Section 4
Health effects

Test Guideline No. 492

Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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, which is not fully reversible, occurring after exposure of the eye to a test chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). Also according to UN GHS, eye irritation refers to the production of changes in the eye, which are fully reversible, occurring after exposure of the eye to a test chemical. 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 (TG) 405; adopted in 1981 and revised in 1987, 2002, 2012 and 2017) (2). The choice of the most appropriate test method and the use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment (IATA) for Serious Eye Damage and Eye irritation (3).

3. 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. Four other in vitro test methods have been validated, considered scientifically valid and adopted as OECD Test Guidelines (TGs) 437 (4), 438 (5), 460 (6) and 491 (7) to address the human health endpoint serious eye damage or no classification.

4. Four validated test methods using commercially available RhCE models are included in this Test Guideline. Validation studies for assessing eye irritation/serious eye damage have been conducted (8)(9)(10)(11)(12)(13)(14)(15) using the EpiOcular™ Eye Irritation Test (EIT), the SkinEthic™ Human Corneal Epithelium (HCE) EIT, the LabCyte CORNEA-MODEL24 EIT and the MCTT HCE™ EIT. Each of these methods makes use of commercially available RhCE tissue constructs as test system, two of them are referred to in the following text as the Validated Reference Methods – EpiOcular™ EIT (VRM1) and SkinEthic™ HCE EIT (VRM2), respectively. From their validation studies and their independent peer review (10)(13)(16)(17) it was concluded that EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and the MCTT HCE™ EIT are 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 methods were recommended as scientifically valid for that purpose. Annexes II-VII provide a synopsis of the important elements of the test methods, as well as flowcharts providing guidance for specific situations.

5. The test method(s) described in this Test Guideline cannot be used on their own to replace the in vivo Draize eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. It is therefore recommended to make use of alternative testing strategies such as those described in TG 467 and 492B to address the required ranges of irritation potential. Strategic combinations of several alternative test methods within (tiered) testing strategies such as the Bottom-Up/Top-Down approach may be able to fully replace the Draize eye test (19). The Bottom-Up approach 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 is designed to be used when, based on existing information, a chemical is expected to cause serious eye damage. The EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT are 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. However, the EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT are 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 (3). A test chemical that is identified as requiring classification for eye irritation/serious eye damage with EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT or MCTT HCE™ EIT will thus require additional testing (in vitro and/or in vivo) to reach a definitive conclusion (UN GHS No Category, Category 2 or Category 1), using e.g., TG 437, 438, 460, 491, or as a last option TG 405.

6. The purpose of this Test Guideline is to describe the procedure used to evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in a RhCE tissue construct, as measured by the tetrazolium dye {TD; e.g., MTT [3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS RN 298-93-1] for VRM1 and VRM2, WST-8 [2-(2-methoxy-4- nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; CAS RN 193149-74-5] for LabCyte CORNEA-MODEL24 EIT, or WST-1 [4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; CAS RN 150849-52-8] for MCTT HCE™ EIT} assay (20)(21)(22) (see paragraph 23). 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 (23) are available to facilitate the validation of new or modified in vitro RhCE-based test methods similar to EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT, in accordance with the principles of Guidance Document No. 34 (24), 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.

8. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

9. This Test Guideline is based on commercial three-dimensional RhCE tissue constructs that are produced using either primary human epidermal keratinocytes (i.e., EpiOcular™ OCL-200), human immortalized corneal epithelial cells (i.e., SkinEthic™ HCE/S), or primary human corneal epithelial cells (i.e., LabCyte CORNEA-MODEL24 or MCTT HCE™). The EpiOcular™ OCL-200, SkinEthic™ HCE/S, LabCyte CORNEA-MODEL24, and MCTT HCE™ RhCE tissue constructs are similar to the in vivo corneal epithelium three-dimensional structure and are produced using cells from the species of interest (25)(26)(27)(28). Moreover, the test methods directly measure cytotoxicity resulting from penetration of the chemical through the cornea and production of cell and tissue damage following chemical exposure, which determines the overall in vivo serious eye damage/eye irritation response. Cell damage can occur by several modes of action (see paragraph 22), but cytotoxicity plays an important, if not the primary, mechanistic role in determining the overall serious eye damage/eye irritation response of a chemical, manifested in vivo mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, regardless of the physicochemical processes underlying tissue damage.

10. A wide range of chemicals, covering a variety of chemical types, chemical classes, molecular weights, LogPs, chemical structures, etc., have been tested in the validation study underlying this Test Guideline. The EpiOcular™ EIT validation database contained 113 chemicals in total, covering 95 different organic functional groups according to an OECD QSAR toolbox analysis (9). The majority of these chemicals represented mono-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 113 tested chemicals were distributed as follows: 13 Category 1 liquids, 15 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 No Category solids (8). The SkinEthic™ HCE EIT validation database contained 200 chemicals in total, covering 165 different organic functional groups (9)(11)(12). The majority of these chemicals represented mono-constituent substances, but several multi-constituent substances (including 10 polymers) were also included in the study. In terms of physical state and UN GHS Categories, the 200 tested chemicals were distributed as follows: 27 Category 1 liquids, 24 Category 1 solids, 19 Category 2A liquids, 10 Category 2A solids, 9 Category 2B liquids, 8 Category 2B solids, 50 No Category liquids and 53 No Category solids (11)(12). The LabCyte CORNEA-MODEL24 EIT catch-up validation database contained 30 reference chemicals listed in the performance standards (23) of this Test Guideline 492. The MCTT HCE™ EIT me-too validation database contained 30 reference chemicals listed in the performance standards (23) of this Test Guideline 492.

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 aerosols. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

12. Test chemicals absorbing light in the same range as formazan dye (FD, naturally or after treatment) and test chemicals able to directly reduce the vital dye TD (to FD) may interfere with the tissue viability measurements and need the use of adapted controls for corrections. The type of adapted controls

that may be required will vary depending on the type of interference produced by the test chemical and the procedure used to quantify each FD (see paragraphs 38-44).

13. Results generated in pre-validation (29)(30)(31)(32) and validation (9)(11)(12)(14)(15) studies have demonstrated that EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT are transferable to laboratories considered to be naïve in the conduct of the assays 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 from data on 113 chemicals is in the order of 95% within laboratories and 93% between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from SkinEthic™ HCE EIT from data on 120 chemicals is in the order of 92% within laboratories and 95% between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from LabCyte CORNEA-MODEL24 EIT from data on 30 reference chemicals listed in the performance standards (22) is in the order of 96% within laboratories and 87% between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from MCTT HCE™ EIT from data on 30 reference chemicals listed in the performance standards (23)is in the order of 93% within laboratories and 90% 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). Considering the data obtained in the validation study (9), the EpiOcular™ EIT has an overall accuracy of 80% (based on 112 chemicals), sensitivity of 96% (based on 57 chemicals), false negative rate of 4% (based on 57 chemicals), specificity of 63% (based on 55 chemicals) and false positive rate of 37% (based on 55 chemicals), when compared to reference in vivo rabbit eye test data (OECD TG 405) (2)(18) classified according to the UN GHS classification system (1). A study where 97 liquid agrochemical formulations were tested with EpiOcular™ EIT demonstrated a similar performance of the test method for this type of mixtures as obtained in the validation study (33). The 97 formulations were distributed as follows: 21 Category 1, 19 Category 2A, 14 Category 2B and 43 No Category, classified according to the UN GHS classification system (1) based on reference in vivo rabbit eye test data (OECD TG 405) (2)(18). An overall accuracy of 82% (based on 97 formulations), sensitivity of 91% (based on 54 formulations), false negative rate of 9% (based on 54 formulations), specificity of 72% (based on 43 formulations) and false positive rate of 28% (based on 43 formulations) were obtained (33).

15. The SkinEthic™ HCE 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). Considering the data obtained in the validation study (11)(12), the SkinEthic™ HCE EIT has an overall accuracy of 84% (based on 200 chemicals), sensitivity of 95% (based on 97 chemicals), false negative rate of 5% (based on 97 chemicals), specificity of 72% (based on 103 chemicals) and false positive rate of 28% (based on 103 chemicals), when compared to reference in vivo rabbit eye test data (OECD TG 405) (2)(18) classified according to the UN GHS classification system (1).

16. The LabCyte CORNEA-MODEL 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). Considering the data obtained in the catch-up validation study (14), the LabCyte CORNEA-MODEL EIT meets the criteria for reproducibility and reproductive capacity, as required by the Performance Standards of this Guideline (23). Additionally, a database consisting of 139 chemicals was tested by the tissue construct developer (unpublished data).

17. The MCTT HCE™ 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). Considering the data obtained in the catch-up validation study (15), the MCTT HCE™ EIT meets the criteria for reproducibility

and predictive capacity, as required by the Performance Standards of this Guideline (23). Additionally, a set consisting of 141 chemicals was tested (15), which showed that MCTT HCE™ EIT has an overall accuracy of 86% (based on 141 chemicals), sensitivity of 99% (based on 80 chemicals), false negative rate of 1% (based on 80 chemicals), specificity of 69% (based on 61 chemicals) and false positive rate of 31% (based on 61 chemicals), when compared to reference in vivo rabbit eye test data (OECD TG 405) (2)(18) classified according to the UN GHS classification system (1).

18. The false negative rates obtained with these RhCE test methods with either substances or mixtures are within the in vivo Draize eye test within-test variability of 12% (34). The false positive rates obtained with any RhCE test methods with either substances or mixtures are not critical in the context of this Test Guideline since all test chemicals that produce a tissue viability equal or lower than the established cut-offs (see paragraph 46) will require further testing with (an)other in vitro test method(s), or as a last option in rabbits, depending on regulatory requirements, according to the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (3). These test methods can be used for all types of chemicals, whereby a negative result 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, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT under classification schemes other than UN GHS.

19. 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 as set out in the OECD Guidance Document on An Integrated Approach for Testing and Assessment for Serious Eye Damage and Eye Irritation, is required (3).

20. 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 RhCE test method to the testing of substances and/or mixtures.

PRINCIPLE OF THE TEST

21. The test chemical is applied topically to a minimum of two three-dimensional RhCE tissue constructs and tissue viability is measured following exposure and a post- treatment incubation period. The RhCE tissues are reconstructed from primary human epidermal keratinocytes, human immortalized corneal epithelial cells, or primary culture human corneal epithelial 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™, LabCyte CORNEA-MODEL24, and MCTT HCE™ RhCE tissue construct consists of at least 3 viable layers of cells and a non-keratinized surface, showing a corneal-like structure analogous to that found in vivo (27). The SkinEthic™ HCE RhCE tissue construct consists of at least 4 viable layers of cells including columnar basal cells, transitional wing cells and superficial squamous cells similar to that of the normal human corneal epithelium (26)(35).

22. 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/or conjunctiva 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) (19)(36)(37). 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 (38)(39). Moreover, the serious eye damage/eye irritation potential of a chemical is principally determined by the extent of initial injury (36), which correlates with the extent of cell death (38) and with the extent of the subsequent responses and eventual outcomes (40)(41). 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 (39)(42). The measurement of viability of the RhCE tissue construct after topical exposure to a test chemical to identify chemicals not requiring classification for serious eye damage/eye irritancy (UN GHS No Category) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium and/or conjunctiva.

23. RhCE tissue viability is classically measured by enzymatic conversion of TD (MTT for VRM1 and VRM2, WST-8 for LabCyte CORNEA-MODEL24 EIT or WST-1 for MCTT HCE™ EIT) by the viable cells of the tissue into coloured FD (blue MTT formazan, or yellow WST-8 and WST-1 formazan). Blue MTT FD is quantitatively measured after extraction from tissues (20), whereas yellow WST-8 FD or WST-1 FD does not require extraction because of its water solubility and is quantitatively measured directly from the WST-8 or WST-1 solution on which the tissues are incubated during the WST-8 (21) or WST-1 assay (22). Chemicals not requiring classification and labelling according to UN GHS (No Category) are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 60% for EpiOcular™ EIT and SkinEthic™ HCE EITL ; > 50% for SkinEthic™ HCE EITS ; > 40% for LabCyte CORNEA-MODEL24 EIT or > 35% for liquids and > 60% for solids in MCTT HCE™ EIT) (see paragraph 46).

DEMONSTRATION OF PROFICIENCY

24. Prior to routine use of RhCE test methods for regulatory purposes, laboratories should demonstrate technical proficiency by correctly predicting the fifteen proficiency chemicals listed in Table 1. These chemicals were selected from the chemicals used in the validation studies of the VRM1 and VRM2 (9)(11)(12). The selection includes, to the extent possible, chemicals that: (i) cover different physical states; (ii) cover the full range of in vivo serious eye damage/eye irritation responses based on high quality results obtained in the reference in vivo rabbit eye test (OECD TG 405) (2)(16) and the UN GHS classification system (i.e., Categories 1, 2A, 2B, or No Category) (1); (iii) cover the various in vivo drivers of classification (34)(43); (iv) are representative of the chemical classes used in the validation study (9)(11)(12); (v) cover a good and wide representation of organic functional groups (9)(11)(12); (vi) have chemical structures that are well-defined (9)(11)(12); (vii) are coloured and/or direct TD reducers; (viii) produced reproducible results in RhCE test methods during their validations; (ix) were correctly predicted by RhCE test methods during their validation studies; (x) cover the full range of in vitro responses based on high quality RhCE test methods data (0 to 100% viability); (xi) are commercially available; and (xii) are not associated with prohibitive acquisition and/or disposal costs. In situations where a listed chemical is unavailable or cannot be used for other justified reasons, another chemical fulfilling the criteria described above, e.g. from the chemicals used in the validation of the VRMs, could be used. Such deviations should however be justified.

Table 1: List of proficiency chemicals

Chemical Name	CAS RN	Organic Functional Group[1]	Physic al state	VRM1 viabili ty	VRM2 viability (%)[3]	LabCyte viability (%)[3-1]	MCTT HCE™ viability (%)[3-2]	VRM Prediction[7]	Colo ur inte
				In Vivo Category 1[4]					
Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	10.9±6.4	5.5±7.4	1.7±1.2	25.3±6.0	No prediction can be made	N
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	L	7.5±4.75[5]	1.6±1.0	7.5±4.75	9.8±7.7	No prediction can be made	N
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	2.3±0.2	0.2±0.1	2.8±2.6	0.5±0.1	No prediction can be made	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	5.3±4.1	3.7±1.5	30.6±6.3	No prediction can be made	N
				***In Vivo* Category 2A[4]**					
2,4,11,13-Tetraazatetradecane-diimidamide, N,N''- bis(4-chlorophenyl)- 3,12-diimino-, di-D- gluconate (20%, aqueous)[6]	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	4.0±1.1	1.3±0.6	0.4±0.4	1.8±0.1	No prediction can be made	Y (weak)
Sodium benzoate	532-32-1	Aryl; Carboxylic acid	S	3.5±2.6	0.6±0.1	2.9±2.6	1.1±0.5	No prediction can be made	N
				***In Vivo* Category 2B[4]**					
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	2.8±0.9	32.4±9.3	2.3±2.2	No prediction can be made	N
2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Alkane, branched with tertiary carbon; Alkene; Bicycloheptane; Bridged-ring carbocycles; Cycloalkane	S	4.7±1.5	15.8±1.1	2.2±2.6	22.9±11.5	No prediction can be made	N
				***In Vivo* No Category[4]**					
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.9±6.4	79.4±6.2	48.0±8.9	56.8±3.4	No Cat	N
Dicaprylyl ether	629-82-3	Alkoxy; Ether	L	97.8±4.3	95.2±3.0	92.7±5.0	89.9±8.9	No Cat	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	96.5±3.5	95.6±14.0	82.4±14.5	No Cat	N
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	77.6±5.4	89.1±2.9	62.6±11.5	72.1±14.8	No Cat	N
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Aromatic heterocyclic halide; Aryl halide; Urea derivatives	S	106.7±5.3	101.9±6.6	77.8±9.0	94.5±5.9	No Cat	N
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)- 4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl	S	102.7±13.4	97.7±5.6	90.2±5.8	98.8±10.1	No Cat	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	92.9±5.1	66.6±0.2	90.5±6.3	No Cat	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (1); VRM1 = Validated Reference Method, EpiOcular™ EIT; VRM2 = Validated Reference Method, SkinEthic™ HCE EIT; Colour interf. = colour interference with the standard absorbance (Optical Density (OD)) measurement of FD.

[1]Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (9).

[2]Based on results obtained with EpiOcular™ EIT in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (9).

[3] Based on results obtained with SkinEthic™ HCE EIT in the validation study (11)(12).

[3-1] Based on results obtained with LabCyte CORNEA-MODEL24 EIT in the validation study (14).

[3-2] Based on results obtained with MCTT HCE™ EIT in the validation study (15).

[4]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (2)(18) and using the UN GHS (1). [5]Based on results obtained in the CEFIC Consortium for *in vitro* Eye Irritation testing strategy (CON4EI) Study.

[6]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.

[7] VRM prediction: see paragraph 46 for interpretation of the prediction model.

25. 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 tissue construct producer (see paragraphs 27, 29 and 32). 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

26. The test methods currently covered by this Test Guideline are the scientifically valid EpiOcular™ EIT, SkinEthic™ HCE EIT, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT (10)(13)(14) (15), the first two being referred as the Validated Reference Methods (VRM1 and VRM2, respectively). The Standard Operating Procedures (SOP) for the RhCE test methods are available and should be employed when implementing and using the test methods in a laboratory (44)(45)(46)(47). The following paragraphs and Annex II describe the main components and procedures of the RhCE test methods.

RhCE TEST METHOD Components

GENERAL CONDITIONS

27. Relevant human-derived cells should be used to reconstruct the cornea-like epithelium three-dimensional tissue, which should be composed of progressively stratified but not cornified cells. The RhCE tissue construct 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 cornea-like epithelium. The RhCE tissue construct should have the epithelial surface in direct contact with 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 construct should form a functional barrier with sufficient robustness to resist rapid penetration of cytotoxic benchmark substances, e.g., Triton X-100 or sodium dodecyl sulphate (SDS). The barrier function should be demonstrated and may be assessed by determination of either the exposure time required to reduce tissue viability by 50% (ET50) upon application

of a benchmark substance at a specified, fixed concentration (e.g., 50-100 µL of 0.3% (v/v) Triton X-100), or the concentration at which a benchmark substance reduces the viability of the tissues by 50% (IC50) following a fixed exposure time (e.g., 30 minutes treatment with 50 µL SDS or 60 minutes treatment with 25 µL SDS) (see paragraph 32). The containment properties of the RhCE tissue construct should prevent the passage of test chemical around the edge of the viable tissue, which could lead to poor modelling of corneal exposure. The human derived cells used to establish the RhCE tissue construct should be free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the tissue construct should be checked by the supplier for absence of contamination by fungi and bacteria.

FUNCTIONAL CONDITIONS

Viability

28. The assay used for quantifying tissue viability is the tetrazolium dye (TD) (MTT for VRM1 and VRM2, WST-8 for LabCyte CORNEA-MODEL24 EIT or WST-1 for MCTT HCE™ EIT) assay (19)(21). Viable cells of the RhCE tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). Alternatively, viable cells of the RhCE tissue construct reduce the vital dye WST-8 or WST-1 into a water soluble yellow formazan. The extracted formazan dye (FD) may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (48). The OD of the blank solution alone (which is the extraction solvent for MTT assay, the diluted WST-8 medium for the WST-8 assay or the diluted WST-1 medium for the WST-1 assay) should be sufficiently small, i.e., OD < 0.1. Users of the RhCE tissue construct should ensure that each batch of the RhCE tissue construct used meets defined criteria for the negative control. Acceptability ranges for the negative control OD values for the VRMs, LabCyte CORNEA-MODEL24, and MCTT HCE™ are given in Table 2. An HPLC/UPLC-spectrophotometry user should use the negative control OD ranges provided in Table 2 as the acceptance criterion for the negative control. It should be documented in the test report 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. A similar procedure should be followed by the tissue producer as part of the quality control tissue batch release, but in this case different acceptance criteria than those specified in Table 2 may apply. An acceptability range (upper and lower limit) for the negative control OD values (in the QC test method conditions) should be established by the RhCE tissue construct developer/supplier.

Table 2. Acceptability ranges for negative control OD values (for the test method users)

TestMethod	Loweracceptancelimit	Upperacceptancelimit
EpiOcular™ EIT(OCL-200)– VRM1 (forboththeliquidsandthesolidsprotocols)	> 0.8[1]	< 2.8
SkinEthic™ HCE EIT(HCE/S)– VRM2 (forboththeliquidsandthesolidsprotocols)	> 1.0	≤ 2.5
LabCyteCORNEA-MODEL24 EIT (forboththeliquidsandthesolidsprotocols)	≥ 0.5	≤ 1.6
MCTT HCE™ EIT (forboththeliquidsandthesolidsprotocols)	≥ 1.6	≤ 3.0

[1]This acceptance limit considers the possibility of extended shipping/storage time (e.g. > 4 days), which has been shown not to impact on the performance of the test method (44).

Barrier function

29. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, as estimated e.g. by ET50 (Triton X-100) or by IC50 (SDS) (Table 3). The barrier function of each batch of the RhCE tissue construct used should be demonstrated by the RhCE tissue construct developer/vendor upon supply of the tissues to the end user (see paragraph 32).

Morphology

30. Histological examination of the RhCE tissue construct should demonstrate human cornea-like epithelium structure (including at least 3 layers of viable epithelial cells and a non-keratinized surface). For the three test methods, appropriate morphology has been established by the developer/supplier and therefore does not need to be demonstrated again by a test method user for each tissue batch used.

Reproducibility

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

Quality control (QC)

32. The RhCE tissue construct should only be used if the developer/supplier demonstrates that each batch of the RhCE tissue construct used meets defined production release criteria, among which those for viability (paragraph 28) and barrier function (see paragraph 29) are the most relevant. An acceptability range (upper and lower limits) for the barrier functions as measured by the ET50 or IC50 (see paragraphs 27 and 29) should be established by the RhCE tissue construct developer/supplier. The ET50 and IC50 acceptability range used as QC batch release criterion by the developer/supplier of the RhCE tissue constructs used in the test methods is given in Table 3. Data demonstrating compliance with all production release criteria should be provided by the RhCE tissue construct developer/supplier to the test method users so that they are able to include this information in the test report. Only results produced with tissues fulfilling all of these production release criteria 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.

Table 3. QC batch release criteria

TestMethod	Lower acceptance limit	Upper acceptance limit
EpiOcular™EIT(OCL-200)– VRM1 (100 µL of 0.3%(v/v)Triton X-100)	ET_{50}= 12.2 minutes	ET_{50} =37.5 minutes
SkinEthic™ HCE EIT (HCE/S) – VRM2 (30 minutes treatmentwith 50µL SDS)	IC_{50} = 1.0mg/mL	IC_{50} =3.2 mg/mL
LabCyte CORNEA-MODEL24 EIT (60 minutes treatment with 25µL SDS)	IC_{50} =1.0 mg/mL	IC_{50} =4.0 mg/mL
MCTT HCE™ EIT – (50 µL of 0.3%(v/v) Triton X-100)	ET_{50}= 17.6 minutes	ET_{50} = 41.0 minutes

Application of the Test Chemical and Control Substances

33. At least two tissue replicates should be used for each test chemical and each control substance in each run. Two different treatment protocols are used, one for liquid test chemicals and one for solid test chemicals (44)(45)(46)(47). For the two VRMs, and MCTT HCE™ EIT, the tissue construct surface should be moistened with calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (Ca2+/Mg2+-free DPBS) before application of test chemicals, to mimic the wet conditions of the human eye. The treatment of the tissues is initiated with exposure to the test chemical(s) and control substances. For any treatment protocols in any of the two VRMs, and MCTT HCE™ EIT, a sufficient amount of test chemical or control substance should be applied to uniformly cover the epithelial surface while avoiding an infinite dose (see paragraphs 34 and 35) (Annex II).

34. 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 four test methods, otherwise they should be treated as solids (see paragraph 35). In the test methods, liquid test chemical are evenly spread over the tissue surface (i.e. a minimum of 60 µL/cm2 application) (see Annex II, (44)(45)(46)(47). Capillary effects (surface tension effects) that may occur due to the low volumes applied to the insert (on the tissue surface) should be avoided to the extent possible to guarantee the correct dosing of the tissue. Tissues treated with liquid test chemicals are incubated for 1 minute (LabCyte CORNEA-MODEL24 EIT), 10 minutes (MCTT HCE™ EIT) or 30 minutes (VRM1 and VRM2) at the standard conditions of each method. At the end of the exposure period, the liquid test chemical and the control substances should be carefully removed from the tissue surface by extensive rinsing with Ca2+/Mg2+-free DPBS at room temperature. For the two VRMs, this rinsing step should be followed by a post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used. For VRM1, LabCyte CORNEA-MODEL24 EIT and for MCTT HCE™ EIT, a post-exposure incubation in fresh medium at standard culture conditions is applied prior to performing the TD assay (see Annex II, (44)(45)(46)(47)).

35. Test chemicals that cannot be pipetted at temperatures up to 37°C are treated as solids in the four test methods. The amount of test chemical applied should be sufficient to cover the entire surface of the tissue, i.e. a minimum of 33 mg/cm2 application should be used (Annex II). Whenever possible, solids should be tested as a fine powder. Tissues treated with solid test chemicals are incubated for a pre-defined period of time (depending on the method used) at standard culture conditions (see Annex II,

(44)(45)(46)(47)). At the end of the exposure period, the solid test chemical and the control substances should be carefully removed from the tissue surface by extensive rinsing with Ca2+/Mg2+-free DPBS at room temperature. For the two VRMs, this rinsing step should be followed by a post- exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) for a pre-defined period of time that varies depending on the VRM used, followed by a post-exposure incubation in fresh medium at standard culture conditions, prior to performing the TD assay (see Annex II, (44)(45)(46)(47)).

36. Concurrent negative and positive controls should be included in each run to demonstrate that the viability (determined with the negative control) and the sensitivity (determined with the positive control) of the tissues are within acceptance ranges defined based on historical data. 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 (%Viabilitytest). The recommended positive control substance to be used with the VRMs, and MCTT HCE™ EIT is neat methyl acetate (CAS RN. 79-20-9, commercially available from e.g., Sigma-Aldrich, Cat# 45997; liquid) for both liquids and solids protocols. The recommended positive control substances to be used with the LabCyte CORNEA- MODEL24 EIT are ethanol (CAS RN. 64-17-5) for liquids protocol and lauric acid (CAS RN. 143-07-7) for solids protocol. The recommended negative control substance to be used with the VRM1 is ultrapure H2O for both liquids and solids protocols. The recommended negative control substance to be used with the VRM2 and MCTT HCE™ EIT is Ca2+/Mg2+-free DPBS for both liquids and solids protocols. The recommended negative control to be used with the LabCyte CORNEA-MODEL24 EIT is Ca2+/Mg2+-free DPBS (CAS RN. 64-17-5) for liquids protocol and no treatment for solids protocol. These were the control substances used in the validation studies of the VRMs and are those for which most historical data exist. The use of suitable alternative positive or negative control substances should be scientifically and adequately justified. Negative and positive controls should be tested with the same protocol(s) as the one(s) used for the test chemicals included in the run (i.e. for liquids and/or solids). This application should be followed by the treatment exposure, rinsing, a post-exposure immersion, and post-exposure incubation where applicable, as described for controls run concurrently to liquid test chemicals (see paragraph 34) or for controls run concurrently to solid test chemicals (see paragraph 35), prior to performing the TD assay (see paragraph 37) (44)(45)(46)(47) . One single set of negative and positive controls is sufficient for all test chemicals of the same physical state (liquids or solids) included in the same run.

Tissue Viability Measurements

37. The TD assay is a standardised quantitative method (20)(21)(22) that should be used to measure tissue viability under this Test Guideline. It is compatible with use in a three-dimensional tissue construct. The TD assay is performed immediately following the post- exposure procedures. The VRMs use the MTT assay. In the VRMs, the RhCE tissue construct sample is placed in 0.3 mL of MTT solution at 1 mg/mL for 180±15 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 construct. The precipitated blue MTT formazan product is then extracted from the tissue using an appropriate volume of isopropanol (or a similar solvent) (44)(45). 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). Tissues tested with liquid test chemicals that are not readily washed off may also be extracted from the bottom of the tissue only. LabCyte CORNEA-MODEL24 EIT uses the WST-8 assay. In the LabCyte CORNEA-MODEL24 EIT, the RhCE tissue construct sample is placed in 0.3 mL of diluted WST-8 solution, prepared according to the standard operating

procedures (46), for 240 minutes at standard culture conditions and the vital dye WST-8 is reduced into a yellow WST-8 formazan by the viable cells of the RhCE tissue construct, which is dissolved into the diluted WST-8 solution (46). MCTT HCE™ EIT uses the WST-1 assay. In the MCTT HCE™ EIT, the RhCE tissue construct sample is placed in 0.3 mL of diluted WST-1 solution, prepared according to the standard operating procedures (47), for 180 minutes at standard culture conditions and the vital dye WST-1 is reduced into a yellow WST-1 formazan by the viable cells of the RhCE tissue construct, which is dissolved into the diluted WST-1 solution (47). The concurrently tested negative and positive control substances should be treated similarly to the tested chemical. In the VRM1 and VRM2, 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-spectrophotometry procedure (see paragraph 44) (10)(48). In the LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT, the WST-8/-1 formazan may be directly quantified (i.e. without the need of an extraction procedure) either by a standard absorbance (OD) measurement at 450 nm using a filter band pass of maximum ±30 nm or by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 44).

38. Optical properties of the test chemical or its chemical action on TD (MTT for VRM1 and VRM2, WST-8 for LabCyte CORNEA-MODEL24 EIT or WST-1 for MCTT HCE™ EIT) may interfere with the measurement of FD leading to a false estimate of tissue viability, i.e., under-prediction of eye irritation. Test chemicals may interfere with the measurement of FD by direct reduction of the TD into coloured FD (blue MTT formazan or yellow WST-8/-1 formazan) and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as FD (i.e., MTT formazan: around 570 nm; WST-8/-1 formazan: around 450 nm). Potential of chemicals to directly reduce TD and/or interfere with colour measurement (only necessary for coloured test chemicals) should be checked before testing (for VRM1, VRM2, LabCyte CORNEA-MODEL24 EIT and MCTT HCE™ EIT). In case of FD interference, additional controls should be used to correct for potential interference from such test chemicals (see paragraphs 39-43 and Annexes 3-6). This is especially important when a specific test chemical is not completely removed from the RhCE tissue construct by rinsing or when it penetrates the cornea-like epithelium and is therefore present in the RhCE tissue constructs when the TD assay is performed. For test chemicals absorbing light in the same range as FD (naturally or after treatment), which are not compatible with the standard absorbance (OD) measurement of FD due to strong interference, i.e., strong absorption at 570±30 nm (with MTT formazan) or 450±30 nm (with WST-8/-1 formazan), an HPLC/UPLC-spectrophotometry procedure to measure FD may be employed (see paragraphs 43 and 44) (10)(48). A detailed description of how to detect and correct for direct TD reduction and interferences by colouring agents is available in the test methods' respective SOPs (44)(45)(46)(47). Illustrative flowcharts providing guidance on how to identify and handle direct TD-reducers and/or colour interfering chemicals for VRM1, VRM2, LabCyte CORNEA-MODEL24 EIT, and MCTT HCE™ EIT are also provided in Annexes III, IV, V and VI respectively.

39. To identify potential interference by test chemicals absorbing light in the same range as FD (naturally or after treatment) and decide on the need for additional controls, the test chemical is added to water and/or isopropanol and incubated for an appropriate time at room temperature (see Annex II, (44)(45)(46)(47). If the test chemical in water and/or isopropanol absorbs sufficient light in the range of 570±20 nm for VRM1 (see Annex III), or if a coloured solution is obtained when mixing the test chemical with water for VRM2 (see Annex IV), LabCyte CORNEA-MODEL24 EIT(see Annex V), and MCTT HCE™ EIT (see Annex VI), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of FD and further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 43 and 44 and Annexes III, IV, V and VI) (44)(45)(46)(47). When performing the standard absorbance (OD) measurement, each interfering test chemical should be applied on at least two viable

tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of TD solution during the TD incubation step, to generate a non-specific colour in living tissues (NSCliving) control (44)(45)(46)(47). The NSCliving control needs to be performed concurrently to the testing of the coloured test chemical and, in case of multiple testing, an independent NSCliving control needs to be conducted with each test performed (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 the MTT or WST-8/-1 solution (%Viabilitytest) minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT or WST-8/-1, run concurrently to the test being corrected (%NSCliving), i.e., True tissue viability = [%Viabilitytest] - [%NSCliving].

40. To identify direct MTT or WST-8/-1 reducers, each test chemical should be added to freshly prepared TD. An appropriate amount of test chemical is added to a TD solution and the mixture is incubated for approximately 3 or 4 hours at standard culture conditions (see Annexes III, IV, V and VI) (44)(45)(46)(47) . If the TD mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple (for MTT solution) or yellow/orange (for WST-8/-1 solution), the test chemical is presumed to directly reduce the TD and a further functional check on non-viable RhCE tissue constructs should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb and retain the test chemical in a similar way as viable tissues. Killed tissues of VRM1, and MCTT HCE™ EIT are prepared by exposure to low temperature ("freeze-killed"). Killed tissues of VRM2 are prepared by prolonged incubation (e.g., at least 24±1 hours) in water followed by storage to low temperature ("water-killed"). Killed tissues of LabCyte CORNEA-MODEL24 EIT are prepared by freezing tissues at -80°C or below, for 30 minutes twice ("freeze-killed"). Each TD reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure, to generate a non-specific TD reduction (NSMTT or NSWST) control (44)(45)(46)(47) . A single NSMTT or NSWST 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 TD reducer (%Viabilitytest) minus the percent non-specific TD reduction obtained with the killed tissues exposed to the same reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT or %NSWST), i.e. true tissue viability = [%Viabilitytest] - [%NSMTT or %NSWST].

41. Test chemicals that are identified as producing both colour interference (see paragraph 39) and direct TD reduction (see paragraph 40) will also require a third set of controls when performing the standard absorbance (OD) measurement, apart from the NSMTT or NSWST, and NSCliving controls described in the previous paragraphs. This is usually the case with darkly coloured test chemicals absorbing light in the range of 570±30 nm for MTT formazan (e.g., blue, purple, black) or with lightly coloured test chemicals absorbing light in the range of 450±30 nm for WST-8/-1 formazan (e.g. yellow, orange) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT or WST-8/-1 as described in paragraph 40. This forces the use of NSMTT or NSWST controls, by default, together with the NSCliving controls. Test chemicals for which both NSMTT or NSWST, and NSCliving controls are performed may be absorbed and retained by both living and killed tissues. Therefore, in this case, the NSMTT or NSWST control may not only correct for potential direct TD reduction by the test chemical, but also for colour interference arising from the absorption and retention of the test chemical by killed tissues. This could lead to double correction for colour interference since the NSCliving control already corrects for colour interference arising from the absorption and retention of the test chemical by living tissues. To avoid a possible double correction for colour interference, a third control for non-specific colour in killed tissues (NSCkilled) needs to be performed (see Annexes III, IV, V and VI) (44)(45)(46)(47). 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 TD solution during the TD incubation step. A single NSCkilled control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT or NSWST 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 (%Viabilitytest) minus %NSMTT or %NSWST minus %NSCliving plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without TD, calculated relative to the negative control ran concurrently to the test being corrected (%NSCkilled), i.e., True tissue viability = [%Viabilitytest] - [%NSMTT or %NSWST] - [%NSCliving] + [%NSCkilled].

42. It is important to note that non-specific TD reduction and non-specific colour interferences may increase the OD (when performing standard absorbance measurements) of the sample above the linearity range of the spectrophotometer and that non-specific TD reduction can also increase the FD peak area (when performing HPLC/UPLC- spectrophotometry measurements) of the sample above the linearity range of the spectrophotometer. On this basis, when using RhCEs, it is important for each laboratory to determine the OD/peak area linearity range of their spectrophotometer with MTT formazan (CAS RN. 57360-69-7) which is commercially available from e.g., Sigma-Aldrich (Cat# M2003), WST-8 formazan (CAS RN 193149-76-7), commercially available from Dojindo Molecular Technologies or WST-1 formazan (CAS RN 150849-53-9), available from e.g., Toronto Research Chemicals (Cat# I718750).

43. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct TD-reducers and colour interfering test chemicals, when the observed interference with the measurement of FD is not strong (i.e., the ODs of the samples obtained with the test chemical without any correction for direct TD reduction and/or colour interference are within the linear range of the spectrophotometer). Nevertheless, results for test chemicals producing %NSMTT or %NSWST and/or %NSCliving ≥ 60% (VRM1, and VRM2 for liquids protocol, and MCTT HCE™ EIT for solids) or 50% (VRM2 for solids protocol) or 40% (LabCyte CORNEA-MODEL24 EIT) or 35% (MCTT HCE™ EIT for liquids) of the negative control should be taken with caution as this is the established cut-off used in the VRMs to distinguish classified from not classified chemicals (see paragraph 46). Standard absorbance (OD) can however not be measured when the interference with the measurement of FD is strong (i.e., leading to uncorrected ODs of the test samples falling outside of the linear range of the spectrophotometer). Coloured test chemicals or test chemicals that become coloured in contact with water or isopropanol that interfere strongly with the standard absorbance (OD) measurement of FD may still be assessed using HPLC/UPLC-spectrophotometry (see Annexes III, IV,V and VI). This is because the HPLC/UPLC system allows for the separation of the FD from the chemical before its quantification (48). For this reason, NSCliving or NSCkilled controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT or NSWST controls should nevertheless be used if the test chemical is suspected to directly reduce TD (following the procedure described in paragraph 40). NSMTT or NSWST 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 TD as described in paragraph 40. When using HPLC/UPLC-spectrophotometry to measure FD, the percent tissue viability is calculated as percent FD peak area obtained with living tissues exposed to the test chemical relative to the FD peak obtained with the concurrent negative control. For test chemicals able to directly reduce TD, true tissue viability is calculated as: %Viabilitytest minus %NSMTT or %NSWST, as described in the last sentence of paragraph 39. Finally, it should be noted that direct TD-reducers or direct TD-reducers that are also colour interfering, which are retained in the tissues after treatment and reduce TD so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC- spectrophotometry) of the

tested samples that fall outside of the linearity range of the spectrophotometer cannot be assessed with RhCE test methods, although these are expected to occur in only very rare situations.

44. HPLC/UPLC-spectrophotometry may be used with all types of test chemicals (coloured, non-coloured, TD-reducers and non-TD reducers) for measurement of FD (10)(48). Due to the diversity of HPLC/UPLC-spectrophotometry systems, it is not feasible for each user to establish the exact same system conditions. As such, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify TD from samples by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bioanalytical method validation (48)(49). These key parameters and their acceptance criteria are shown in Annex VII. Once the acceptance criteria defined in Annex VII have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure FD under the experimental conditions described in this Test Guideline.

Acceptance Criteria

45. For each run using RhCE tissue batches that met the quality control (see paragraph 32), 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 28). Similarly, tissues treated with the positive control substance, i.e., methyl acetate (for VRM1, VRM2, and MCTT HCE™ EIT), ethanol (for LabCyte CORNEA-MODEL24 EIT with liquid protocol) or lauric acid (LabCyte CORNEA-MODEL24 EIT with solid protocol), should show a mean tissue viability < 50% relative to the negative control in the VRM1 with either the liquids or the solids protocols, ≤ 30% (liquids protocol) or ≤ 20% (solids protocol) relative to the negative control in the VRM2, ≤ 35% relative to the negative control in the MCTT HCE™ EIT and ≤ 40% relative to the negative control in the LabCyte CORNEA-MODEL24 EIT, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (44)(45)(46)(47). 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 standard deviation (SD) between three tissue replicates should not exceed 18%). If either the negative control or positive control included in a run is outside of the accepted ranges, the run is considered "non-qualified" and should be repeated. If the variability between tissue replicates of a test chemical is outside of the accepted range, the test must be considered "non-qualified" and the test chemical should be re-tested.

Interpretation of Results and Prediction Model

46. The OD values/peak areas obtained with the replicate samples 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 for identifying test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) is given in Table 4. 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 (>) the established percentage tissue viability cut-off value, as shown in Table 4. In this case no further testing in other test methods is required.
- If the mean percent tissue viability after exposure and post-exposure incubation is less than or equal (≤) to the established percentage tissue viability cut-off value, no prediction can be made from this result in isolation, as shown in Table 4. This is because in case of a true positive, the methods cannot resolve between UN GHS Categories 1 and 2 (see paragraph 19). Furthermore,

RhCE test methods show a high percentage of false positive results (see paragraphs 14-17). In both cases, further information will be required for classification purposes according to the IATA guidance document (3).

Table 4. Prediction Models according to UN GHS classification

Test Method	No Category	No prediction can be made
EpiOcular™ EIT (for both protocols)	Mean tissue viability> 60%	Mean tissue viability ≤ 60%
SkinEthic™HCE EIT (for the liquids' protocol)	Mean tissue viability> 60%	Mean tissue viability ≤ 60%
SkinEthic™HCE EIT (for the solids' protocol)	Mean tissue viability> 50%	Mean tissue viability ≤ 50%
LabCyteCORNEA-MODEL24 EIT (for both protocols)	Mean tissue viability > 40%	Mean tissue viability ≤ 40%
MCTT HCE™ EIT (for the liquids' protocol)	Mean tissue viability > 35%	Mean tissue viability ≤ 35%
MCTT HCE™ EIT (for the solids' protocol)	Mean tissue viability > 60%	Mean tissue viability ≤ 60%

47. 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% (VRM1, and VRM2 for liquids' protocol, and MCTT HCE™ EIT for solids' protocol), 50±5% (VRM2 for solids' protocol), 40±5% (LabCyte CORNEA-MODEL24 EIT), or 35±5% (MCTT HCE™ EIT for liquids' protocol), a second test should be considered, as well as a third one in case of discordant results between the first two tests.

Benchmark chemicals may be useful for evaluating the serious eye damage/eye irritation potential of unknown test chemicals, or product class, or for evaluating the relative ocular toxicity potential of a classified chemical within a specific range of positive response

DATA AND REPORTING

Data

48. Data from individual replicate tissues in a run (e.g., OD values/FD peak areas and calculated percent tissue viability data for the test chemical and controls, and the final RhCE test method prediction) should be reported in tabular form for each test chemical, including data from repeat tests, as appropriate. In addition, mean percent tissue viability and difference of viability between two tissue replicates (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 measurement of FD through direct TD reduction and/or coloured interference should be reported for each tested chemical.

Test report

49. 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;
- Justification for the use of a different negative control than those referenced in Annex II , if applicable;
- Justification for the use of a different positive control than those referenced in Annex II , if applicable;
- 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 Tissue Construct and Protocol Used (providing rationale for the choices, if applicable)

Test Method Conditions

- RhCE tissue construct used, including batch number;
- Wavelength and band pass (if applicable) used for quantifying FD, and linearity range of measuring device (e.g., spectrophotometer);
- Description of the method used to quantify FD
- Description of the HPLC/UPLC-spectrophotometry system used, if applicable;
- Complete supporting information for the specific RhCE tissue construct used including its performance. This should include, but is not limited to:
  - i. Viability quality control (supplier)
  - ii. Viability under test method conditions (user);
  - iii. Barrier function quality control (for positive and negative control);
  - iv. Morphology, if available;
  - v. Other quality controls (QC) of the RhCE tissue construct, if available;
- Reference to historical data of the RhCE tissue construct. This should include, but is not limited to: Acceptability of the QC data with reference to historical batch data;
- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the 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 TD-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT or NSWST, NSCliving and NSCkilled, if applicable);

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 value or FD 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 TD-reducers and/or coloured test chemicals, including OD value or FD peak area, %NSMTT or %NSWST, %NSCliving, %NSCkilled, 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, e.g., colouration of the tissues by a coloured test chemical;

Discussion of the results

Conclusion

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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 (24).

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

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) (3).

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, which are fully reversible, occurring after the exposure of the eye to a substance or mixture. Interchangeable with "Reversible effects on the eye" and with "UN GHS Category 2" (1).

ET50: 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.

Formazan dye (FD): Chromogenic product of the reduction of MTT, WST-8 or WST-1.

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.

HCE: SkinEthic™ Human Corneal Epithelium

HPLC: High Performance Liquid Chromatography.

IC50: Concentration at which a benchmark chemical reduces the viability of the tissues by 50% following a fixed exposure time (e.g., 30 or 60 minutes treatment with SDS).

Infinite dose: Amount of test chemical applied to the RhCE tissue construct 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

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. (CAS RN 298-93-1)

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”.

NSCkilled: Non-Specific Colour in killed tissues. NSCliving: Non-Specific Colour in living tissues. NSMTT: Non-Specific MTT reduction.

NSWST: Non-Specific WST-8 or WST-1 reduction.

OD: Optical Density.

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 (24).

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples. 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 (22).

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

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 (24).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (24).

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 (24).

Serious eye damage: refers to the production of tissue damage in the eye, or serious physical decay of vision, which is not fully reversible, occurring after exposure of the eye to a substance or mixture. Interchangeable with “Irreversible effects on the eye” and with “UN GHS Category 1” (1).

Standard Operating Procedures (SOP): Formal, written procedures that describe in detail how specific routine, and test-specific, laboratory operations should be performed. They are required by GLP.

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 (24).

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.

Tetrazolium dye (TD): Tetrazolium salts MTT, WST-8 and WST-1.

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) (3).

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 (24).

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 (24).

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 (24).

VRM: Validated Reference Method.

VRM1: EpiOcular™ EIT is referred as the Validated Reference Method 1.

VRM2: SkinEthic™ HCE EIT is referred to as the Validated Reference Method 2.

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 chemical.

WST: Water soluble tetrazolium salt.

WST-1: Water soluble tetrazolium salt-1 [4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; CAS RN 150849-52-8]

WST-8: Water soluble tetrazolium salt-8. [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt. (CAS RN 193149-74-5).

ANNEX II - MAIN TEST METHOD COMPONENTS OF THE RhCE TEST METHODS VALIDATED FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SERIOUS EYE DAMAGE

Nr.	1		2		3		4	
Test Method Component	EpiOcular™ EIT (VRM 1)		SkinEthic™ HCE EIT (VRM 2)		LabCyte CORNEA-MODEL24 EIT		MCTT HCE™ EIT	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Model surface	0.6 cm^2	0.6 cm^2	0.5 cm^2	0.5 cm^2	0.3 cm^2	0.3 cm^2	0.6 cm^2	0.6 cm^2
Number of tissue	At least 2	At least 2	At least 2	At least 2	3 tissues	3 tissues	At least 2	At least 2

[1] For details of the protocol, the latest version of the Standard Operating Procedures (SOP) for each test method should be consulted. The references of the SOP are available in the bibliography.

Nr.	1		2		3		4	
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**		**MCTT HCE™ EIT**	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Precheck for colour interference	50 µL + 1 mL H_2O for 60 min at 37±2ºC, 5±1% CO_2, ≥95% RH (non-coloured test chemicals), or 50 µL + 2 mL isopropanol mixed for 2-3h at RT (coloured test chemicals) → If the OD of the test chemical at 570±20 nm, after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	50 mg + 1 mL H_2O for 60 min at 37±2ºC, 5±1% CO_2, ≥95% RH (non-coloured test chemicals) and/or 50 mg + 2 mL isopropanol mixed for 2-3h at RT (coloured and non-coloured test chemicals) → If the OD of the test chemical at 570±20 nm after subtraction of the OD for isopropanol or water is > 0.08 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	10 µL + 90 µL H2O mixed for 30±2 min at Room Temperature (RT, 18-28oC) → If test chemical is coloured, living adapted controls should be performed	10 mg + 90 µL H2O mixed for 30±2 min at RT → If test chemical is coloured, living adapted controls should be performed	50 µL + 0.5 mL H2O water mixed for 15 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. If test chemical is coloured, living adapted controls should be performed	10 mg + 0.5 mL H2O mixed for 15 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. If test chemical is coloured, living adapted controls should be performed	40 µL + 1 mL H2O mixed for 60 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. → If the OD of the test chemical at 450±20 nm after subtraction of the OD for water is > 0.1 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.	40 mg + 1 mL H2O mixed for 60 minutes at 37±2ºC, 5±1% CO_2, ≥95% RH. → If the OD of the test chemical at 450±20 nm after subtraction of the OD for water is > 0.1 (which corresponds to approximately 5% of the mean OD of the negative control), living adapted controls should be performed.

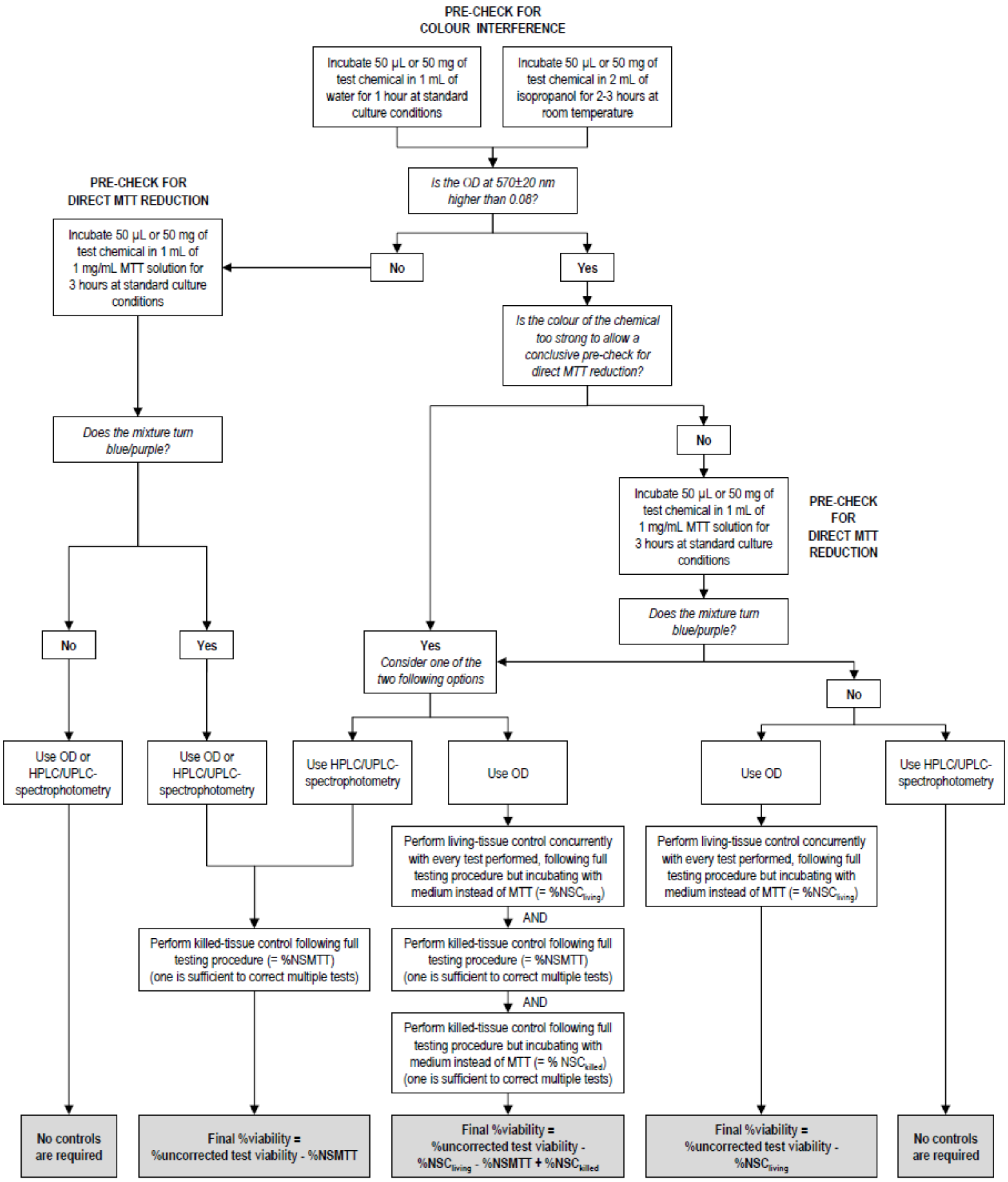
Nr.	1		2		3		4	
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**		**MCTT HCE™ EIT**	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Pre- check for direct tetrazolium reduction	50 µL + 1 mL MTT 1 mg/mL solution for 180±15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple,freeze-killed adapted controls should be performed (50 µL of sterile deionized water in MTT solution is used as negative control)	50 mg + 1 mL MTT 1 mg/mL solution for 180±15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed (50 µL of sterile deionized water in MTT solution is used as negative control)	30 µL + 300 µL MTT 1 mg/mL solution for 180± 15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µL of sterile deionized water in MTT solution is used as negative control)	30 mg + 300 µL MTT 1 mg/mL solution for 180± 15 min at 37±2ºC, 5±1% CO_2, ≥95% RH → if solution turns blue/purple, water-killed adapted controls should be performed (30 µL of sterile deionized water in MTT solution is used as negative control)	50 µL + 300 µL diluted WST-8 solution for 240±20 min at 37±2ºC, 5±1% CO_2, ≥95% RH → If solution turns yellow, freeze-killed adapted controls should be performed.	10 mg + 300 µL diluted WST-8 solution for 240±20 min at 37±2ºC, 5±1% CO_2, ≥95% RH → If solution turns yellow, freeze-killed adapted controls should be performed.	40 µL + 1 mL diluted WST-1 solution for 180±10 min at 37±2ºC, 5±1% CO_2, ≥95% RH → If solution turns yellow, freeze-killed adapted controls should be performed.	40 mg + 1 mL diluted WST-1 solution for 180±10 min at 37±2ºC, 5±1% CO_2, ≥95% RH → If solution turns yellow, freeze-killed adapted controls should be performed.
Pre-treatment	20 µL Ca2+/Mg2+-free DPBS for 30±2 min at 37±2ºC, 5±1% CO_2, ≥95% RH, protected from light.	20 µL Ca2+/Mg2+ -free DPBS for 30±2 min at 37±2ºC, 5±1% CO_2, ≥95% RH, protected from light.	-	-	-	-	-	-
Treatment doses and application	50 µL (83.3 µL/cm^2)	50 mg (83.3 mg/cm^2) using a calibrated tool (e.g., a levelled spoonful calibrated to hold 50 mg of sodium chloride).	10 µL Ca2+/Mg2+-free DPBS + 30±2 µL (60 µL/cm^2) For viscous, use a nylon mesh	30 µL Ca2+/Mg2+-free DPBS + 30±2 mg (60mg/cm^2)	50 µL (167 µL/cm^2)	10 mg (33 mg/cm^2)	40 µL (67 µL/cm^2)	40 µL Ca2+/Mg2+-free DPBS + 40 ±1 mg (67 mg/cm^2)

Nr.	1		2		3		4	
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**		**MCTT HCE™ EIT**	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Exposure time and temperature	30 min (±2 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	6 hours (±0.25 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	30 min (±2 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	4 hours (± 0.1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	1 min (± 5 second) in culture medium at RT	24 hours (± 1 hr) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	10 min (±1 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	3 hours (± 5min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH
Rinsing at room temperature	3 times in 100 mL of Ca^{2+}/Mg^{2+}-free DPBS	3 times in 100 mL of Ca^{2+}/Mg^{2+}-free DPBS	20 mL of Ca^{2+}/Mg^{2+}-free DPBS	25 mL of Ca^{2+}/Mg^{2+}-free DPBS	10 times or over by stream of Ca^{2+}/Mg^{2+} -free DPBS	10 times or over by stream of Ca^{2+}/Mg^{2+} -free DPBS	4 time with DPBS	4 times with 10 mL of DPBS and shaking in 30 mL of DPBS in the beaker
Post-exposure immersion	12 min (±2 min) at RT in culture medium	25 min (±2 min) at RT in culture medium	30 min (±2 min) at 37oC, 5% CO_2, 95% RH in culture medium	30 min (±2 min) at RT in culture medium	-	-	-	-
Post-exposure incubation	120 min (±15 min) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	18 h (±0.25 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	none	18 h (± 0.5 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	24 h (± 1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	none	16 h (± 1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	16 h (± 1 h) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH
Negative control	50 µL H_2O Tested concurrently	50 µL H_2O Tested concurrently	30 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently	30 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently	50 µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently	No treatment Tested concurrently	40 µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently	40 µL Ca^{2+}/Mg^{2+}-free DPBS Tested concurrently
Positive control	50 µL Methyl acetate Tested concurrently	50 µL Methyl acetate Tested concurrently	30 ± 2µL Methyl acetate Tested concurrently	30 ± 2µL Methyl acetate Tested concurrently	50 µL Ethanol Tested concurrently	10 mg Lauric acid Tested concurrently	40 µL Methyl acetate or 2% SDS Tested concurrently	40 µL Methyl acetate or 2% SDS Tested concurrently
Tetrasolium salt solution	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL 1 mg/mL	300 µL of diluted WST-8 solution (10 fold dilution of CCK-8)	300 µL of diluted WST-8 solution (10 fold dilution of CCK-8)	300 µL of diluted WST-1 solution (25 fold dilution of WST-1)	300 µL of diluted WST-1 solution (25 fold dilution of WST-1)

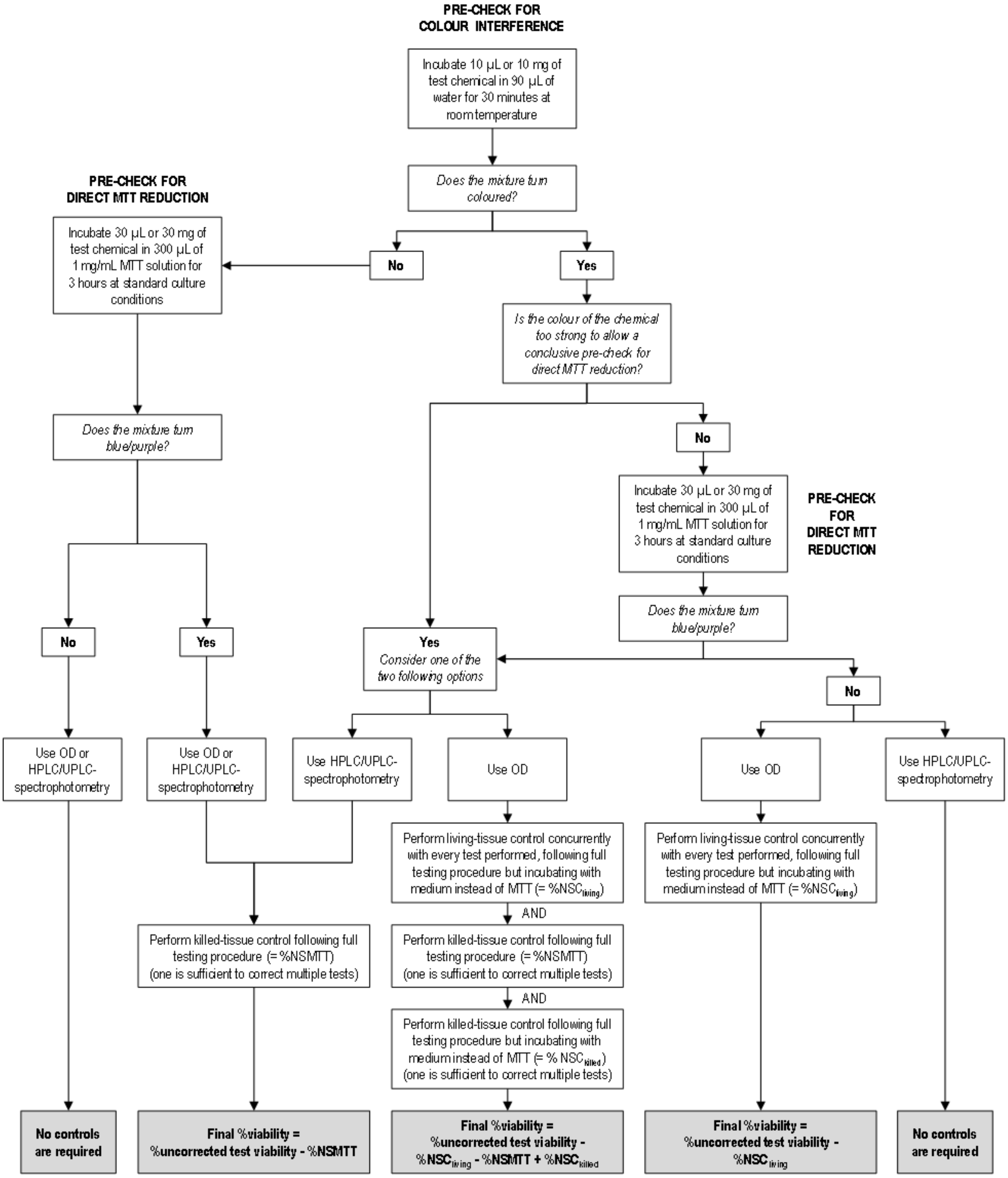
Nr.	1		2		3		4	
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**		**MCTT HCE™ EIT**	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Tetrazolium salt incubation and temperature	180 min (±15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (±15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (±15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (±15 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	240 minutes (± 15 minutes) at 37±2ºC, 5±1% CO_2, ≥95% RH	240 minutes (± 15 minutes) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (±5 min) at 37±2ºC, 5±1% CO_2, ≥95% RH	180 min (±5 min) at 37±2ºC, 5±1% CO_2, ≥95% RH
Extraction solvent	2 mL isopropanol (extraction from top and bottom of insert by piercing the tissue)	2 mL isopropanol (extraction from top and bottom of insert by piercing the tissue)	1.5 mL isopropanol (extraction from top and bottom of insert)	1.5 mL isopropanol (extraction from top and bottom of insert)	Not required	Not required	Not required	Not required
Extraction time and temperature	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	2-3 h with shaking (~120 rpm) at RT or overnight at 4-10°C	4 h with shaking (~120 rpm) at RT or at least overnight without shaking at 4-10°C	At least 2 h with shaking (~120 rpm) at RT	Not required	Not required	Not required	Not required
OD reading	570 nm (550 - 590 nm) without reference filter	570 nm (550 - 590 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	570 nm (540 - 600 nm) without reference filter	450 nm with reference filter (650 nm)	450 nm with reference filter (650 nm)	450 nm (420-480 nm) without reference filter	450 nm (420-480 nm) without reference filter
Tissue Quality Control	Treatment with 100 µL of 0.3% (v/v) Triton X-100 12.2 min ≤ ET50 ≤ 37.5 min	Treatment with 100 µL of 0.3% (v/v) Triton X-100 12.2 min ≤ ET50 ≤ 37.5 min	30 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC50 ≤ 3.5 mg/mL	30 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC50 ≤ 3.5 mg/mL	60 minutes treatment with SDS (25 µL) 1.0 mg/mL ≤ IC50 ≤ 4.0 mg/mL	60 minutes treatment with SDS (25 µL) 1.0 mg/mL ≤ IC50 ≤ 4.0 mg/mL	Treatment with 50 µL of 0.3% (v/v) Triton X-100 17.6 min ≤ ET50 ≤ 41.0 min	Treatment with 50 µL of 0.3% (v/v) Triton X-100 17.6 min ≤ ET50 ≤ 41.0 min

Nr.	1		2		3		4	
Test Method Component	**EpiOcular™ EIT (VRM 1)**		**SkinEthic™ HCE EIT (VRM 2)**		**LabCyte CORNEA-MODEL24 EIT**		**MCTT HCE™ EIT**	
Protocol[1]	Liquids (pipetteable at 37±1°C or lower temperatures for 15min)	Solids (not pipetteable)	Liquids and viscous (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)	Liquid (pipetteable)	Solids (not pipetteable)
Acceptance Criteria	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.8 2. Mean viability of the tissue replicates exposed for 30 minutes with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 0.8 and < 2.8 2. Mean viability of the tissue replicates exposed for 30 minutes with the positive control, expressed as % of the negative control, should be < 50% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 30 minutes with the positive control, expressed as % of the negative control, should be ≤ 30% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control, expressed as % of the negative control, should be ≤ 20% 3. The difference of viability between two tissue replicates should be less than 20%.	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 0.5 and ≤ 1.3. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 40% 3. The standard deviation (SD) between three tissue replicates should not exceed 18%	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 0.5 and ≤ 1.3. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 40% 3. The standard deviation (SD) between three tissue replicates should not exceed 18%	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 1.6 and ≤ 3.0. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 35% 3. The difference of viability between two tissue replicates should not exceed 20%	1. Mean OD of the tissue replicates treated with the negative control should be ≥ 1.6 and ≤ 3.0. 2. Mean viability of the tissue replicates treated with the positive control should be ≤ 35% 3. The difference of viability between two tissue replicates should not exceed 20%

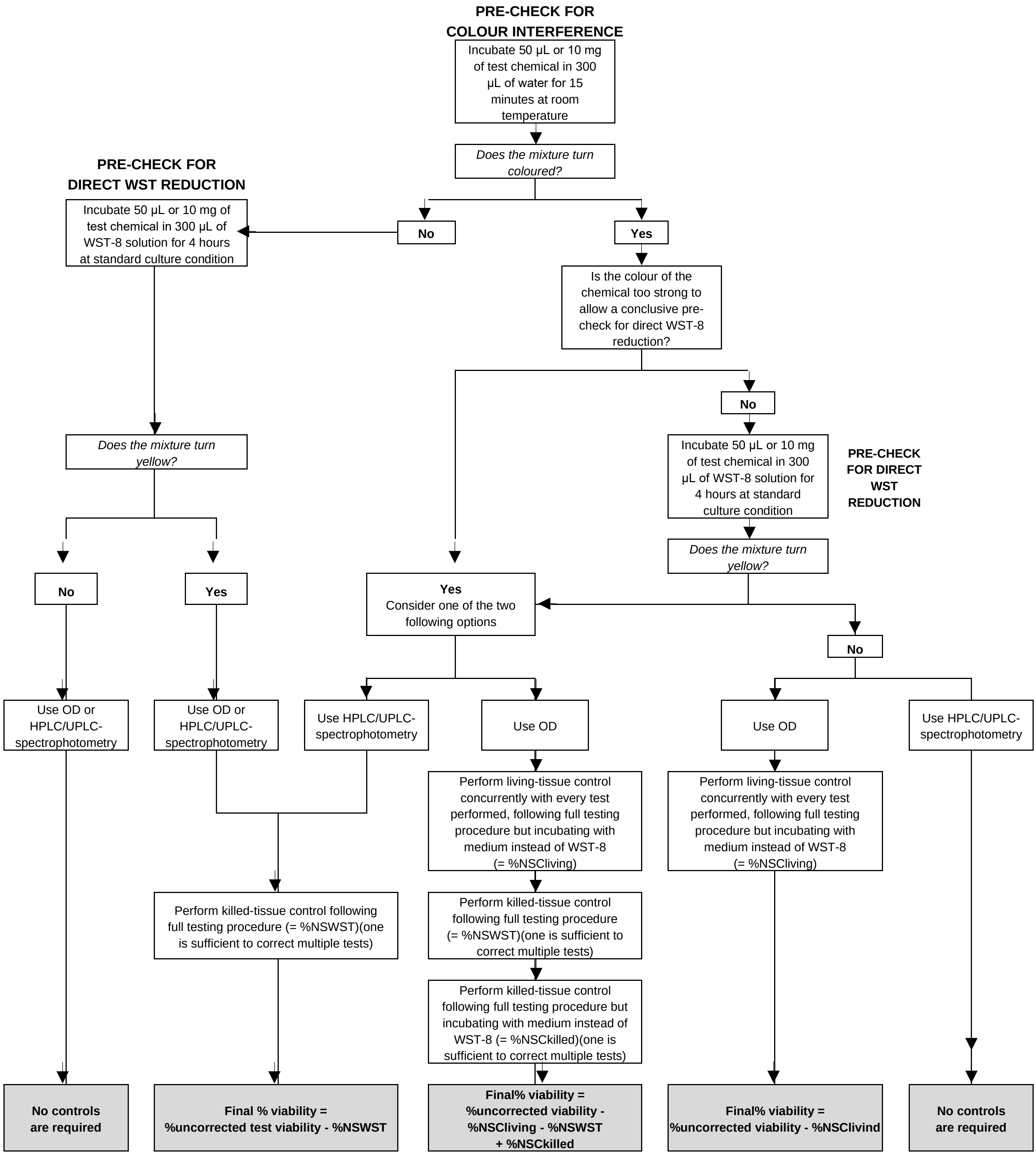
ANNEX III - ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM1 SOP



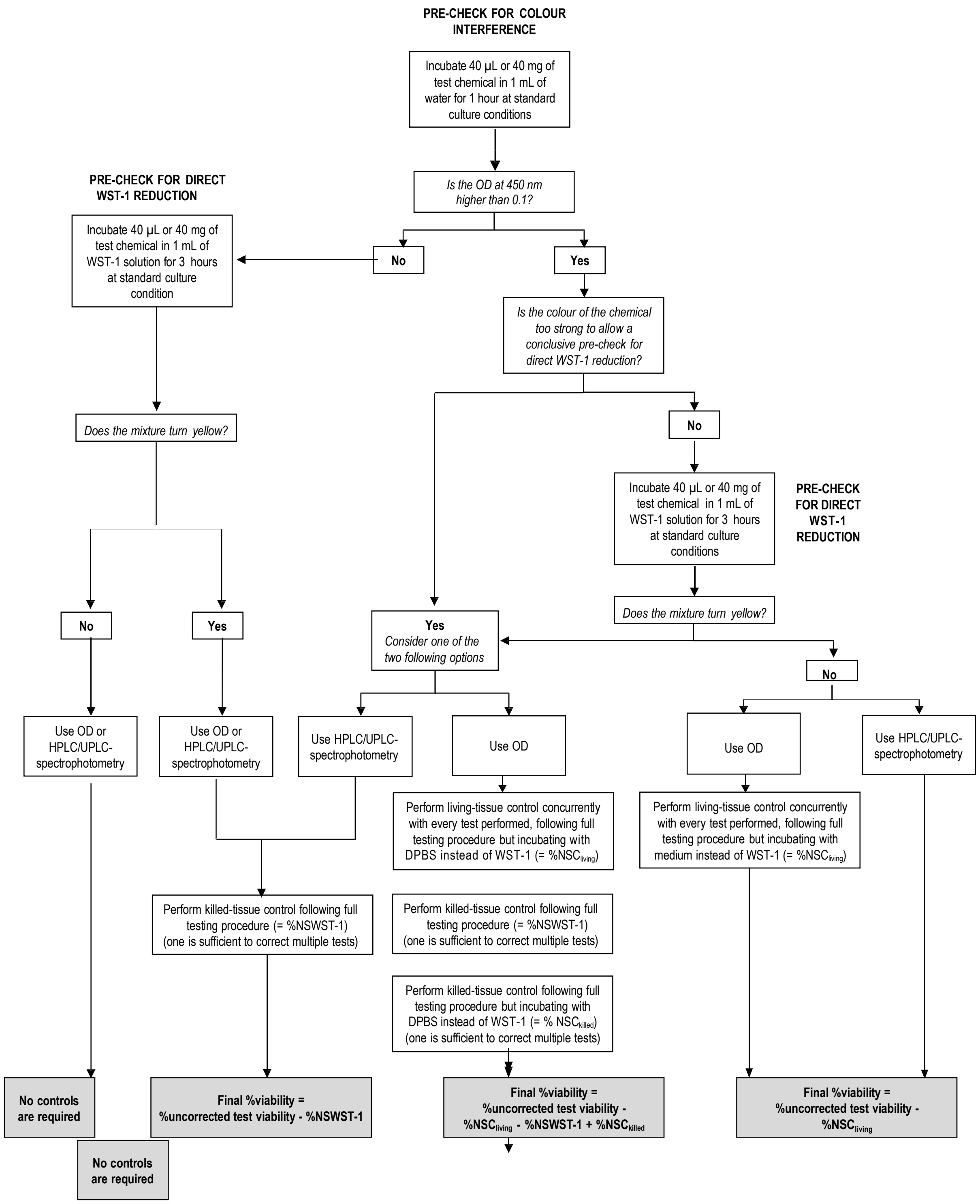
ANNEX IV - ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE VRM2 SOP



ANNEX V - ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT WST-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE LABCYTE CORNEA-MODEL24 EIT SOP



ANNEX VI - ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT WST-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE MCTT HCE™ EIT SOP



ANNEX VII - KEY PARAMETERS AND ACCEPTANCE CRITERIA FOR QUALIFICATION OF AN HPLC/UPLC-SPECTROPHOTOMETRY SYSTEM FOR MEASUREMENT OF MTT FORMAZAN EXTRACTED FROM RhCE TISSUE CONSTRUCTS

Parameter	Protocol Derived from FDA Guidance (43)(45)	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living RhCE tissue constructs without any treatment), dead blank (isopropanol extract from killed RhCE tissue constructs without any treatment), and of a dye (e.g., methylene blue)	$Area_{interference}$ ≤ 20% of AreaLLOQ[1]
Precision	Quality Controls (i.e., MTT formazan at 1.6 µg/mL, 16 µg/mL and 160 µg/mL) in isopropanol (n=5)	CV ≤ 15% or ≤ 20% for the LLOQ
Accuracy	Quality Controls in isopropanol (n=5)	%Dev ≤ 15% or ≤ 20% for LLOQ
Matrix Effect	Quality Controls in living blank (n=5)	85% ≤ %Matrix Effect ≤ 115%
Carryover	Analysis of isopropanol after an ULOQ[2] standard	$Area_{interference}$ ≤ 20% of $Area_{LLOQ}$
Reproducibility (intra-day)	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)	Calibration Curves: %Dev ≤ 15% or ≤ 20% for LLOQ Quality Controls: %Dev ≤ 15% and CV ≤ 15%
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in RhCE Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	%Dev ≤ 15%
Long Term Stability of MTT Formazan in RhCE Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C	%Dev ≤ 15%

[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.