Section 4
Health effects

Test Guideline No. 492B
Reconstructed Human Cornea-like Epithelium (RHCE) Test Method for Eye Hazard Identification

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Reconstructed Human Cornea-like Epithelium (RHCE) Test Method for Eye Hazard Identification

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 (No Cat).

2. The assessment of serious eye damage/eye irritation has initially involved the use of laboratory animals (OECD Test Guideline (TG) 405; adopted in 1981 and revised in 1987, 2002, 2012 and 2017) (2). *In vitro* or *ex vivo* test methods have been adopted as OECD Test Guidelines (TGs) 437 (4), 438 (5), 460 (6), 491 (7), 492 (8), 494 (9) and 496 (10) to identify either chemicals for serious eye damage potential and/or to identify chemicals not requiring classification for eye hazard potential. The choice of the most appropriate test method to be used should be considered 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 TG describes an *in vitro* procedure allowing the identification on its own of chemicals (substances and mixtures) not requiring classification (No Cat), requiring classification for eye irritation (Cat 2) and requiring classification for serious eye damage (Cat 1) according to the UN GHS ocular hazard categories (1).

4. This TG describes a validated test method, namely the SkinEthic™ Human Corneal Epithelium (HCE) Time-to-Toxicity (TTT) test using a commercially available reconstructed Human Cornea-like Epithelium (HCE). The RhCE is designed to closely mimic the histological, morphological, biochemical and physiological properties of the human corneal epithelium. A validation study for assessing the three UN GHS ocular hazard categories has been conducted (11)(12)(13) on this method, referred to in the following text as the Validated Reference Method (VRM). From the validation study and its independent peer review (14) it was concluded that the SkinEthic™ HCE TTT is able to correctly identify chemicals (both substances and mixtures) by discriminating the three UN GHS categories for serious eye damage/eye irritation, i.e. UN GHS Cat. 1, Cat. 2 and No Cat chemicals (1), and the test method was recommended as a full replacement to the *in vivo* Draize acute eye irritation test for classification of chemicals. It is recognized that the use of this test guideline is subject to national and international regulatory considerations and conditions. The Guidance Document No. 263 on IATA should be consulted for further testing with other adequate *in vitro* tests in a weight-of-evidence approach, if deemed necessary (3). Annexes II-V provide

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a synopsis of the important elements of the test method, as well as flowcharts providing guidance for specific situations.

5. The purpose of this TG 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 reduction of a vital dye (MTT [3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide; CAS RN 298-93-1]) hereafter designated as the tetrazolium dye (TD) (15) (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) for the two or three exposure times, and is then used to predict the eye hazard potential of the test chemical.

6. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. The SkinEthic™ HCE tissue construct is a three-dimensional model produced using cells from the species of interest which mimics the *in vivo* corneal epithelium(17). The test method directly measures cytotoxicity resulting from penetration of the chemical through the corneal epithelium and production of cell and tissue damage following chemical exposure, which is used to predict the eye hazard identification of a test chemical. Cell damage can occur by several modes of action (see paragraph 16), 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.

8. A total of 151 chemicals covering a variety of chemical types, chemical classes, chemical structures, as well as molecular weights, LogP and other physical-chemical properties have been tested in the validation study underlying this TG. The validation database covered 134 different organic functional groups (11)(12)(13) and all key *in vivo* drivers of classification (26)(27). The majority of these chemicals represented mono-constituent substances (a total of 151 substances, of which 16 were tested in dilution), but several multi-constituent substances (including surfactants or polymers) were also included in the study. In terms of physical state and according to UN GHS Categories (1), the 151 tested chemicals were distributed as follows: 70 liquids, comprising 21 Cat. 1, 25 Cat. 2 (incl. 16 Cat. 2A and 9 Cat. 2B) and 24 No Cat, and 81 solids, comprising 29 Cat. 1, 19 Cat.2, and 33 No Cat. (11)(12)(13).

9. The SkinEthic™ HCE TTT is not intended to discriminate between UN GHS Category 2A (eye irritation, effects fully reversible within 21 days) and UN GHS Category 2B (mild eye irritation, effects fully reversible within 7 days). This differentiation needs to be addressed by other methods or approaches, if discrimination is deemed necessary (3).

10. This TG is applicable to substances and mixtures, to solids, liquids, semi-solids and waxes. The liquids may be aqueous or non-aqueous, solids may be soluble or insoluble in water. Caution should be used when testing solid chemicals with poor water solubility (< 0.014 mg/mL) as they were frequently underpredicted by SkinEthic™ HCE TTS in the validation study (e.g., 5 out of 9 Cat.1 chemicals were underpredicted to be Cat.2). 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 TG does not allow testing of gases and aerosols. Limited information is currently available on the applicability of the test method to multi-constituent substances/mixtures (11). The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. In cases where evidence demonstrates the non-applicability of the TG to specific formulations, the TG should not be used for those formulations. When considering testing of difficult-to-test

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chemicals (e.g. unstable substances), or test chemicals or mixtures 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.

11. 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 MTT (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 33-38).

12. The validation study carried out in three laboratories demonstrated that SkinEthic™ HCE TTT is transferable to laboratories considered to be naïve in the conduct of the assays and also is reproducible within- and between laboratories (13)(14). The within-laboratory reproducibility (WLR) for SkinEthic™ was 85-95% for TTL (20 chemicals) and 100% for TTS (20 chemicals). The between-laboratory reproducibility (BLR) was 90% for TTL and 100% for TTS.

13. The SkinEthic™ HCE TTT test 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 (13)(14) (Table 1), the SkinEthic™ HCE TTT test has a balanced accuracy of 74.4% (based on 151 chemicals) with correct predictions of 79% for Cat 1 (based on 50 chemicals), 69% for Cat 2 (based on 44 chemicals) and 75% for No Cat (based on 57 chemicals), when compared to reference *in vivo* rabbit eye test data (OECD TG 405) (2)(16) and classified according to the UN GHS classification system (1). The performance for SkinEthic™ HCE TTL and TTS is different (Annex V).

Table 1. The weighted[1] performance of the SkinEthic™ HCE TTT using the 3x3 matrix showing correct, under- and over- predictions per UN GHS category

UN GHS categories	SkinEthic HCE TTT - Predicted categories (n/N%)		
	Cat 1 (n)	Cat 2 (n)	No Cat (n)
Cat 1 (N=50)	79.2% (39.60)	20.8% (10.40)	0% (0.00)
Cat 2 (N=44)	18.3% (8.06)	69.2% (30.46)	12.5% (5.48)
No Cat (N=57)	1.8% (1.00)	23.3% (13.33)	74.9% (42.67)

14. The term "test chemical" is used in this TG 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

15. 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-soak

[1] In the weighted calculation, each chemical has an equal weight of 1 in the performance regardless of the number of test runs (i.e., in 3 overall test runs, if chemical A is tested Cat.1 two times and Cat.2 one time, a fractional weight of 0.66 (2/3) and 0.33 (1/3) is assigned to the number of predictions (n) for Cat.1 and Cat.2, respectively (11).

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incubation period. The SkinEthic™ HCE tissues are reconstructed from human immortalized 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 SkinEthic™ HCE 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 (17)(18).

16. 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)(20)(21). 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 (22). Moreover, the serious eye damage/eye irritation potential of a chemical is principally determined by the extent of initial injury (20), which correlates with the extent of cell death (22) and with the extent of the subsequent responses and eventual outcomes (23)(24). Thus, slight irritants generally only affect the superficial conjunctival and 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 endothelium (22)(25). 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.

17. RhCE tissue viability is classically measured by enzymatic conversion of MTT for the SkinEthic™ HCE TTT by the viable cells of the tissue into a formazan dye (FD), which is quantitatively measured after extraction from tissues (15).

18. The SkinEthic™ HCE TTT Test is based on two protocols, one for liquids (SkinEthic™ HCE TTL) and one for solids (SkinEthic™ HCE TTS). The SkinEthic™ HCE TTT uses three exposure times for TTL and two exposure times for TTS (see paragraphs 29-30). The SkinEthic™ HCE TTL and TTS protocols make use of different prediction models. For SkinEthic™ HCE TTL, a chemical that results in a mean viability below or equal to 50% within all time treatments will be classified as a Cat 1, and one that results in a mean viability strictly above 50% as a No Cat. Any other combination of mean viability values will lead to a Cat 2 classification (see paragraph 43). For SkinEthic™ HCE TTS, a chemical that results in a mean viability below or equal to 40% after 30 minutes. exposure and below or equal to 60% after 120 minutes. exposure will be classified as a Cat 1. A mean viability strictly above these cut-offs within the two time treatments will classify the chemical as a No Cat. Any other combination of mean viability values will classify the chemical as a Cat 2 (see paragraph 43).

DEMONSTRATION OF PROFICIENCY

19. Prior to routine use of the test method for regulatory purposes, laboratories should demonstrate technical proficiency by correctly predicting the fourteen proficiency chemicals listed in Table 2. These chemicals were selected from the chemicals used in the validation studies of the SkinEthic™ HCE TTT (13)(14). The selection includes, to the extent possible, chemicals that: (i) cover different physical states; (ii) cover the range of *in vivo* serious eye damage/eye irritation responses based on high quality results obtained in the reference *in*

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vivo rabbit eye test (OECD TG 405) (2)(16) and the UN GHS classification system (i.e., Categories 1, 2, or No Category) (1); (iii) cover the key *in vivo* drivers of classification (26)(27); (iv) are representative of the chemical classes used in the validation study (13); (v) cover a good and wide representation of organic functional groups (11)(12)(13); (vi) have chemical structures that are well-defined (11)(12)(13); (vii) produced reproducible results in the RhCE test method during the validation study; (viii) were correctly predicted by the RhCE test method during the validation study; (ix) cover the range of *in vitro* responses based on high quality of the test method data; (x) are commercially available; and (xi) 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 SkinEthic™ HCE TTT, could be used. Such deviations should however be justified.

Table 2: List of proficiency chemicals for SkinEthic™ HCE TTL (Table 2A) and for SkinEthic™ HCE TTS (Table 2B)

Table 2A. List of Proficiency Chemicals for SkinEthic™ HCE TTL

ChemicalName	CASRN	OrganicFunctionalGroup [1]	Physicalstate	Viability 1 5 min. (%) [2,3]	Viability 2 16 min. (%) [2,3]	Viability 3 120 min. (%) [2,3]	VRM Prediction
***In Vivo*Category1**							
N.N--Diethylethanolamine	100-37-8	Alcohol. Aliphatic Amine. tertiary	L	2.9±1.7	1.0±1.3	0.6±0.5	Cat 1
Acetic acid (10%)	64-19-7	Carboxylic acid	L	4.2±0.4	25.9±12.7	2.8±0.3	Cat 1
***In Vivo*Category2**							
2-Butanone	78-93-3	Ketone	L	21.1±5.4	90.8±13.7	87.6±12.3	Cat 2
Acetone	67-64-1	Ketone	L	6.4±2.6	97.1±3.9	99.1±3.5	Cat 2
Hexadecyltrimethylammonium chloride (2%)	112-02-7	Ammonium salt. Alkylammonium salt	L	58.0±10.7	62.1±9.2	2.4±0.8	Cat 2
***In Vivo*NoCategory**							
1.3-Diisopropylbenzene	99-62-7	Cyclic. Phenyl. Aromatic	L	100.5±8.5	94.5±6.3	96.5±9.3	No Cat
Dodecane	112-40-3	Methyl. Methylene	L	98.3±6.9	103.1±5.6	98.9±5.1	No Cat

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Table 2B. List of Proficiency Chemicals for SkinEthic™ HCE TTS

ChemicalName	CASRN	OrganicFunctionalGroup[1]	Physicalstate	Viability 1 30 min. (%)[2,3]	Viability 2 120 min. (%)[2,3]		VRM Prediction
***In Vivo*Category1**							
1-Naphthalene acetic acid Na salt	61-31-4	Benzyl. Carboxylic aromatic. Naphthalene	S	1.6±0.7	1.6±0.4		Cat 1
1.2-Benzisothiazol-3(2H)-one	2634-33-5	Benzothiazole/ Benzoisothiazole	S	4.0±1.0	4.0±0.4		Cat 1
***In Vivo*Category2**							
4-Carboxybenzaldehyde	619-66-9	Aldehyde. Aryl. Carboxylic acid	S	80.1±12.3	5.0±1.4		Cat 2
2-Hydroxy-1.4-naphthoquinone	83-72-7	Enol. Naphthoquinone	S	82.1±8.9	4.5±1.5		Cat 2
Ammonium nitrate	6484-52-2	Amine. Nitrate. Ammonium	S	61.0±4.8	1.8±0.4	-	Cat 2
***In Vivo*NoCategory**							
Magnesium carbonate	56378-72-4	Magnesium. Carbonate	S	98.3±7.1	93.4±11.7		No Cat
Anthracene	120-12-7	Anthracene. Carboxylic aromatic	S	97.7±6.6	98.1±4.7		No Cat

1. 3-Diisopropylbenzene
2. 2-Benzisothiazol-3(2H)-one

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; VRM = Validated Reference Method, SkinEthic™ HCE TTT.

[1] Organic functional group assigned according to an OECD QSAR Toolbox analysis (version 3.2;https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm).

[2] Based on results obtained with SkinEthic™ HCE TTT in the validation study (13)(14).

[3] For liquids, the SkinEthic™ HCE TTL uses three exposure-times : Test chemicals are applied neat for 5 minutes (viability 1), and diluted at 20% (w/v) for 16 minutes (viability 2) and 120 minutes (viability 3). For solids, the SkinEthic™ HCE TTS uses two exposure-times: Test chemicals are applied neat for 30 minutes (viability 1) and 120 minutes (viability 2) (see paragraph 29).

20. 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 24, 25 and 27). 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

21. The test method currently covered by this TG is the scientifically validated SkinEthic™ HCE TTT Test (14). The Standard Operating Procedures (SOP) for both SkinEthic™ HCE TTL and TTS are available and should be employed when implementing and using the test method in a laboratory (28)(29). The following paragraphs and Annex II describe the main components and procedures of the RhCE test method.

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RhCE TEST METHOD COMPONENTS

General Conditions

22. 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., sodium dodecyl sulphate (SDS). For example, the barrier function should be demonstrated and may be assessed by determination of 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 50 µL SDS (see paragraph 27). 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

The assay used for quantifying tissue viability is the tetrazolium dye (MTT) assay (15). 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). The extracted formazan dye may be quantified using either a standard absorbance (Optical Density (OD)) measurement or an HPLC/UPLC-spectrophotometry procedure (28)(29). The OD of the blank solution alone (which is the extraction solvent for MTT 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 SkinEthic™ HCE TTT are given in

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Table 3. For HPLC/UPLC-spectrophotometry, the negative control OD ranges provided in Table 3 should be used as 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

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23. Table 3 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.

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Table 3. Acceptability ranges for negative control OD values (for the test method users)

Test Method	Lower acceptance limit	Upper acceptance limit
SkinEthic™ HCE TTT(HCE/S) - VRM (for both TTL and TTS protocols)	> 1.0	≤ 2.5

Barrier function

24. The RhCE tissue construct should be sufficiently thick and robust to resist the rapid penetration of cytotoxic benchmark substances, as estimated e.g. by IC_{50} (SDS) (Table 4). 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 27).

Morphology

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

Reproducibility

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

Quality control (QC)

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, the most relevant among which are those for viability (paragraph 0) and barrier function. An acceptability range (upper and lower limits) for the barrier functions as measured by the IC_{50} should be established by the RhCE tissue construct supplier. The IC_{50} acceptability range used as QC batch release criterion by the supplier of the RhCE tissue constructs used in the test method is given in

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27. Table 4. Data demonstrating compliance with all production release criteria should be provided by the RhCE tissue construct 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 for eye hazard identification in accordance with UN GHS.

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Table 4. QC batch release criteria of the SkinEthic™ HCE tissue construct

Test Method	Lower acceptance limit	Upper acceptance limit
SkinEthic™ HCE TTT(HCE/S) - VRM (30 minutes treatment with 50 µL SDS)	IC50 = 1.0 mg/mL	IC50 =3.2 mg/mL

Application of the Test Chemical and Control Substances

28. At least two tissue replicates for each time-treatment point 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 (28)(29). For the SkinEthic™ HCE TTT, the tissue construct surface should be moistened with calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (Ca^{2+}/Mg^{2+}-free DPBS) for liquids' protocol before application of test chemicals or with water for solids' protocol, 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, a sufficient amount of test chemical or control substance should be applied to uniformly cover the epithelial surface.

29. Test chemicals that can be pipetted at 37°C or lower (using a positive displacement pipette, if needed) are treated as liquids in the test method, otherwise they should be treated as solids (see paragraph 30). Specific procedures for liquids that do not form a solution or a stable suspension are included in the SOP (28). In the test method, liquid test chemicals are evenly spread over the tissue surface (i.e. 160±2 µL/cm^2 application) (Annex II). SkinEthic™ HCE tissues are topically exposed for three different treatment times: Test chemicals are applied neat for 5 minutes, and diluted at 20% (w/v) in distilled water for 16 minutes and 120 minutes at the pre-defined conditions of the method (28). 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 Ca^{2+}/Mg^{2+}-free DPBS at room temperature. This rinsing step should be followed by a 10 minute post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue) prior to performing the MTT assay (Annex II)(28).

30. Test chemicals that cannot be pipetted (for example, highly viscous liquids) are treated as solids in the test method. The amount of test chemical applied should be sufficient to cover the entire surface of the tissue, i.e. a minimum of 160±2 mg/cm^2 application should be used (Annex II). Whenever possible, solids should be tested as a fine powder. SkinEthic™ HCE tissues are topically exposed to test chemicals for 30 and 120 minutes at standard culture conditions (see Annex II)(29). 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 Ca^{2+}/Mg^{2+}-free DPBS at room temperature. This rinsing step should be followed by a 30 minute post-exposure immersion in fresh medium at room temperature (to remove any test chemical absorbed into the tissue), prior to performing the TD assay (Annex II)(29).

31. 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 (%$Viability_{test}$). The recommended positive control substance to be used with the SkinEthic™ HCE TTT is neat methyl acetate (CAS RN. 79-20-9, commercially available) for liquids' protocol, and lactic acid at 1% (W/V) in water (CAS RN. 50-21-5, commercially available) for solids' protocol. The recommended negative control substance to be used is

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Ca^{2+}/Mg^{2+}-free DPBS for both liquids and solids protocols. These were the control substances used in the validation studies of the SkinEthic™ HCE TTT 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, and a post-exposure immersion, as described for controls run concurrently to liquid test chemicals (see paragraph 29) or for controls run concurrently to solid test chemicals (see paragraph 30), prior to performing the MTT assay (see paragraph 32) (26)(27). 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

32. The MTT assay is a standardised quantitative method (15) that should be used to measure tissue viability under this TG. It is compatible with use in a three-dimensional tissue construct. The MTT assay is performed immediately following the post-soak procedure. 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) (28)(29). 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 or 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. 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 (see paragraph 40).

33. Optical properties of the test chemical or its chemical action on MTT may interfere with the measurement of formazan dye (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 MTT into coloured FD (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 FD (i.e., MTT formazan: around 570 nm). Potential of chemicals to directly reduce TD and/or interfere with colour (only necessary for coloured test chemicals) should be checked before testing. In case of FD interference, additional controls should be used to correct for potential interference from such test chemicals (see paragraphs 34-36 and Annexes III). 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 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), an HPLC/UPLC-spectrophotometry procedure to measure FD may be employed (see paragraphs 39 and 40) (28). A detailed description of how to detect and correct for direct MTT reduction and interferences by colouring agents is available in the test method SOPs (28)(29). Illustrative flowcharts providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals are also provided in Annexes III.

34. 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

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test chemical is added to water and incubated for an appropriate time at room temperature (Annex II) (28)(29). If a coloured solution is obtained when mixing the test chemical with water (see Annex III), the test chemical is presumed to interfere with the standard absorbance (OD) measurement of FD; in such a case, further colorant controls should be performed or, alternatively, an HPLC/UPLC-spectrophotometry procedure should be used, which do not require these controls (see paragraphs 39 and 40 and Annexes III and IV). 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 (28)(29). 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 the MTT ($\%Viability_{test}$) 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 = [$\%Viability_{test}$] - [$\%NSC_{living}$].

35. To identify direct MTT reducers, each test chemical should be added to freshly prepared MTT solution. An appropriate amount of test chemical is added to a MTT solution and the mixture is incubated for approximately 3 hours at standard culture conditions (see Annex III) (28)(29). 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 the 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. 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 SkinEthic™ HCE TTT are prepared by prolonged incubation (e.g., at least 24±1 hours) in water followed by storage at low temperature ("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 (28)(29). 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 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), i.e. true tissue viability = [$\%Viability_{test}$] - [%NSMTT].

36. Test chemicals that are identified as producing both colour interference (see paragraph 34) and direct MTT reduction (see paragraph 35) will 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 for MTT formazan (e.g., blue, purple, black) because their intrinsic colour impedes the assessment of their capacity to directly reduce MTT as described in paragraph 35. 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 (Annex III) (28)(29). In this additional

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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] - [$\%NSC_{living}$] + [$\%NSC_{killed}$].

37. It is important to note that non-specific MTT 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 MTT 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.

38. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals, when the observed interference with the measurement of FD is not strong (i.e., the ODs of the samples obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer). Nevertheless, results for test chemicals producing %NSMTT and/or $\%NSC_{living} \geq 50\%$ for liquids protocol or 60% for solids protocol of the negative control should be taken with caution. 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 that interfere strongly with the standard absorbance (OD) measurement of FD may still be assessed using HPLC/UPLC-spectrophotometry (Annex III). This is because the HPLC/UPLC system allows for the separation of the FD from the chemical before its quantification. For this reason, NSC_{living} or NSC_{killed} controls are never required when using HPLC/UPLC-spectrophotometry, independently of the chemical being tested. NSMTT control should nevertheless be used if the test chemical is suspected to directly reduce MTT (following the procedure described in paragraph 35). NSMTT control 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 35. 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 MTT, true tissue viability is calculated as: $\%Viability_{test}$ minus %NSMTT as described in the last sentence of paragraph 35.

39. Finally, it should be noted that 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 samples that fall outside of the linearity range of the spectrophotometer cannot be assessed with the RhCE test method, although these are expected to occur in only very rare situations.

40. HPLC/UPLC-spectrophotometry may be used with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of FD. 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

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HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT 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 (30)(31). These key parameters and their acceptance criteria are shown in Annex IV. Once the acceptance criteria defined in Annex IV have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure FD under the experimental conditions described in this TG.

Acceptance Criteria

41. For each run using RhCE tissue batches that met the quality control (see paragraph 27), 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 3(see paragraph23). For the SkinEthic™ HCE TTL protocol, tissues treated with the positive control substance, i.e., methyl acetate, should show a mean tissue viability ≤ 50% at the time exposure of 5 minutes and > 50% at both times 16 and 120 minutes, relative to the negative control. For the SkinEthic™ HCE TTS protocol, tissues treated with the positive control substance, i.e., lactic acid at 1% (W/V) in water, should show a mean tissue viability > 40% relative to the negative control at the time exposure of 30 minutes and 20%< %Viability ≤ 70% after 120 minutes treatment, thus reflecting the ability of the tissues to respond to an irritant test chemical under the conditions of the test method (28)(29).

42. 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

43. 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 values used to identify and classify test chemicals for ocular hazard categories according to UN GHS classification; i.e. No Category (not classified), Category 2 (eye irritation) and Category 1 (serious eye damage) are given in Table 5. Results should thus be interpreted as follows:

- The test chemical is identified as a chemical not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and a post-soak incubation is more than (>) the established percentage tissue viability cut-off value within all-time treatments.
- The test chemical is identified as a chemical inducing serious eye damage according to UN GHS (Category 1) if the mean percent tissue viability after exposure and a post-soak incubation is less than or equal to (≤) the established percentage tissue viability cut-off value within all-time treatments.
- The test chemical is identified as a chemical inducing eye irritation according to UN GHS (Category 2) if the combination of mean percent tissue viability within all-time treatment fall outside of the criteria established to identify the test chemical as a No Cat or a Cat 1, as shown in Table 5.

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Table 5. Prediction Model according to UN GHS classification

	No Category	Category 2	Category 1
SkinEthic HCE TTL (for the liquids protocol)	Mean tissue viability > 50% within all-time treatments	Any other combination of values[1]	Mean tissue viability ≤ 50% within all-time treatments
SkinEthic HCE TTS(for the solids protocol)	Mean tissue viability > 40% after 30 minutes and > 60% after 120 minutes	Any other combination of values[1]	Mean tissue viability ≤ 40% after 30 minutes and ≤ 60% after 120 minutes

[1]Any combination of values other than those defined for No Cat or Cat 1.

44. 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 established percentage tissue viability cut-off value ±5% within the time treatments, a second test should be considered, as well as a third one in case of discordant results between the first two tests.

DATA AND REPORTING

Data

45. 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 MTT reduction and/or coloured interference should be reported for each tested chemical.

Test report

46. 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;

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

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)

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

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- Description of the HPLC/UPLC-spectrophotometry system used, if applicable; LLOQ, ULOQ, and results of calibration curves and QCs using the same type of fitting and weighting as in the validation of the HPLC/UPLC should be included in the reporting.
- 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 (supplier);
  - iv. Morphology, if available (supplier);
  - v. Other quality controls (QC) of the RhCE tissue construct, if available (supplier);
- 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 (e.g., version of the SOP);
- Doses of test chemical and control substances used;
- Duration and temperature of exposure, post-exposure immersion and post-exposure incubation periods (where applicable);
- Description of any modifications to the test procedure;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (positive control, negative control, NSMTT, 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 MTT-reducers and/or coloured test chemicals, including OD value or FD peak area, %NSMTT, %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;

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Discussion of the results

Conclusion

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Annex A. DEFINITIONS

Balanced Accuracy: Average of the proportion of the correct predictions of each category. The closeness of agreement between test method results and accepted reference values taking into account imbalance of the class distribution.

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.

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.

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) with the optional Categories 2A (effects fully reversible within 21 days) and 2B (effects fully reversible within 7 days).

Formazan dye (FD): Chromogenic product of the reduction of MTT.

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.

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

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

LLOQ: Lower Limit of Quantification.

LogP: Logarithm of the octanol-water partition 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).

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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”.

NSC_{killed}: Non-Specific Colour in killed tissues. NSC_{living}: Non-Specific Colour in living tissues. NSMTT: Non-Specific MTT reduction.

OD: Optical Density.

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 (32).

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 (32).

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 (32).

Reproducibility: The agreement among results obtained from repeated testing of the same test chemical using the same test protocol (See "Reliability") (32).

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.

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).

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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.

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

Test: A single test chemical concurrently tested in a minimum of two tissue replicates as defined in the corresponding SOP.

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

Test chemical: The term "test chemical" is used to refer to what is being tested.

TTL: SkinEthic™ HCE Time-to-Toxicity for Liquids chemicals

TTS: SkinEthic™ HCE Time-to-Toxicity for Solids chemicals

TTT: SkinEthic™ HCE Time-to-Toxicity test method

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 (31).

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 (31).

VRM: Validated Reference Method.

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

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Annex B. MAIN TEST METHOD COMPONENTS OF THE SKINETHIC™ HCE TTT TEST METHOD VALIDATED FOR EYE HAZARD IDENTIFICATION

Test Method Component	SkinEthic™ HCE TTT (VRM)	
Protocol[1]	TTL protocol: Liquid and viscous test chemicals (pipettable)	TTS protocol: Solid test chemicals (not pipettable)
Model surface	0.5 cm^2	0.5 cm^2
Number of tissue replicates	At least 2	At least 2
Pre- or post-check for colour interference	10 µL + 90 µL H_2O mixed for 30±2 min at Room Temperature (RT[2]) → If test chemical is coloured, living adapted controls should be performed	10 mg + 90 µL H_2O mixed for 30±2 min at RT → If test chemical is coloured, living adapted controls should be performed
Pre- or post-check for direct tetrazolium reduction	50-80 µL chemical + 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	30-80 mg chemical + 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

[1] For details of the protocol, the latest version of the Standard Operating Procedures (SOP) should be consulted and employed when implementing the test method in a laboratory (28-29).

[2] Room Temperature (RT): 18-28°C

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Test Method Component	SkinEthic™ HCE TTT (VRM)	
Protocol[1]	TTL protocol: Liquid and viscous test chemicals (pipettable)	TTS protocol: Solid test chemicals (not pipettable)
	adapted controls should be performed (50-80 µL of sterile deionized water in MTT solution is used as negative control)	adapted controls should be performed (30-80 µL of sterile deionized water in MTT solution is used as negative control)
Treatment doses and application	10 µL Ca^{2+}/Mg^{2+}-free DPBS + 80±2 µL (160 µL/cm^2) For viscous, use a nylon mesh	80±2 mg (160 mg/cm^2) (grounded if needed to cover the whole tissue surface) + 80 µL distilled water Use a nylon mesh
Exposure time and temperature	5 min (±15 sec) (neat), 16 min (±1 min) (at 20 % (w/v) in distilled water) in culture medium at RT and 120 min (±2 min) (at 20 % (w/v) in distilled water) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH	30 min (±2 min) and 120 min (±5 min) (neat) in culture medium at 37±2ºC, 5±1% CO_2, ≥95% RH
Rinsing at room temperature	25 mL of Ca^{2+}/Mg^{2+}-free DPBS (2 mL per push)	25 mL of Ca^{2+}/Mg^{2+}-free DPBS (2 mL per push)
Post-soak immersion	10 min (±1 min) at RT in 4 mL culture medium	30 min (±2 min) at RT in 4 mL culture medium
Negative control	80 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS tested concurrently	80 ± 2µL Ca^{2+}/Mg^{2+}-free DPBS tested concurrently
Positive control	80 ± 2µL Methyl acetate tested concurrently (CAS RN 79-20-9)	80 ± 2µL Lactic acid 1% in water (w/v) tested concurrently (CAS RN 50-21-5)
Tetrasolium salt solution	300 µL 1 mg/mL	300 µL 1 mg/mL
Tetrazolium salt	180 min (±15 min) at 37±2ºC, 5±1% CO_2,	180 min (±15 min) at 37±2ºC, 5±1% CO_2,

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Test Method Component	SkinEthic™ HCE TTT (VRM)	
Protocol[1]	TTL protocol: Liquid and viscous test chemicals (pipettable)	TTS protocol: Solid test chemicals (not pipettable)
incubation and temperature	≥95% RH	≥95% RH
Extraction solvent	1.5 mL isopropanol (750µL under and 750µL over) (extraction from top and bottom of insert)	1.5 mL isopropanol (extraction from bottom of insert)
Extraction time and temperature	At least 2 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
OD reading	570 nm (540 - 600 nm) without reference filter	570 nm (540 - 600 nm) without reference filter
Tissue Quality Control	30±2 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC_{50} ≤ 3.2 mg/mL	30±2 min treatment with SDS (50 µL) 1.0 mg/mL ≤ IC_{50} ≤ 3.2 mg/mL
Acceptance Criteria	1. Mean OD of the tissue replicates treated with the negative control should be > 1.0 and ≤ 2.5 2. Mean viability of the PC, expressed as % of the NC, is ≤ 50% at the time exposure of 5 minutes and > 50% at both times 16 and 120 minutes. 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 PC, expressed as % of the NC, is > 40% at the time exposure of 30 minutes and 20%< PC ≤ 70% at the time exposure of 120 minutes. 3. The difference of viability between two tissue replicates should be less than 20%.

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Annex C. ILLUSTRATIVE FLOWCHART PROVIDING GUIDANCE ON HOW TO IDENTIFY AND HANDLE DIRECT MTT-REDUCERS AND/OR COLOUR INTERFERING CHEMICALS, BASED ON THE SKINETHIC™ HCE TTT SOP

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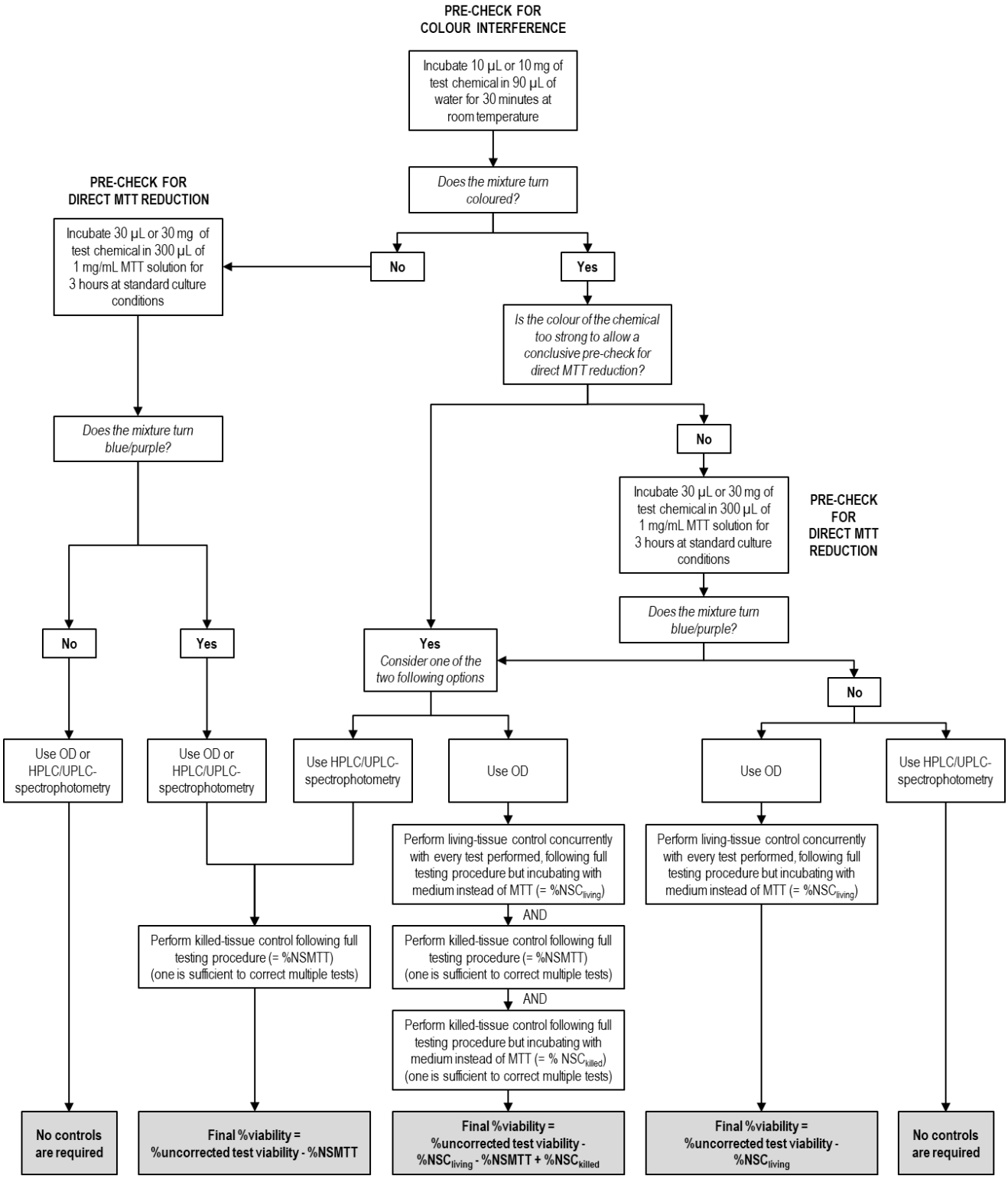
PRE-CHECK FOR COLOUR INTERFERENCE
Incubate 10 µL or 10 mg of test chemical in 90 µL of water for 30 minutes at room temperature
Does the mixture turn coloured?
No
Yes
PRE-CHECK FOR DIRECT MTT REDUCTION
Incubate 30 µL or 30 mg of test chemical in 300 µL of 1 mg/mL MTT solution for 3 hours at standard culture conditions
Is the colour of the chemical too strong to allow a conclusive pre-check for direct MTT reduction?
Does the mixture turn blue/purple?
No
Yes
Use OD or HPLC/UPLC-spectrophotometry
Use OD or HPLC/UPLC-spectrophotometry
No
Incubate 30 µL or 30 mg of test chemical in 300 µL of 1 mg/mL MTT solution for 3 hours at standard culture conditions
PRE-CHECK FOR DIRECT MTT REDUCTION
Does the mixture turn blue/purple?
Yes
Consider one of the two following options
No
Use HPLC/UPLC-spectrophotometry
Use OD
Use OD
Use HPLC/UPLC-spectrophotometry
Perform living-tissue control concurrently with every test performed, following full testing procedure but incubating with medium instead of MTT (= %NSC living)
AND
Perform killed-tissue control following full testing procedure (= %NSMTT) (one is sufficient to correct multiple tests)
AND
Perform killed-tissue control following full testing procedure but incubating with medium instead of MTT (= % NSC killed) (one is sufficient to correct multiple tests)
Perform killed-tissue control following full testing procedure (= %NSMTT) (one is sufficient to correct multiple tests)
Perform living-tissue control concurrently with every test performed, following full testing procedure but incubating with medium instead of MTT (= %NSC living)
No controls are required
Final %viability = %uncorrected test viability - %NSMTT
Final %viability = %uncorrected test viability - %NSC living - %NSMTT + %NSC killed
Final %viability = %uncorrected test viability - %NSC living
No controls are required

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Annex D. 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%

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[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.

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Annex E. PERFORMANCE OF THE SKINETHIC™ HCE TTT FOR THE LIQUIDS AND SOLIDS USING THE 3X3 MATRIX SHOWING CORRECT, UNDER- AND OVER- PREDICTIONS PER UN GHS CATEGORY[4]

Table 1: Performance of the SkinEthic™ HCE TTL protocol

UN GHS categories	SkinEthic™ HCE TTL - Predicted categories		
	Cat 1	Cat 2	No Cat
Cat 1 (N=21)	85.4%	14.6%	0.0%
Cat 2 (N=25)	20.2%	79.8%	0.0%
No Cat (N=24)	0.0%	20.8%	79.2%

Table 2: Performance of the SkinEthic™ HCE TTS protocol

UN GHS categories	SkinEthic™ HCE TTS - Predicted categories		
	Cat 1	Cat 2	No Cat
Cat 1 (N=29)	74.7%	25.3%	0.0%
Cat 2 (N=19)	15.8%	55.3%	28.9%
No Cat (N=33)	3.0%	25.3%	71.7%

[4] The between-test variability of the Draize eye test was assessed by Barroso et al. (2017) for chemicals for which more than one independent study was conducted by different laboratories. For chemicals for which at least one of the studies resulted in Cat.1 classification among all repeated studies, an overall concordance of classification of 62.5%(10/16) was found for UN GHS Cat.1. For chemicals for which at least one of the studies resulted in Cat.2 classification among all repeated studies, an overall concordance of classifications of 38.5% (5/13) was found for Un GHS Cat.2. Regarding chemicals with at least one No Cat. Classification among all repeated studies, 89.5% (17/19) of the chemicals with two repeated studies available showed a concordant UN GHS No Cat. Classification.

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