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REVISED PERFORMANCE STANDARDS FOR THE ASSESSMENT OF PROPOSED SIMILAR OR MODIFIED IN VITRO RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM (RHCE) TEST METHODS FOR EYE HAZARD

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No. 216

PERFORMANCE STANDARDS FOR THE ASSESSMENT OF PROPOSED SIMILAR OR MODIFIED *IN VITRO* RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM (RHCE) TEST METHODS FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SERIOUS EYE DAMAGE, BASED ON THE VALIDATED REFERENCE METHOD EPIOCULAR™ EYE IRRITATION TEST DESCRIBED IN TG 492

(Intended for the developers of new or modified similar test methods)

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
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Paris 2015

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FOREWORD

This document includes Performance Standards (PS) *in vitro* Reconstructed Human Cornea-like epithelium (RhCE) test methods for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage, based on the validated reference method Epiocular™ EIT described in TG 492. The PS for TG 492 were initially developed by the European Union Reference Laboratory –European Centre for the Validation of Alternative Methods. The PS are intended for the developers of new or modified similar test methods to the validated reference method. They have been reviewed by the OECD Expert Group on Eye Irritation/Serious Eye Damage in November 2014. The present document was approved by the WNT in April 2015, declassified and published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides, and Biotechnology on 10 July 2015.

PERFORMANCE STANDARDS FOR THE ASSESSMENT OF PROPOSED SIMILAR OR MODIFIED *IN VITRO* RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM (RHCE) TEST METHODS FOR IDENTIFYING CHEMICALS NOT REQUIRING CLASSIFICATION AND LABELLING FOR EYE IRRITATION OR SERIOUS EYE DAMAGE, BASED ON THE VALIDATED REFERENCE METHODS EPIOCULAR™ EIT AND SKINETHIC™ HCE EIT DESCRIBED IN TG 492

- Second Edition -

(Intended for the developers of new or modified similar test methods)

These Performance Standards Document was approved by the 29th Meeting of the WNT in April 2017.

INTRODUCTION

1. The purpose of Performance Standards (PS) is to provide the basis by which new or modified test methods, both proprietary (*i.e.* copyrighted, trademarked, registered) and non-proprietary, can be deemed to be structurally and mechanistically similar to a Validated Reference Method (VRM) and demonstrate to have sufficient reliability and relevance for specific testing purposes (i.e., scientifically valid), in accordance with the principles of Guidance Document No. 34 (1). The PS, based on valid and accepted test method(s), can be used to evaluate the reliability and relevance of new test methods that are based on similar scientific principles and measure or predict the same biological or toxic effect (1). Such methods are referred to as *simila*r or "*me-too*" test methods. Moreover, the PS may be used to evaluate *modified* test methods, which may propose potential improvements in comparison to an approved earlier version of the test method. In such cases the PS should be used to determine the effect of the proposed changes on the test method's performance and the extent to which such changes may affect other information available from the validation process (e.g., relating to the definition of essential test method components). However, depending on the number and nature of the proposed changes as well as the data and documentation available in relation to these changes, modified test methods may be found unsuitable for a PS-based validation (e.g., if the changes are so substantial that the method is not any longer deemed sufficiently similar with regard to the PS). In such cases they should be subjected to the same validation process as described for a new test method (1). Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to Performance Standards, if those test methods have been reviewed and included in the respective Test Guideline by the OECD. Proposed new (me-too) or modified test methods considered similar to the VRMs according to these PS should therefore be submitted to the OECD for adoption and inclusion into Test Guideline 492 before being used for regulatory purposes.

2. New (me-too) or modified test methods proposed for use under Test Guideline 492 should be evaluated prior to their use for regulatory purposes to establish their similarity to the VRMs and to

determine their reliability and relevance to identify chemicals not requiring classification for serious eye damage/eye irritation according to UN GHS (2). The reliability and relevance of new (me-too) or modified test methods should be determined using Reference Chemicals (Table 3) representing the full range of the TG 405 *in vivo* serious eye damage/eye irritation responses, *i.e.*, Serious Eye Damage (UN GHS Category 1), Eye Irritation (UN GHS Categories 2A and 2B) and not-classified (UN GHS No Category) (2)(3). The proposed similar or modified test methods should have reliability and predictive capacity, which are equal to or better than defined minimum values derived from the VRMs [VRM1, EpiOcular™ Eye Irritation Test (EIT) (OCL-200) and VRM2, SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)] as described in paragraphs 26 to 28 of these PS (4)(5)(6)(7)(8)(9)(10).

3. The PS consist of (1): (i) Essential Test Method Components; (ii) Minimum List of Reference Chemicals, and; (iii) Defined Reliability and Accuracy Values that the proposed test method should meet or exceed.

ESSENTIAL TEST METHOD COMPONENTS

4. The Essential Test Method Components consist of essential structural, functional, and procedural elements of scientifically valid test method (VRMs) that should be included in the protocol of a proposed, mechanistically and functionally similar or modified test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a similar or modified proposed test method is based on the same concepts as the corresponding VRMs (10). The essential test method components are described in detail in the following paragraphs.

General conditions

5. Relevant human-derived cells (e.g., human corneal epithelial cells or keratinocytes) 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 the exposure time required to reduce tissue viability by 50% (ET_{50}) upon application of a benchmark substance at a specified, fixed concentration (e.g., 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% (IC_{50}) following a fixed exposure time (e.g., 30 minutes treatment with 50µL SDS) (see paragraph 10). 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. Furthermore the absence of fungi and bacteria contamination should be ensured for the RhCE tissue constructs.

Functional conditions

Viability

6. The assay used for quantifying tissue viability is the MTT assay (11). Viable cells of the RhCE tissue construct reduce the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS number 298-93-1] into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent). The extracted MTT formazan may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (9)(12). The OD of the extraction solvent alone should be sufficiently small, *i.e.*, OD < 0.1 at 570 nm using a filter band pass of maximum ±30 nm. Users of the RhCE tissue construct should ensure that each batch of the RhCE tissue construct used meets defined criteria for the negative control. As examples, acceptability ranges for the negative control OD values for the VRM1 and VRM2 are given in Table 1 (10). Adequate ranges should be provided for any new similar or modified test method. These may vary depending on the specific test method. An HPLC/UPLC-spectrophotometry user should use the defined negative control OD ranges as the acceptance criterion for the negative control. It should be documented that the tissues treated with the negative control substance are stable in culture (provide similar tissue viability measurements) for the duration of the test exposure period. 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 1 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 1: Acceptability ranges for negative control OD values of the VRMs (for the test method users)

Test Methods	Lower acceptance limit	Upper acceptance limit
EpiOcular™ EIT (OCL-200) – VRM1 (for both the liquids and the solids protocols)	> 0.8[1]	< 2.5
SkinEthic™ HCE EIT (HCE/S) – VRM2 (for both the liquids and the solids protocols)	> 1.0	≤ 2.5

[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 (13).

Barrier function

7. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, as estimated by e.g., ET_{50} (Triton X-100) or by IC_{50} (SDS) (Table 2). 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 users (see paragraph 10).

Morphology

8. 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).

Reproducibility

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

Quality control (QC)

10. 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 6) and barrier function (see paragraph 7) are the most relevant. An acceptability range (upper and lower limits) for the barrier function as measured either by the ET_{50} or by the IC_{50} (see paragraphs 5 and 7) should be established by the RhCE tissue construct developer/supplier. As examples, the ET_{50} and the IC_{50} acceptability range used as QC batch release criterion by the developers/suppliers of the RhCE tissue constructs (used in the VRMs) are given in Table 2. Adequate ranges should be provided for any new similar or modified test method. These may vary depending on the specific test method. 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 2: QC batch release criterion of the VRMs

Test Methods	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 min	ET_{50} = 37.5 min
SkinEthic™ HCE EIT (HCE/S) – VRM2 (30 minutes treatment with 50 µL SDS)	IC_{50} = 1 mg/mL	IC_{50} = 3.2 mg/mL

Procedural Conditions

11. At least two tissue replicates should be used for each test chemical and each control substance in each run. A sufficient amount of test chemical or control substance should be applied to uniformly cover the epithelial surface while avoiding an infinite dose (e.g., about 60-80 µL/cm^2 or 60-80 mg/cm^2). Whenever possible, solids should be tested as a fine powder. Two different treatment protocols may be used for different types of chemicals, e.g. for liquid test chemicals or for solid test chemicals. It may also be important that the tissue viability measurements are not made immediately after exposure to the test chemical, but rather after a sufficient post-exposure incubation period (in fresh medium) after the test chemical has been rinsed from the tissue. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects. If different protocols are used to test different types of chemicals (e.g., liquids and solids) they may differ in terms of their exposure, post-exposure immersion (if applicable) and post-exposure incubation (if applicable) periods.

12. 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 (%Viability$_{test}$). For example, the positive control substance used in the VRMs is neat methyl acetate (CAS No. 79-20-9; commercially available from e.g., Sigma-Aldrich, Cat# 186325; liquid). The negative control substances used in the VRM1 and the VRM2 are ultrapure H_2O, and calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (Ca^{2+}/Mg^{2+}-free DPBS), respectively. One single set of negative and positive controls is sufficient for all test chemicals included in the same run and tested with the same protocol.

13. The MTT assay is a standardised quantitative method (11) that should be used to measure tissue viability. It is compatible with use in a three-dimensional tissue construct. 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 isopropanol (or a similar solvent). There may be different ways to proceed for the extraction and what follows is an example of the procedure used in the VRMs for both the solids and the liquids protocols. In the VRMs, 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) while tissues tested with liquid test chemicals should be extracted from both the top and the bottom of the tissues. Tissues tested with liquid test chemicals that are not readily washed off may also be extracted from the bottom of the tissue only. The concurrently tested negative and positive control substances should be treated similarly to the tested chemical. The extracted MTT formazan may be quantified either by a standard absorbance (OD) measurement at 570 nm using a filter band pass of maximum ±30 nm or by using an HPLC/UPLC-spectrophotometry procedure (10)(12).

14. Optical properties of the test chemical or its chemical action on MTT may interfere with the measurement of MTT formazan leading to a false estimate of tissue viability. Test chemicals may interfere with the measurement of MTT formazan by direct reduction of the MTT into blue MTT formazan and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures, in the same OD range as MTT formazan (i.e., around 570 nm). Pre-checks should be performed before testing to allow identification of potential direct MTT reducers and/or colour interfering chemicals and additional controls should be used to correct for potential interference from such test chemicals (see paragraphs 15-18). Adherence to essential test method components will help to assure that a similar or modified proposed test method is based on the same concepts as the corresponding VRMs (10). As examples, illustrative flowcharts providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals for VRMs are described in Annexes III-IV of the TG 492 (10). 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 MTT assay is performed. For test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment), which are not compatible with the standard absorbance (OD) measurement of MTT formazan due to too strong interference, i.e., strong absorption at 570±30 nm, an HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed (12). A detailed description of how to detect and correct for direct MTT reduction and interferences by colouring agents should be available in the test method's SOP.

15. To identify potential interference by test chemicals absorbing light in the same range as MTT formazan (naturally or after treatment) and decide on the need for additional controls, spectral analysis of a test chemical in water (environment during exposure) and/or isopropanol (extraction solvent) should be performed. If a test chemical in water and/or isopropanol absorbs sufficient light in the range of 570±30 nm or if a coloured solution is obtained, the test chemical is presumed to interfere with the standard absorbance (OD) measurement of MTT formazan and further colourant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required (see paragraphs 18). 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 MTT solution during the MTT incubation step, to generate a non-specific colour in living tissues (NSC_{living}) control (14)(15)(16). The NSC_{living} control needs to be performed concurrently to the testing of the coloured test chemical and, in case of multiple testing, an independent NSC_{living} 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 MTT solution ($\%Viability_{test}$) minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected ($\%NSC_{living}$), i.e., True tissue viability = $[\%Viability_{test}] - [\%NSC_{living}]$.

16. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. If the MTT mixture containing the test chemical (or suspension for insoluble test chemicals) turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhCE tissue constructs should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (12). 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 are usually prepared by exposure to low temperature ("freeze-killed"), but may also be prepared by prolonged incubation (e.g., at least 24 hours) in water ("water-killed"). Each MTT reducing test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure, to generate a non-specific MTT reduction (NSMTT) control (15)(16). A single NSMTT control is sufficient per test chemical regardless of the number of independent tests/runs

performed. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the MTT reducer (%$Viability_{test}$) minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT), i.e., True tissue viability = [%$Viability_{test}$] - [%NSMTT].

17. Test chemicals that are identified as producing both colour interference (see paragraph 15) and direct MTT reduction (see paragraph 16) should also require a third set of controls when performing the standard absorbance (OD) measurement, apart from the NSMTT and NSC_{living} 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 (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 16. This forces the use of NSMTT controls, by default, together with the NSC_{living} controls. Test chemicals for which both NSMTT and NSC_{living} controls are performed may be absorbed and retained by both living and killed tissues. Therefore, in this case, the NSMTT control may not only correct for potential direct MTT reduction by the test chemical, but also for colour interference arising from the absorption and retention of the test chemical by killed tissues. This could lead to double correction for colour interference since the NSC_{living} 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 (NSC_{killed}) needs to be performed. In this additional control, the test chemical is applied on at least two killed tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. A single NSC_{killed} control is sufficient per test chemical regardless of the number of independent tests/runs performed, but should be performed concurrently to the NSMTT control and with the same tissue batch. True tissue viability is calculated as: the percent tissue viability obtained with living tissues exposed to the test chemical (%$Viability_{test}$) minus %NSMTT minus %NSC_{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control ran concurrently to the test being corrected (%NSC_{killed}), i.e., True tissue viability = [%$Viability_{test}$] - [%NSMTT] - [%NSC_{living}] + [%NSC_{killed}].

18. NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT (following the procedure described in paragraph 16). NSMTT controls should also be used with test chemicals having a colour (intrinsic or appearing when in water) that impedes the assessment of their capacity to directly reduce MTT as described in paragraph 16. When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent negative control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as: %$Viability_{test}$ minus %NSMTT, as described in the last sentence of paragraph 16.

19. Non-specific MTT reduction and non-specific colour interferences may increase the OD (when performing standard absorbance measurements) of the tissue extract above the linearity range of the spectrophotometer. Non-specific MTT reduction can also increase the MTT formazan peak area (when performing HPLC/UPLC-spectrophotometry measurements) of the tissue extract above the linearity range of the spectrophotometer. It is thus important to determine the OD/peak area linearity range of the spectrophotometer before use with e.g., MTT formazan (CAS #57360-69-7), commercially available from e.g., Sigma-Aldrich (Cat# M2003). It should also be noted that direct MTT-reducers or direct MTT-reducers that are also colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-

spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed, although these are expected to occur in only very rare situations.

Acceptance Criteria

20. For each run, 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 paragraph 6. Similarly, tissues treated with the positive control substance, should show a mean tissue viability (relative to the negative control) within an historically established range, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method. The variability between tissue replicates of test chemicals and control substances should fall within the accepted limits also established from historical data. If either the negative control or positive control included in a run is outside of the accepted ranges, the run must be 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. Paragraph 29 provides more details on re-testing in case of non-qualified tests/runs during validation studies. Importantly, an increased frequency of non-qualified runs/tests may indicate problems with either the test system (i.e., the intrinsic RhCE tissue quality) or with the handling (e.g., shipment of the tissues, SOP execution). Therefore, occurrence of non-qualified runs/tests in validation studies should be carefully monitored and all non-qualified runs and non-qualified tests need to be reported.

Interpretation of Results and Prediction Model

21. The OD values/peak areas obtained with the replicate tissue extracts for each test chemical should be used to calculate the mean percent tissue viability (mean between tissue replicates) normalised to the negative control, which is set at 100%. A percentage tissue viability cut-off value for identifying test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) should be established. 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 cut-off. 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. In this case, further testing with other test methods will be required because RhCE test methods show a certain number of false positive results and cannot resolve between UN GHS Categories 1 and 2.

MINIMUM LIST OF REFERENCE CHEMICALS

22. Reference Chemicals are used to determine if the reliability (reproducibility) and relevance (predictive capacity) of a proposed similar or modified test method, proven to be structurally and functionally sufficiently similar to the VRMs, or representing a minor modification of the VRMs, are equal to or better than defined minimum values derived from the VRMs (4)(7)(8)(9). The 30 Reference Chemicals listed in Table 3 were mainly selected from the chemicals used in the validation studies of the VRMs (4)(8)(9). 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)(17) and the UN GHS

classification system (i.e., Categories 1, 2A, 2B, or No Category) (2); (iii) cover the various *in vivo* drivers of classification (18)(19); (iv) are representative of the chemical classes used in the validation studies (4)(8)(9); (v) cover a good and wide representation of organic functional groups (4)(8)(9); (vi) have chemical structures that are well-defined (4)(8)(9); (vii) are coloured and/or direct MTT reducers (4)(8)(9); (viii) cover the full range of *in vitro* responses based on high quality EpiOcular™ EIT and SkinEthic™ HCE EIT data (0 to 100% viability) (4)(8)(9); (ix) are commercially available; and (x) are not associated with prohibitive acquisition and/or disposal costs.

23. The 30 Reference Chemicals listed in Table 3 include chemicals representing different chemical classes (*i.e.* chemical categories based on organic functional groups), and are representative of the full range of TG 405 *in vivo* responses. The Reference Chemicals included in this list comprise 7 UN GHS Category 1, 4 UN GHS Category 2A, 4 UN GHS Category 2B and 15 UN GHS No Category (not-classified) chemicals. They represent the minimum number of chemicals that should be used to evaluate the reliability (reproducibility) and relevance (predictive capacity) of a proposed similar or modified test method able to identify chemicals not requiring classification and labelling for serious eye damage/eye irritation according to UN GHS (2). The exclusive use of these Reference Chemicals for the development/optimisation of new similar test methods should be avoided to the extent possible and the identity of all additional chemicals used for test method development (e.g., for setting the prediction model or exposure times) should be reported when submitting a PS-based validation study. In situations where a listed chemical is unavailable or cannot be used for other justified reasons, another chemical fulfilling the criteria described in paragraph 22 above, e.g., from the chemicals used in the validation of the VRMs, could be used. Such deviations should however be justified. To further evaluate the accuracy of the proposed test method, additional chemicals representing other chemical classes and for which adequate *in vivo* reference data are available may be tested in addition to the Reference Chemicals listed in Table 3.

Table 3: Minimum list of Reference Chemicals for determination of Reliability and Predictive Capacity for similar or modified *in vitro* RhCE-based test methods

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM1 Viability (%)[2]	VRM2 Viability (%)[3]	Prediction of VRM1[4]	Prediction of VRM2[4]	MTT Reducer in VRM	Colour Interfer. in VRM
***In Vivo* Category 1[5]**									
(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	Aliphatic amine, primary; Aliphatic amine, secondary; Alkoxy silane	L	22.7±8.4	36.1±17.3[10]	No prediction can be made	No prediction can be made	N	N
Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	10.9±6.4	5.5±7.4	No prediction can be made	No prediction can be made	Y (strong)	N
Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	L	7.5±4.7[6]	1.6±1.0	No prediction can be made	No prediction can be made	N	N
1,2-Benzisothiazol-3(2H)-one	2634-33-5	Benzo-thiazolinone; Benzo-isothiazolinone	S	2.3±0.3	0.6±0.2	No prediction can be made	No prediction can be made	N	N
2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	S	2.3±0.2	0.2±0.1	No prediction can be made	No prediction can be made	N	N
Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	Alkene; Biphenyl; Sulfonic acid	S	16.2±3.1	14.6±6.7	No prediction can be made	No prediction can be made	N	N
Sodium oxalate	62-76-0	Oxocarboxylic acid	S	29.0±1.2	5.3±4.1	No prediction can be made	No prediction can be made	N	N
***In Vivo* Category 2A[5]**									
2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)[7]	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	4.0±1.1	1.3±0.6	No prediction can be made	No prediction can be made	N	Y (weak)

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM1 Viability (%)[2]	VRM2 Viability (%)[3]	Prediction of VRM1[4]	Prediction of VRM2[4]	MTT Reducer in VRM	Colour Interfer. in VRM
gamma-Butyrolactone	96-48-0	Lactone; Oxolane; Saturated heterocyclic fragment	L	10.4±4.7	5.5±3.0	No prediction can be made	No prediction can be made	N	N
2-Amino-3-hydroxy pyridine	16867-03-1	Heterocyclic Phenol	S	30.9±18.0	23.0±17.3	No prediction can be made	No prediction can be made	Y (weak)	N
Sodium benzoate	532-32-1	Aryl; Carboxylic acid	S	3.5±2.6	0.6±0.1	No prediction can be made	No prediction can be made	N	N
***In Vivo* Category 2B[5]**									
2-Methyl-1-pentanol	105-30-6	Alcohol; Alkane, branched with tertiary carbon	L	15.1±7.2	10.5±15.8[9]	No prediction can be made	No prediction can be made	N	N
Diethyl toluamide	134-62-3	Benzamide	L	15.6±6.3	2.8±0.9	No prediction can be made	No prediction can be made	N	N
1,4-Dibutoxy benzene	104-36-9	Alkoxy; Aryl; Ether	S	107.0±9.3	108.4±10.7	No Cat	No Cat	N	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	No prediction can be made	No prediction can be made	N	N
***In Vivo* No Category[5]**									
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.9±6.4	79.4±6.2	No Cat	No Cat	N	N
2-Ethoxyethyl methacrylate	2370-63-0	Alkoxy; Ether; Methacrylate	L	49.4±10.3[8]	21.8±17.9	No prediction can be made	No prediction can be made	N	N
3-Phenoxybenzyl alcohol	13826-35-2	Alcohol; Benzyl; Ether	L	34.3±12.0	2.2±0.8	No prediction can be made	No prediction can be made	Y (weak)	N

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM1 Viability (%)[2]	VRM2 Viability (%)[3]	Prediction of VRM1[4]	Prediction of VRM2[4]	MTT Reducer in VRM	Colour Interfer. in VRM
4-(Methylthio)-benzaldehyde	3446-89-7	Aldehyde; Aryl; Sulfide	L	63.1±17.0[10]	67.5±8.4[11]	No Cat	No Cat	N	N
Dipropyl disulphide	629-19-6	Disulfide	L	81.7±6.4	58.3±8.3	No Cat	No prediction can be made	N	N
Ethyl thioglycolate	623-51-8	Carboxylic acid ester; Thioalcohol	L	23.2±15.2	2.2±3.1	No prediction can be made	No prediction can be made	Y (strong)	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	104.2±4.2	96.5±3.5	No Cat	No Cat	N	N
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	77.6±5.4	89.1±2.9	No Cat	No Cat	N	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	No Cat	No Cat	N	N
2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	3179-89-3	Alcohol; Aromatic amine; Azo; Nitrobenzene	S	8.3±8.9	112.9±5.5	No prediction can be made	No Cat	Y (weak)	Y (medium)
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	No Cat	No Cat	N	N
4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	Benzyl; Phenol; tert-Butyl	S	106.7±13.7	97.6±5.7	No Cat	No Cat	N	N

Chemical Name	CASRN	Organic Functional Group[1]	Physical State	VRM1 Viability (%)[2]	VRM2 Viability (%)[3]	Prediction of VRM1[4]	Prediction of VRM2[4]	MTT Reducer in VRM	Colour Interfer. in VRM
Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	Alcohol; Ammonium salt; Ether	S	62.6±3.1[12]	72.3±4.3	No Cat	No Cat	N	N
Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	S	88.6±3.3	92.9±5.1	No Cat	No Cat	N	N
Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	Dihydroxyl group; Enol; Furanone; Furanondione; Phosphate ester	S	3.4±0.8	3.9±3.1	No prediction can be made	No prediction can be made	N	N

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; VRM1 = Validated Reference Method, EpiOcular™ EIT; VRM2 = Validated Reference Method, SkinEthic™ HCE EIT; Colour Interfer. in VRM = colour interference with the standard absorbance (OD) measurement of MTT formazan in validated reference methods.

[1]Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis (4).

[2]Based on results obtained with EpiOcular™ EIT in the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) (4).

[3] Based on results obtained with SkinEthic™ HCE EIT in its validation study (8)(9).

[4]When discordant results were obtained within and/or between laboratories in the validation study, the prediction of the VRM1 and VRM2 indicated in the table is based on the mode of all predictions (see footnotes 8, 9, 10 and 11 below). Incorrect VRM predictions (false positives or false negatives) are underlined.

[5]Based on results from the *in vivo* rabbit eye test (OECD TG 405) (3)(17) and using the UN GHS (2).

[6]Based on results obtained in the CEFIC CONsortium for *in vitro* Eye Irritation testing strategy (CON4EI) Study (manuscript in preparation).

[7]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.

[8]The VRM prediction is based on the mode of all predictions obtained in the validation study. Discordant results obtained in one of three laboratories (laboratory 1) and between laboratories.

[9]The VRM prediction is based on the mode of all predictions obtained in the validation study. Discordant results obtained in one of three laboratories (laboratory 2) and between laboratories

[10]The VRM prediction is based on the mode of all predictions obtained in the validation study. Discordant results obtained in one of three laboratories (laboratory 3) and between laboratories.

[11]The VRM prediction is based on the mode of all predictions obtained in the validation study. Discordant results obtained in two of three laboratories (laboratories 1 and 2) and between laboratories

[12]The VRM prediction is based on the mode of all predictions obtained in the validation study. Discordant results obtained in one laboratory, assumed to be non-reproducible in other laboratories as well as between laboratories.

DEFINED RELIABILITY AND ACCURACY VALUES

24. For purposes of establishing the reliability and relevance of proposed similar or modified RhCE test methods to be used by several independent laboratories, all of the 30 Reference Chemicals listed in Table 3 should be tested in at least three laboratories. In each laboratory, all Reference Chemicals should be tested in three independent runs performed with different tissue batches and at sufficiently spaced time points. Each run should consist of at least two concurrently tested tissue replicates for each test chemical, negative control substance, positive control substance and adapted controls for direct MTT reduction and/or colour interference, when applicable.

25. The calculation of the within-laboratory reproducibility, between-laboratory reproducibility, accuracy, sensitivity and specificity values of the proposed test method should be done according to the rules described below to ensure that a predefined and consistent approach is used:

1. Within-laboratory reproducibility (WLR) should be calculated based on concordance of classifications using only qualified tests obtained with Reference Chemicals for which at least two qualified tests are available. In addition, it should be reported the number and identity of the Reference Chemicals which per laboratory have none or only one qualified test (omitted from WLR calculations), as well as how many and which Reference Chemicals per laboratory have two or three qualified tests (used for WLR calculations).

2. For the calculation of between-laboratory reproducibility (BLR) the final classification for each Reference Chemical in each participating laboratory should be obtained by using the arithmetic mean value of viability over the different qualified tests performed. BLR should be calculated based on concordance of classifications using only qualified tests from Reference Chemicals for which at least one qualified test per laboratory is available. It should be reported how many and which Reference Chemicals do not have at least one qualified test per laboratory (omitted from BLR calculations), as well as how many and which Reference Chemicals have 3, 4, 5, 6, 7, 8 or 9 qualified tests that can be used to calculate BLR (with at least one qualified test per laboratory).

3. The calculation of predictive capacity (i.e., sensitivity, false negatives, specificity, false positives and accuracy) should be done using all qualified tests obtained for each Reference Chemical in each of at least three laboratories. The calculations should be based on the individual predictions of each qualified test for each Reference Chemical in each laboratory and neither on the arithmetic mean values of viability over the different qualified tests performed nor on the mode of all predictions obtained (or any other procedure used to summarise the multiple test results obtained into a single prediction per Reference Chemical). The predictive capacity should be determined using a weighted calculation in which the final outcome of each individual qualified test obtained for each Reference Chemical (from all laboratories participating in the validation study) is captured as an independent prediction in the calculations and correction factors are applied so that all Reference Chemicals have an equal weight in the calculations, even if it wasn't possible to obtain the same number of qualified tests for all Reference Chemicals during the validation study after re-testing (see paragraph 29). In summary, the "classified" and "not-classified" predictions for each Reference Chemical (obtained by the various laboratories participating in the study) should be divided by the total number of available predictions to determine the number of correct and under- or over-predictions for that chemical (as fractions of 1) and these should be used to calculate sensitivity, false negatives, specificity, false positives and accuracy so that all chemicals contribute with an equal weight of 1 in the calculations.

In this context, a qualified test consists of a test that meets the criteria for an acceptable test, as defined in the corresponding SOP, and is within a qualified run. Otherwise, the test is considered as non-qualified. A

qualified run consists of a run that meets the test acceptance criteria for the negative control and positive control, as defined in the corresponding SOP. Otherwise, the run is considered as non-qualified.

Within-laboratory reproducibility

26. The within-laboratory reproducibility of the proposed similar or modified test method should be equal to or better than the defined value given below, which was derived from the VRMs. An assessment of within-laboratory reproducibility should show in every laboratory a concordance of predictions (classified or not-classified) obtained in different, independent tests of the 30 Reference Chemicals listed in Table 3 equal or higher (≥) than 90% (actual for EpiOcular™ EIT: 93.3%, 93.3% and 96.7% and for SkinEthic™ HCE EIT: 96.7%, 93.3% and 90.0% in each laboratory, respectively).

Between-laboratory reproducibility

27. The between-laboratory reproducibility of the proposed similar or modified test method should be equal to or better than the defined value given below, which was derived from the VRM. An assessment of between-laboratory reproducibility should show a concordance of classifications (classified or not-classified) obtained for the 30 Reference Chemicals listed in Table 3 between a minimum of three laboratories equal or higher (≥) than 85% (actual for EpiOcular™ EIT and SkinEthic™ HCE EIT: 90.0%).

Predictive capacity

28. The predictive capacity of the proposed similar or modified test method should be equal to or better than the defined values given below, which were derived from the VRM. The sensitivity, specificity and accuracy obtained with the 30 Reference Chemicals listed in Table 3 should be equal or higher (≥) than 90%, 60% and 75%, respectively (Table 4). Furthermore, none of the UN GHS Category 1 Reference Chemicals should be under-predicted in the majority of the qualified tests obtained by the laboratories participating in the validation study.

Table 4: Required sensitivity, specificity and accuracy for similar or modified RhCE test method to be considered valid to identify chemicals not requiring classification for serious eye damage or eye irritation. Values are based on the results of the VRM1: EpiOcular™ EIT and VRM2: SkinEthic™ HCE EIT for the 30 Reference Chemicals listed in Table 3

Sensitivity	Specificity	Accuracy
≥ 90% (actual values: 93.3% (VRM1) - 91.3% (VRM2))	≥ 60% (actual values: 63.3% (VRM1) – 73.0% (VRM2))	≥ 75% (actual values: 78.2% (VRM1) – 82.2% (VRM2))

Study Quality Criteria

29. It is possible that one or several tests pertaining to one or more Reference Chemicals does/do not meet the test acceptance criteria (non-qualified tests), or is/are not acceptable for technical reasons or because they were obtained in a non-qualified run (due to failure of the concurrent positive and/or negative controls). To complement missing data, a maximum of two additional tests for each Reference Chemical is admissible per laboratory ("re-testing"). More precisely, since in case of re-testing also the positive and negative control substances have to be concurrently tested, a maximum number of two additional runs may be conducted for each Reference Chemical in each laboratory. Non-qualified tests should be documented

and reported. Importantly, each laboratory should not produce more than three qualified tests per Reference Chemical. Excess production of data and subsequent data selection are regarded as not appropriate. All tested tissues should be reported. The extent of unacceptable tests/runs should be documented and the basis for the likely cause of each should be provided.

30. It is conceivable that even after re-testing, three qualified tests are not obtained for every Reference Chemical in every participating laboratory, leading to an incomplete data matrix. In such cases the following three criteria should all be met in order to consider the datasets acceptable for purposes of PS-based validation studies:

1. All Reference Chemicals should have at least one complete test sequence in one laboratory.
2. Each of at least three participating laboratories should have a minimum of 85% complete test sequences (for 30 Reference Chemicals: 4 incomplete test sequences are allowed per laboratory).
3. At least 90% of all test sequences from at least three laboratories need to be complete (for 30 Reference Chemicals tested in 3 laboratories: a total of 9 incomplete test sequences are allowed).

In this context, a test sequence consists of the total number of independent tests performed for a single Reference Chemical in a single laboratory, including any re-testing (a total of 3 to 5 tests). A complete test sequence consists of a test sequence containing three qualified tests. A test sequence containing less than 3 qualified tests is considered as incomplete.

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