test exposure period.

<table>
<thead>
<tr>
<th>Test Method</th>
<th>Lower acceptance limit</th>
<th>Upper acceptance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpiOcular™ EIT (OCL-200) – VRM (for both the liquids and the solids protocols)</td>
<td>&gt; 0.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&lt; 2.5</td>
</tr>
</tbody>
</table>

<sup>1</sup>This lower acceptance limit already considers extended shipping time (e.g., > 4 days). For tissues shipped to the US and Europe the negative control OD values are expected to be > 1.0.

**Barrier function**

27. The RhCE tissue should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, e.g., Triton X-100, as estimated by e.g., ET<sub>50</sub> (Table 3).

**Morphology**

28. Histological examination of the RhCE model should be provided demonstrating human corneal epithelium-like structure (including at least 3 layers of viable epithelial cells and a non-keratinized surface).

**Reproducibility**

7
29. The results of the positive and negative controls of the test method should demonstrate reproducibility over time.

Quality control (QC)

30. The RhCE model should only be used if the developer/supplier demonstrates that each batch of the RhCE model meets defined production release criteria, among which those for viability (see paragraph 26), barrier function (see paragraph 27) and morphology (see paragraph 28) are the most relevant. These data should be provided to the test method users so that they are able to include this information in the test report. An acceptability range (upper and lower limit) for the ET<sub>50</sub> should be established by the RhCE model developer/supplier. Only results produced with qualified tissues can be accepted for reliable prediction of chemicals not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. The acceptability ranges for the VRM are given in Table 3.

<table>
<thead>
<tr>
<th>Test Method</th>
<th>Lower acceptance limit</th>
<th>Upper acceptance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpiOcular™ EIT (OCL-200) - VRM (100 µL of 0.3% (v/v) Triton X-100)</td>
<td>ET&lt;sub&gt;50&lt;/sub&gt; = 12.2 min</td>
<td>ET&lt;sub&gt;50&lt;/sub&gt; = 37.5 min</td>
</tr>
</tbody>
</table>

Application of the Test Chemical and Control Substances

31. At least two tissue replicates should be used for each test chemical and each control substance in each run. Before exposure to test chemicals or a control substances, the tissue surface of the VRM is pretreated with 20 µL of calcium and magnesium-free Dulbecco’s Phosphate Buffered Saline (Ca<sup>2+</sup>/Mg<sup>2+</sup>-free DPBS) and incubated in the dark at 37±1°C in a humidified atmosphere of 5±1% CO<sub>2</sub> in air (standard culture conditions) for 30±2 minutes to mimic the wet conditions of human eye. After this 30±2 minutes pre-treatment, treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. According to the test procedure, it is essential that the tissue viability measurements are not made immediately after exposure to the test chemical, but rather after a sufficiently long post-exposure incubation period (in fresh medium) after the test chemical has been rinsed from the tissue. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects. Two different treatment protocols are used, one for liquid test chemicals and one for solid test chemicals (28). In either case, a sufficient amount of test chemical or control substance should be applied to uniformly cover the epidermis surface while avoiding an infinite dose.

32. Test chemicals that can be pipetted at 37°C or lower temperatures (using a positive displacement pipette, if needed) are treated as liquids in the VRM. Viscous, waxy, resinous and gel-like test chemicals with unclear physical state should be incubated at 37±1°C for 15±1 minutes before deciding which treatment protocol to use. If such test chemicals become pipettable after this incubation period (using a positive displacement pipette, if necessary), they should be treated as liquids and should be applied to the tissues directly from the water bath (at 37±1°C), otherwise they should be treated as solids (see paragraph 33). 50 µL of liquid test chemical are evenly spread over the 0.6 cm<sup>2</sup> of the tissue surface (83.3 µL/cm<sup>2</sup> application). Tissues treated with liquid test chemicals and with control substances tested concurrently to liquid test chemicals are incubated for 30±2 minutes at standard culture conditions. At the end of the exposure period, the test chemical should be carefully removed from the tissue surface by extensive rinsing with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free DPBS at room temperature. This rinsing step is followed by a 12±2 minutes post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) and a 120±15 minutes post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28).
33. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the VRM. Approximately 50 mg of solid test chemical are evenly applied over the 0.6 cm² of the tissue surface using a calibrated tool (approximately 83.3 mg/cm² application). Whenever possible, solids should be tested as a fine powder. Tissues treated with solid test chemicals and with control substances tested concurrently to solid test chemicals are incubated for 6±0.25 hours at standard culture conditions. At the end of the exposure period, the test chemical should be carefully removed from the tissue surface by extensive rinsing with Ca²⁺/Mg²⁺-free DPBS at room temperature. This rinsing step is followed by a 25±2 minutes post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) and an 18±0.25 hours post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28).

34. Concurrent negative and positive controls should be included in each run to demonstrate that the viability (determined with the negative control), the barrier function and the resulting tissue sensitivity (determined with the positive control) of the tissues are within defined historical acceptance ranges. The concurrent negative control also provides the baseline (100% tissue viability) to calculate the relative percent viability of the tissues treated with the test chemical. The suggested positive control substance is neat methyl acetate (CAS No. 79-20-9; Sigma-Aldrich, Cat# 186325; liquid). The suggested negative control substance is ultrapure H₂O. Separate negative and positive controls should be tested concurrently to each liquid and solid test chemical. For controls performed concurrently to liquid test chemicals, 50 μL of the negative control and positive control substance should be applied to the tissues exactly as for the liquid test chemicals followed a 30±2 minutes exposure at standard culture conditions, rinsing, a 12±2 minutes post-exposure immersion in fresh medium at room temperature and a 120±15 minutes post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay. For controls performed concurrently to solid test chemicals, 50 μL of the negative control and positive control substance should be applied to the tissues (as described for the liquid test chemicals) followed a 6±0.25 hours exposure at standard culture conditions, rinsing, a 25±2 minutes post-exposure immersion in fresh medium at room temperature and an 18±0.25 hours post-exposure incubation in fresh medium at standard culture conditions, prior to performing the MTT assay (28).

Tissue Viability Measurements

35. The MTT assay is a validated quantitative method (14) that should be used to measure tissue viability under this Test Guideline. It is compatible with use in a three-dimensional tissue construct. The MTT assay is performed immediately following the post-exposure incubation period. In the VRM, the RhCE tissue sample is placed in 0.3 mL of MTT solution at 1 mg/mL for 180±10 minutes at standard culture conditions. The vital dye MTT is reduced into a blue MTT formazan precipitate by the viable cells of the RhCE tissue. The precipitated blue MTT formazan product is then extracted from the tissue using 2 mL of isopropanol (or a similar solvent). Tissues tested with liquid test chemicals should be extracted from both the top and the bottom of the tissues, while tissues tested with solid test chemicals and coloured liquids should be extracted from the bottom of the tissue only (to minimise any potential contamination of the isopropanol extraction solution with any test chemical that may have remained on the tissue). The corresponding negative and positive controls should be treated similarly to the test chemical. The extracted MTT formazan may be quantified either by a standard absorbance (OD) measurement at 570 nm using a filter band pass of maximum ± 30 nm or by using an HPLC/UPLC-photometry procedure (see paragraph 42) (18).

36. Optical properties of the test chemical or its chemical action on MTT may interfere with the MTT assay leading to a false estimate of tissue viability. Test chemicals may interfere with the MTT assay by direct reduction of the MTT into blue MTT formazan and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as MTT formazan (i.e., around 570 nm). Additional controls should be used to detect and correct for potential interference from such test
chemicals (see paragraphs 37-41). This is especially important when a specific test chemical is not completely removed from the RhCE tissue by rinsing or when it penetrates the corneal epithelium and is therefore present in the RhCE tissues when the MTT assay is performed. A detailed description of how to correct for direct MTT reduction and interferences by colouring agents is available in the VRM SOP (28). For coloured test chemicals or test chemicals that become coloured in contact with water or isopropanol, which are not compatible with the standard absorbance (OD) measurement due to too strong interference with the MTT assay, the alternative HPLC/UPLC-photometry procedure to measure MTT formazan may be employed (see paragraphs 41 and 42) (18).

37. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. In the VRM, 50 µL or 50 mg of test chemical are added to 1 mL of 1 mg/mL MTT solution and the mixture is incubated for approximately 3 hours at standard culture conditions (28). 50 µL of sterile deionized water in MTT solution is used as negative control. If the MTT mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhCE tissues should be performed. This additional functional check employs killed tissues that possess no metabolic activity but absorb and bind the test chemical in a similar way as viable tissues. Each MTT reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure (28). A single non-specific MTT reduction (NSMTT) control is sufficient per test chemical regardless of the number of independent tests/runs performed. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

38. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol and decide on the need for additional controls, spectral analysis of a test chemical in water (environment during exposure) and/or isopropanol (extraction solvent) should be performed. In the VMR, 50 µL or 50 mg of test chemical are added to (i) 1 mL of water and incubated for approximately 1 hour at standard culture conditions and/or (ii) 2 mL of isopropanol and incubated for 2-3 hours at room temperature. If the test chemical in water and/or isopropanol absorbs sufficient light in the range of 570±30 nm (for the VRM, if the OD of the test chemical solution is > 0.08 after subtraction of the OD for isopropanol or water, which corresponds to approximately 5% of the mean OD of the negative control), the test chemical is presumed to interfere with the MTT assay and further colorant controls should be performed. Each interfering test chemical is applied on at least two viable tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. An independent non-specific colour in living tissues (NSC_{living}) control needs to be performed concurrently per coloured test chemical (in each run) due to the inherent biological variability of living tissues. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSC_{living}).

39. Test chemicals that are identified as producing both colour interference (see paragraph 38) and direct MTT reduction (see paragraph 37) will also require a third set of controls, apart from the NSMTT and NSC_{living} controls described in the previous paragraphs. This is usually the case with darkly coloured test chemicals interfering with the MTT assay (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 37. This forces the use of NSMTT controls, by default, together with the NSC_{living} Controls. Test chemicals for which both NSMTT and NSC_{living} controls are performed may bind to both living and killed tissues. Therefore, in this case, the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the binding of the test chemical to killed tissues. This could lead to double correction for colour interference since the NSC_{living} control already corrects for colour interference.
arising from the binding of the test chemical to living tissues. Still, both controls are required because the

test chemical may not necessarily bind in the same amount and with the same strength to living and killed
tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour

in killed tissues (NSC\textsubscript{killed}) needs to be performed. In this additional control, the test chemical is applied on

at least two killed tissue replicates, which undergo the entire testing procedure but are incubated with

medium instead of MTT solution during the MTT incubation step. A single NSC\textsubscript{killed} control is sufficient

per test chemical regardless of the number of independent tests/runs performed, but should be performed

concurrently to the NSMTT control and with the same tissue batch. True tissue viability is calculated as:

the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT minus
%NS\textsubscript{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test

chemical and incubated with medium without MTT, calculated relative to the negative control ran

concurrently to the test being corrected (%NS\textsubscript{killed}).

40. It is important to note that non-specific MTT reduction and non-specific colour interferences may

increase the OD of the tissue extract above the linearity range of the spectrophotometer. On this basis, it is

important for each laboratory to determine the OD linearity range of their spectrophotometer with e.g.,

MTT formazan (CAS \# 57360-69-7), commercially available from Sigma-Aldrich (Cat\# M2003), before

initiating the testing of test chemicals for regulatory purposes.

41. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess
direct MTT-reducers and colour interfering test chemicals, when the observed interference with the

measurement of MTT formazan is not too strong (i.e., the ODs of the tissue extracts obtained with the test
chemical without any correction for direct MTT reduction and/or colour interference are within the linear
range of the spectrophotometer) or when the uncorrected percent viability obtained with the test chemical is
already \( \leq 60\% \) (see paragraph 44). Nevertheless, results for test chemicals producing %NSMTT and/or
%NS\textsubscript{living} \( \geq 60\% \) of the negative control should be taken with caution. Standard absorbance (OD) can
however not be measured when the interference with the measurement of MTT formazan is too strong (i.e.,
leading to uncorrected ODs falling outside of the linear range of the spectrophotometer) and the
uncorrected percent viability obtained with the test chemical is \( > 60\% \). Coloured test chemicals or test
chemicals that become coloured in contact with water or isopropanol that interfere too strongly with the

MTT-reduction assay may still be assessed using HPLC/UPLC-photometry instead of standard absorbance
(OD). This is because the HPLC/UPLC system allows for the separation of the MTT formazan from the
chemical before its quantification (18). For this reason, NSC\textsubscript{living} or NSC\textsubscript{killed} controls are never required
when using HPLC/UPLC-photometry, independently of the chemical being tested. NSMTT controls should
nevertheless be used if the test chemical is suspected to directly reduce MTT (following the procedure
described in paragraph 37). NSMTT controls should also be used with test chemicals having a colour
(intrinsic or appearing when in water) that impedes the assessment of their capacity to directly reduce MTT
as described in paragraph 37. When using HPLC/UPLC-photometry to measure MTT formazan, the
percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues
exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative
control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as: the percent
tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT.

42. HPLC/UPLC-photometry may be used with all types of test chemicals (coloured, non-coloured,
MTT-reducers and non-MTT reducers) for measurement of MTT formazan (18). Due to the diversity of
HPLC/UPLC-photometry systems, it is not feasible for each user to establish the exact same system

conditions. As such, qualification of the HPLC/UPLC-photometry system should be demonstrated before
its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a minimum set
of standard qualification parameters described in the U.S. Food and Drug Administration guidance for
industry on bioanalytical method validation (18)(29). These key parameters and their acceptance criteria
are shown in Table 4. Once the acceptance criteria defined in Table 4 have been met, the HPLC/UPLC-
photometry system is considered qualified and ready to measure MTT formazan under the experimental
conditions described in this Test Guideline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protocol Derived from FDA Guidance (18)(29)</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity</td>
<td>Analysis of isopropanol, living blank (isopropanol extract from living RhCE tissues without any treatment), dead blank (isopropanol extract from killed RhCE tissues without any treatment), and of a dye (e.g., methylene blue)</td>
<td>$\text{Area}<em>{\text{interference}} \leq 20% \text{ of } \text{Area}</em>{\text{LLOQ}}$</td>
</tr>
<tr>
<td>Precision</td>
<td>Quality Controls (i.e., MTT formazan at 1.6 µg/mL, 16 µg/mL and 160 µg/mL in isopropanol (n=5))</td>
<td>CV $\leq 15%$ or $\leq 20%$ for LLOQ</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Quality Controls in isopropanol (n=5)</td>
<td>$%\text{Dev} \leq 15% \text{ or } \leq 20% \text{ for LLOQ}$</td>
</tr>
<tr>
<td>Matrix Effect</td>
<td>Quality Controls in living blank (n=5)</td>
<td>85% $\leq \text{ME%} \leq 115%$</td>
</tr>
<tr>
<td>Carryover</td>
<td>Analysis of isopropanol after an ULOQ$^2$ standard</td>
<td>$\text{Area}<em>{\text{interference}} \leq 20% \text{ of } \text{Area}</em>{\text{ULOQ}}$</td>
</tr>
<tr>
<td>Reproducibility (intra-day)</td>
<td>3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ, i.e., 200 µg/mL) Quality Controls in isopropanol (n=5)</td>
<td>Calibration Curves: $%\text{Dev} \leq 15% \text{ or } \leq 20% \text{ for LLOQ}$</td>
</tr>
<tr>
<td>Reproducibility (inter-day)</td>
<td>Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3)</td>
<td>Quality Controls: $%\text{Dev} \leq 15%$ and CV $\leq 15%$</td>
</tr>
<tr>
<td></td>
<td>Day 2: 1 calibration curve and Quality Controls in isopropanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3: 1 calibration curve and Quality Controls in isopropanol</td>
<td></td>
</tr>
<tr>
<td>Short Term Stability of MTT Formazan in RhCE Tissue Extract</td>
<td>Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature</td>
<td>$%\text{Dev} \leq 15%$</td>
</tr>
<tr>
<td>Long Term Stability of MTT Formazan in RhCE Tissue Extract, if required</td>
<td>Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C</td>
<td>$%\text{Dev} \leq 15%$</td>
</tr>
</tbody>
</table>

$^1$LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability, i.e., 0.8 µg/mL.

$^2$ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls (~70 µg/mL in the VRM), i.e., 200 µg/mL.

Acceptance Criteria

For each run using EpiOcular™ tissue batches that met the quality control (see paragraph 30), tissues treated with the negative control substance should exhibit OD reflecting the quality of the tissues that followed shipment, receipt steps and all protocol processes and should not be outside the historically established boundaries described in Table 2 (see paragraph 26). Similarly, tissues treated with the positive control substance, i.e., methyl acetate, should show a mean tissue viability $< 50\%$ (relative to the negative control) with either the liquids' or the solids' protocols, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (28). The variability between tissue replicates of test chemicals and control substances should fall within the accepted limits (i.e., the difference of viability between two tissue replicates should be less than 20% or the SD between three tissue replicates should not exceed 18%). If either the negative control or positive control included in a run fall out of the accepted ranges, the run is considered as not qualified and should be repeated. If the variability between tissue replicates of a test chemical falls outside of the accepted range, the test chemical should be re-tested.
Interpretation of Results and Prediction Model

44. The OD values obtained with the replicate tissue extracts for each test chemical should be used to calculate the mean percent tissue viability (mean between tissue replicates) normalised to the negative control, which is set at 100%. The percentage tissue viability cut-off value distinguishing classified from non-classified test chemicals is 60%. Results should thus be interpreted as follows:

- The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (> ) 60%. In this case no further testing in other test methods is required.

- The test chemical is identified as potentially requiring classification and labelling according to UN GHS (Category 2 or Category 1) if the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) to 60%. When the final mean percent tissue viability is less than or equal (≤) to 60% further testing with other test methods will be required because EpiOcular™ EIT shows a certain number of false positive results (see paragraph 14) and cannot resolve between UN GHS Categories 1 and 2 (see paragraph 15).

45. A single test composed of at least two tissue replicates should be sufficient for a test chemical when the result is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent tissue viability equal to 60±5%, a second test should be considered, as well as a third one in case of discordant results between the first two tests.

DATA AND REPORTING

Data

46. Data from individual replicate tissues in a run (e.g., OD values/MTT formazan peak areas and calculated percent tissue viability data for the test chemical and controls, and the final EpiOcular™ EIT prediction) should be reported in tabular form for each test chemical, including data from repeat tests, as appropriate. In addition mean percent tissue viability ± Diff (if n=2 replicate tissues) or ± SD (if n=3 replicate tissues) for each individual test chemical and control should be reported. Any observed interferences of a test chemical with the MTT assay through direct MTT reduction and/or coloured interference should be reported for each tested chemical.

Test Report

47. The test report should include the following information:

Test Chemical

- 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;
  - Physical state, volatility, pH, LogP, 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.

- Multi-constituent substance, UVCB and mixture
  - Characterisation 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, 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.

Positive and Negative 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, 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;
- Reference to historical positive and negative control results demonstrating suitable run acceptance criteria.

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility and study director.

RhCE Model and Protocol Used (providing rationale for the choices, if applicable)

Test Method Conditions

- RhCE model used, including batch number;
- Calibration information for measuring device (e.g., spectrophotometer), wavelength and band pass (if applicable) used for measuring OD, and OD linearity range of measuring device;
- Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-photometry system, if applicable;
- Complete supporting information for the specific RhCE model used including its performance. This should include, but is not limited to:
  i) Viability;
  ii) Barrier function;
  iii) Morphology;
  iv) Reproducibility and predictive capacity;
  v) Quality controls (QC) of the model;
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- Reference to historical data of the model. This should include, but is not limited to:
  i) Acceptability of the QC data with reference to historical batch data;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g., testing of proficiency chemicals);

Run and Test Acceptance Criteria

- Positive and negative control means and acceptance ranges based on historical data;
- Acceptable variability between tissue replicates for positive and negative controls;
- Acceptable variability between tissue replicates for the test chemical;

Test Procedure

- Details of the test procedure used;
- Doses of test chemical and control substances used;
- Duration and temperature of exposure, post-exposure immersion and post-exposure incubation periods (where applicable);
- Description of any modifications to the test procedure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT, NSC_{living} and NSC_{killed}, if applicable);
- Description of evaluation criteria used including the justification for the selection of the cut-off point for the prediction model;

Results

- Tabulation of data from individual test chemicals and control substances for each run (including repeat experiments where applicable) and each replicate measurement, including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability, Difference between tissue replicates or SD, and final prediction;
- If applicable, results of controls used for direct MTT-reducers and/or coloured test chemicals, including OD or MTT formazan peak area, %NSMTT, %NSC_{living}, %NSC_{killed}, Difference between tissue replicates or SD, final correct percent tissue viability, and final prediction;
- Results obtained with the test chemical(s) and control substances in relation to the define run and test acceptance criteria;
- Description of other effects observed;

Discussion of the Results

Conclusion
LITERATURE


10. EC EURL ECVAM (2014). Eye Irritation Validation Study (EIVS): statistical analysis of the data
generated under SOP ver 8.0 of EpiOcular™ EIT (solid test substances, laboratory Beiersdorf), pp. 21. Available at: (in publication).

11. EC EURL-ECVAM (2014). Recommendation on the use of the EpiOcular™ Eye Irritation Test (EIT) for identifying chemicals not requiring classification and labelling for serious eye damage/eye irritation according to UN GHS. Available at: (in publication).


Toxicol. **88**, 701-23.


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 (16).

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

Chemical: A substance or mixture.

Concordance: See "Accuracy".

Cornea: The transparent part of the front of the eyeball that covers the iris and pupil and admits light to the interior.

CV: Coefficient of Variation.

Dev: Deviation.

EIT: Eye Irritation Test.

EURL ECVAM: European Union Reference Laboratory for Alternatives to Animal Testing.

Eye irritation: Production of changes in the eye following the application of a test substance 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).

ET₅₀: Can be estimated by determination of the exposure time required to reduce tissue viability by 50% upon application of a benchmark chemical at a specified, fixed concentration.

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.

False positive rate: The proportion of all negative substances 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.

HPLC: High Performance Liquid Chromatography.

Infinite dose: Amount of test chemical applied to the RhCE tissue exceeding the amount required to completely and uniformly cover the epithelial surface.

Irreversible effects on the eye: See “Serious eye damage”.

LLOQ: Lower Limit of Quantification.
**LogP**: Logarithm of the octanol-water partitioning coefficient

**ME**: Matrix Effect.

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

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

**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.

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

**Negative control**: A sample containing all components of a test system and treated with a substance known not to induce a positive response. 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”.

**NSC**<sub>killed</sub>: Non-Specific Colour in killed tissues.

**NSC**<sub>living</sub>: Non-Specific Colour in living tissues.

**NSMTT**: Non-Specific MTT reduction.

**OD**: Optical Density.

**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 (16).

**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.

**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 (16).

**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
Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals (16).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (16).

Reversible effects on the eye: See “Eye irritation”.

RhCE: Reconstructed human Cornea-like Epithelium.

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

SD: Standard Deviation.

Sensitivity: The proportion of all positive/active test 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 (16).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance 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).

SOP: Standard Operating Procedure.

Specificity: The proportion of all negative/inactive test 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 (16).

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including 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: A single test chemical concurrently tested in a minimum of two tissue replicates as defined in the corresponding SOP.

Tissue viability: Parameter measuring total activity of a cell population in a reconstructed tissue as their ability to reduce the vital dye MTT, which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Top-Down approach: Step-wise approach used for a 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).

Test chemical: The term "test chemical" is used to refer to what is being tested.
**Tiered testing strategy:** A stepwise testing strategy, which uses test methods in a sequential manner. All existing information on a test chemical is reviewed at each tier, using a weight-of-evidence process, to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier in the strategy. If the hazard potential/potency of a test chemical can be assigned based on the existing information at a given tier, no additional testing is required (16).

**ULOQ:** Upper Limit of Quantification.

**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 standardised 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 do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B). Interchangeable with “Not Classified”.

**UPLC:** Ultra-High Performance Liquid Chromatography.

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

**Valid test method:** A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (16).

**Validated test method:** A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose.

**VRM:** Validated Reference Method.

**Weight-of-evidence:** The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a test substance.