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DRAFT UPDATED TEST GUIDELINE 496

In vitro Macromolecular Test Methods for Identifying the eye hazard potential of chemicals

INTRODUCTION

1. The *in vitro* macromolecular test methods Ocular Irritection (OI®) and OptiSafe Eye Irritation Test™ (OS) are acellular biochemical *in vitro* test methods that can be used to identify chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage. At this time, only OI® can be used to identify chemicals that have the potential to induce serious eye damage.

2. The test method(s) described in this Test Guideline cannot be used on their own to replace the in vivo Draize eye test to predict across the full range of serious eye damage/eye irritation responses and mechanistic aspects for different chemical classes. It is therefore recommended to make use of alternative testing strategies such as those described in TG 467 or 492B, which can be used on its own, to address the required ranges of irritation potential. Strategic combinations of alternative test methods within a (tiered) testing strategy that combine the strengths of individual *in vitro* test methods to address the required ranges of irritation potential and/or chemical classes with existing knowledge on mechanistic aspects of ocular toxicity within an Integrated Approaches to Testing and Assessment (IATA), may be able to replace the Draize eye test (1)(2) for hazard classification as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1). The Top-Down testing strategy approach is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential, while the Bottom-Up approach is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification (1)(2).

3. The macromolecular test method Ocular Irritection (OI®) is an acellular biochemical *in vitro* test method that can be used, under certain circumstances and with specific limitations as described in Appendix A, paragraphs 4 to 9, for eye hazard classification and labelling of chemicals. While it is not considered valid as a stand-alone replacement for the *in vivo* rabbit eye test, the OI test method is recommended as an initial step of a Top-Down testing strategy approach as described within the OECD Guidance Document (GD) 263 (1) to positively identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 (3) without further testing. The OI® test method is also recommended to identify chemicals that

do not require classification for eye irritation or serious eye damage as defined by the UN GHS (UN GHS No Category) (3), and may therefore be used as an initial step within a Bottom- Up testing strategy approach (OECD GD 263) (1). However, a chemical that is neither predicted to cause serious eye damage (UN GHS Cat. 1) in the OI test method, nor as UN GHS No Cat. (i.e. predicted not to cause eye irritation/serious eye damage) in either the OI® or OS™ test method would require additional information and/or testing to establish a definitive UN GHS classification.

4. The macromolecular test method OptiSafe Eye Irritation Test (OptiSafe EIT) is a acellular biochemical in vitro test method that can be used, under certain circumstances and with specific limitations as described in Appendix B, paragraphs 4 to 8, to identify chemicals that do not require classification for eye irritation or serious eye damage as defined by the UN GHS (UN GHS No Category) (3), and may therefore be used as an initial step within a Bottom- Up testing strategy approach (OECD GD 263) (1).

5. The differences between the OI® and the OS test method include 1) characterization of the test chemical, 2) reagent preparation, 3) physiochemical handling procedures, and 4) results interpretation.

6. The choice of the most appropriate test method and use of this Test Guideline should be seen in the context of the OECD GD 263 where the Top-Down and the Bottom-Up testing approach represent one part of a wider Integrated Approach on Testing and Assessment for Serious Eye Damage and Eye Irritation (1).

7. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical using the individual *in vitro* macromolecular test methods, OI® (Appendix A) and the OS (Appendix B).

INTRODUCTION – LITERATURE

1. OECD (2018). Guidance Document on an Integrated Approach on Testing and Assessment for Serious Eye Damage and Eye Irritation. Series on Testing and Assessment, No. 263. Environment, Health and Safety Publications, Organisation for Economic Cooperation and Development, Paris, France. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2017)15&doclanguage=en

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Appendix A: Ocular Irritection (OI®) method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

1. The OI® test method contains a macromolecular reagent composed of a mixture of proteins, glycoproteins, carbohydrates, lipids and low molecular weight components, that when rehydrated forms a complex macromolecular matrix which mimics the highly ordered structure of the transparent cornea (1, 2). Corneal opacity is described as the most important driver for classification of eye hazard (3). It can result from the disruptive effects test chemicals may have on the highly organized structure of corneal proteins and carbohydrates through e.g. '*coagulation'* described as the precipitation/denaturation of macromolecules (particularly proteins) or '*saponification'* described as the breakdown of lipids (4). Test chemicals presenting an ocular hazard will produce turbidity of the macromolecular reagent by promoting protein denaturation, unfolding and changes in conformation as well as disruption and disaggregation of the macromolecular matrix components. Although the macromolecular OI® test method was originally developed to address the disruptive effects of ocular irritants causing corneal opacity, the validation study suggests that it can also detect irritants that cause only conjunctival injuries as evaluated in the rabbit ocular irritancy test method (OECD TG 405). However, being an acellular biochemical test system, the macromolecular assay does not address the cytotoxicity and reversibility aspects of ocular toxicity. Therefore, consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (5) when deciding on classification.

2. The OI® assay is the first validated macromolecular test assay to identify chemicals inducing serious eye damage (i.e., UN GHS Category 1) and chemicals that do not require classification for eye irritation or serious eye damage as defined by the UN GHS (UN GHS No Category). It is referred to as the Validated Reference Method (VRM) assay, as Performance Standards (6) are available to facilitate the validation of new or modified *in vitro* macromolecular test methods similar to Ocular Irritection®, in accordance with the principles of Guidance Document No. 34 (7), and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the Performance Standards, if these test methods have been reviewed and included in this Test Guideline by the OECD.

3. The term "test chemical" is used in this Test Guideline to refer to what is tested and is not related to the applicability of the *in vitro* macromolecular test method to the testing of substances and/or mixtures. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

4. The *in vitro* macromolecular test method Ocular Irritection® underwent an independent validation study between 2009 and 2012 (8), followed by an independent peer-review by EURL-ECVAM Scientific Advisory Committee (ESAC) in 2016 (9). Additional assessment of supplemental data as recommended by ESAC, regarding the characterisation of the raw material, the macromolecular matrix powder used to perform the assay and its stability over time, was conducted by the OECD expert group. A total of 89 test chemicals, including 13 mixtures and 76 substances, were assessed during the validation study. They covered a broad spectrum of functional groups distributed as 20 UN GHS Cat. 1, 26 UN GHS Cat. 2 and 43 UN GHS No Category test chemicals and including 25 solids, 57 liquids and 7 viscous test chemicals. The Test Guideline is applicable to solid and liquid chemicals whose 10% solution/dispersion (v/v or w/v as appropriate) has a pH in the range $4 \leq pH \leq 9$. The liquids may be viscous or non-viscous. Solids may be soluble or insoluble in water, as they are tested neat unless they have surfactant properties. Gases and aerosols have not been assessed yet in a validation study and are therefore outside of the applicability domain.

5. Specific limitations have been identified from in-house data with earlier versions of the OI® or validation study (10) for some chemicals that fall within the applicability domain as defined within paragraph 6 (e.g. intensely coloured chemicals, chemicals which caused salting-out precipitation, high concentrations of some surfactants, and highly volatile chemicals), that may interfere with the test system. Interference may include inhibition of the proper functioning of the macromolecular matrix reflected as specific OD_{405} readings for sets of controls and test chemicals (paragraphs 19 – 20), or specific observations of the test system considered as an integral part of the result report and analysis (paragraph 22), The set of acceptance criteria outlined in paragraphs 19 – 20 and integrated within the system software, allow continuing identification of such limitations.

6. The OI® is applicable to substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable and polymerising substances such as these containing acrylates), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful, or acceptable for the intended regulatory purpose. In addition, it is important to take into account the mechanistic insight provided by the selected in vitro method and how it covers the mechanisms of the test chemical. If appropriate, the use of an additional *in vitro* method, based if possible on different mechanisms of action may be considered, as outlined in OECD GD263 (5).

7. Performance of the OI® test method was evaluated using weighted calculation of individual predictions from each qualified result for each chemical used in the validation study in each of the participating laboratories, as recommended by EURL-

ECVAM Scientific Advisory Committee (ESAC) (9)[1]. When used to identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1, the *in vitro* macromolecular test method was found to have an overall accuracy of 75% (66.5/89), a specificity of 81% (55.8/69), a sensitivity of 54% (10.7/20), as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (8) (11) with the in vivo rabbit eye test bearing their own uncertainties as summarized elsewhere (5). These results include the UN GHS Cat. 1 chemical Tetraethylene glycol diacrylate (CAS 17831-71-9) which is unstable, light sensitive polymerising agent identified as generating negative results with other adopted eye irritation assays. If this difficult to test chemical was not considered in the performance analysis, the *in vitro* macromolecular test method shows an overall accuracy of 76% (66.5/88), a specificity of 81% (55.8/69), a sensitivity of 56% (10.7/19), a false positive rate of 19% (13.1/69) and a false negative rate of 44% (8.3/19). When used for this purpose, test chemicals classified based only on persistent but non severe effects *in vivo* were found to have higher risks of underprediction (5 out of 7). However, false negative rates in this context (i.e. *in vivo* UN GHS Category 1 identified as not being UN GHS Category 1 by the test) are not critical since all test chemicals that come out negative would be subsequently tested with other adequately validated *in vitro* test(s), or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach according to the OECD GD 263 (5).

8. When used to identify chemicals that do not require classification for eye irritation and serious eye damage, the OI® test method was found to have an overall accuracy of 75% (67.0/89), a sensitivity of 91% (41.7/46) and a specificity of 59% (25.3/43) calculated based on a weighted approach as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (8) (11) with the *in vivo* rabbit eye test bearing their own uncertainties as summarized elsewhere (5). These results include nevertheless the UN GHS Cat. 1 the chemical Tetraethylene glycol diacrylate (CAS 17831-71-9) which is an unstable, light sensitive polymerising agent identified as generating negative results with other adopted eye irritation assays. If this difficult to test diacrylate chemical was not considered in the performance analysis, the *in vitro* macromolecular test method shows an overall accuracy of 76% (67.0/88), a specificity of 59% (25.3/43), a sensitivity of 93% (41.7/45), a false positive rate of 41% (17.7/43) and a false negative rate of 7% (3.3/45), as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (8) (11). However, false positive rates in this context (UN GHS No Category identified as requiring classification) are not critical since all test chemicals that come out positive would be subsequently tested with other adequately validated *in vitro* test(s), or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach according to the OECD GD 263 (5). It should also be noted that under the considerations of the IATA outlined in the OECD GD 263 (5), chemicals containing the acrylate functional group would not be expected to be candidates for testing in the bottom-up approach as this functional group could be associated with skin irritation alerts, thus not consistent with a hypothesis that would initiate a bottom-up approach (see part 2 in the figure 1 in the OECD GD 263 (5)).

[1] Performance calculated based on the majority results is comparable (8)(9)

9. The OI® test method is not recommended for the identification of test chemicals that should be classified as irritating to eyes (i.e., UN GHS Category 2 or Category 2A) or test chemicals that should be classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of *in vivo* UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and of *in vivo* UN GHS No Category chemicals overclassified as UN GHS Category 2, 2A or 2B. For this purpose, further information and/or testing with other test methods will be required for classification purposes according to the IATA guidance document (5).

PRINCIPLE OF THE TEST

10. The *in vitro* macromolecular test method Ocular Irritection® consists of two components: a macromolecular matrix and a membrane disc for the controlled delivery of the test chemical to the macromolecular matrix. It is an acellular biochemical test system and does not address the cytotoxicity aspect of ocular toxicity. The macromolecular matrix serves as the target for the test chemical and is composed of a mixture of proteins, glycoproteins, carbohydrates, lipids and low molecular weight components forming a gel matrix. The protein oligomers which are part of the matrix self-associate to form larger fibrils that are held together by non-covalent forces. The macromolecular matrix, when rehydrated with a buffered salt solution, forms a highly ordered and transparent structure. Test chemicals causing ocular damage are known to produce denaturation of collagen and saponification of lipids (e.g., by alkalis), coagulation and precipitation of proteins (e.g., by acids) and/or dissolvance of lipids (e.g., by solvents) (12). Test chemicals producing protein denaturation, unfolding and changes in conformation will lead to the disruption and disaggregation of the highly organized macromolecular reagent matrix, and produce turbidity of the macromolecular reagent. Such phenomena is quantified, by measuring the changes in light scattering (at a wavelength of 405 nm using a spectrometer), which is compared to the standard curve established in parallel by measuring the increase in OD produced by a set of calibration substances. The standard curve is used for deriving an Irritection Draize Equivalent (IDE) Score for each tested dose/concentration of the test chemical (described in detail in paragraph 18). The highest IDE Score of the five tested doses/concentrations of a test chemical, namely Maximal Qualified Score (MQS), is then used to determine an UN GHS ocular hazard category based on pre-defined cut-off values (see paragraph 21).

DEMONSTRATION OF PROFICIENCY

11. For any laboratory establishing the OI® test method, the proficiency chemicals provided in Appendix 3 – Annex 3 should be used. A laboratory should use these chemicals to demonstrate their technical competence in performing the *in vitro* macromolecular test method prior to submitting its results for regulatory hazard classification purposes.

PROCEDURE

12. The protocol for the OI® test method is available and should be employed when implementing and using the test method in a laboratory (10). The following paragraphs describe the main components and procedures of the in vitro macromolecular test method based on the Ocular Irritection protocol.

Characterisation of the test chemical

13. The pH of a 10% water solution of the test chemical is measured to determine whether it falls within the applicability domain of the test. Detailed procedures for pH measurement for chemicals with different degree of solubility are described in the test protocol (10). In addition, for test chemicals for which surfactant properties have not been clearly identified, the foam test is performed as described in the protocol (10) to determine the appropriate test chemical application procedure described in paragraph 15. The foam test evaluates the proportion and the persistence of the foam layer generated after 10 seconds of vortexing of the 10% solution of the test chemical (10).

Reagent preparation and activation

14. As a basis of the Ocular Irritection® *in vitro* macromolecular test method, a macromolecular matrix is prepared by dissolving the reagent powder provided within the kit into a hydrating solution, and filtering the dissolved reagent. The resulting pH and temperature should fall within pre-established ranges (i.e. pH range of 7.9-8.2 and temperature range of 20-25ºC). Furthermore, the reagent solution (as well as the blanking buffer conducted in parallel for each tested dose/concentration) should be activated using an activator buffered solution, to reduce the pH of the reagent solution and initiate formation of the ordered macromolecular matrix. The resulting pH of the activated reagent solution should fall within pre-established pH ranges (6.4-6.7) at ambient temperature (20-25^{0}C). Aliquots of the activated protein matrix reagent solution are transferred to a 24-well plate.

Application of Test Chemicals

15. Test chemicals are applied at room temperature (20-25°C) directly onto the macromolecular matrix or over a cellulose membrane based on their physico-chemical properties (Figure 1 in Appendix A – Annex 2a). For known non-surfactants or unknown test chemicals characterized as not having surfactant-like properties based on the foam test described in paragraph 13 and in the test protocol (10), a series of five doses (i.e., 25, 50, 75, 100 and 125 µl for liquids and mg for solids) are applied neat onto the membrane disc placed over the matrix reagent. Solids may be ground to ensure the test chemical is evenly spread over the entire surface of the membrane. Known surfactants and unknown test chemicals characterized to have surfactant-like properties based on the foam test (10), are first diluted to form 5% working solutions in distilled water, and 125 µl of a series of five two-fold dilutions (i.e., 0.3125%, 0.625%, 1.25%, 2.5% and 5%) are applied directly into the macromolecular activated reagent followed by the membrane disc which is applied over the well (Appendix A – Annex 2a). Waxy solid (pieces) test chemicals are applied undiluted also directly to the reagent solution and covered by the membrane disc (Appendix A –Annex 2a).

16. The macromolecular matrix of the Ocular Irritection® test method is exposed to the test chemicals and concurrent controls for 24.0 ± 0.5 hours in an incubator maintained at 25 ± 1ºC. Following this exposure period, the test system is checked visually. For non-surfactant test chemicals (or unknown test chemicals characterized not to have surfactant-like properties based on the foam test (10)), the membrane discs should be

intact and not damaged. Furthermore wells with reduced volumes may be indicative of possible hygroscopic effects or technical problems. In this case the experiment shall be repeated once, and if the same effects are observed again, the test chemical is then considered to be excluded or incompatible with the test method.

Control Chemicals

17. Concurrent controls should be tested in parallel to the test chemical. In the case of Ocular Irritection®, these include 4 calibrating chemicals and two quality control (QC) chemicals provided within the commercial kit (see Appendix A – Annex 1 for definitions). The calibrating chemicals include four chemicals with UN GHS classification (11) ranging from No Category to Category 1and cover a defined range of OD responses (Table 1) which are used to derive the standard curve for Irritection Draize Equivalent (IDE) Score determination (described in paragraph 18 and Appendix A – Annex 2b). The two QC chemicals have defined ranges of IDE scores associated with their irritation potential which falls close to the prediction model cut-offs.

IDE and MQS score determination

18. Following incubation the activated protein reagent solution of test chemicals and controls (see paragraph 17) are transferred to a 96-well plate for OD reading at 405nm. The process of transfer is described in detail and illustrated in the protocol within the kit (10). The raw OD readings from each well are obtained and the IDE scores for the QCs and test chemicals are calculated by the software following the formulas outlined in Annex2b. MQS for a test chemical is determined from a single test run qualified as appropriate based on the analysis of the OD scores for the calibrators and QC chemicals (paragraph 18) as well as aspects of the dose response generated with the five tested doses/concentrations of test chemical (paragraph 15).

DATA AND REPORTING

Study Acceptance Criteria

19. Qualified results in the OI® are determined by the software which automatically performs for the following qualification check:

- §Test run qualification check: One of two criteria relating to four calibrators and two Quality Controls must be met for a test run to be accepted as *Qualified* for further data analysis:
  - The values obtained for all four calibrators and for at least one of two Quality Controls are within the pre-established accepted ranges (Table 1); or
  - The values obtained for any three of four calibrators, and for both Quality Controls are within the pre-established accepted ranges (Table 1). If only one calibrator is out of its acceptance range, the OI® software substitutes a pre-defined value for generation of the standard curve

  An OI® test run is considered Non-Qualified (NQ) when either two (or more) calibrators are out of range, or when one calibrator and one Quality Control are out of range.

Table 1. Acceptance criteria for calibrators and quality control chemicals in the Ocular Irritection® test method

Acceptance OD_{405} range	
Calibrator 0	0.062 - 0.262
Calibrator 1	0.089 – 0.315
Calibrator 2	0.351 - 0.945
Calibrator 3	1.277 – 2.127
Acceptance IDE range	
QC 1	7.2-20.8
QC 2	23.6-35.6

20. The following additional checks are performed by the software and prompt further interpretation of the series of the five data points for the test chemical and controls before acceptable MQS can be determination (see paragraph 17) for the test chemical. Result from a qualified test run can be excluded based on consideration of the following checks:

- Net Optical Density Check: The Net OD_{χ} for a test chemical should be greater than the pre-established value (i.e. > -0.015). When a test chemical Net OD_{χ} is < -0.015, a meaningful IDE Score cannot be calculated by linear extrapolation and the test result is excluded from consideration for MQS determination.
- If the Net OD_{χ} for a test chemical in a qualified run is below $OD_{Cal\ 2}$, an additional check is prompted to verify that the macromolecular matrix is responding properly. This check is performed by addition of an inhibition check solution provided in the test kit followed by re-measuring the OD_{χ} which should fall above $OD_{Cal\ 2}$ for the data to qualify/be accepted for further interpretation.
- Blank OD value check: Blank OD corresponding to any of the test chemical dose/concentrations greater than 1.2 indicates interference by the test substance (i.e. intense colouration). The test chemical with the corresponding blank control may be re-tested at least once more to confirm colour interference and excluded test result status.
- Finally, a dose response check is conducted to verify that the test chemical dose response is consistent with a typical pattern characteristic for known types of correctly predicted chemicals, If the dose response for a test chemical has an atypical/irregular pattern, the IDE results should be excluded from consideration for MQS determination. Examples of appropriate qualified dose response curves are presented in the protocol provided with the kit (10).

Interpretation of Results and Prediction Model

21. The optical density (OD_{405}) obtained with a qualified test chemical is compared to the standard curve obtained with the set of calibrators, to derive an Irritection Draize Equivalent (IDE) Score, for each tested dose/concentration. The highest obtained IDE score, named the Maximal Qualified Score (MQS), is then used to predict the ocular hazard potential of the test chemical according to the UN GHS classification system (11). In the case of the Ocular Irritection® *in vitro* macromolecular test method the Prediction Model described in table 2 is used.

Table 2. Ocular Irritection® prediction model

Maximal Qualified Score (MQS)	Predicted UN GHS classification**
0 – 12.5	No Category
> 12.5 – 30.0	No Prediction Can be Made*
> 30.0	Category 1

* If the MQS result is > 12.5 – 30.0 No final Prediction Can be made (NPCM) from this result in isolation. This is because a considerable number of *in vivo* UN GHS Category 1 chemicals showed MQS within this interval (paragraph 7) and were therefore under-predicted with the macromolecular test assay. In addition, considerable number of *in vivo* UN GHS No Category showed MQS within this interval i.e. were over-predicted (paragraph 8). For final classification of chemicals with MQS in the interval > 12.5 – 30.0, further information and/or testing with other test methods will be required according to the IATA guidance document (5).
**Consideration would need to be given to all possible mechanisms of ocular toxicity that may be relevant to the test chemical, based on existing data and knowledge as outlined in GD263 (5) when deriving a classification.

Test report

22. The test report should include the following information relevant to the conduct of the study:

Test and Control Chemicals

- Chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Purity and composition of the test/control substance or mixture (in percentage(s) by weight), to the extent this information is available;
- In case of multi-constituent test chemicals and UVCB: characterization as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study, colour, optical density or absorbance characteristics;

- pH of the 10% solution of the test chemical determined as described in the protocol (10)
- Outcome of the foam test if surfactant properties are not defined by supplier of test chemical;
- Treatment of the test/control chemical prior to testing, if applicable (e.g., warming, grinding);
- Storage conditions and stability to the extent available;

Solvent or Vehicle, if applicable

Information Concerning the Sponsor and the Test Facility

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

Test Method Conditions

- Description of test system used;
- The procedure used to ensure the performance (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency chemicals).

Test Procedure

- Number of test dose/concentrations used;
- Identity of the solvent and benchmark controls, if applicable;
- Test chemical dose, application and exposure time used;
- Description of any modifications to the test procedure, if applicable.

Results

- Tabulation of the OD_{405} for calibrators and Quality Controls with outcome for the acceptance criteria for the test run: Qualified or Not-Qualified assay (Unqualified)
- Tabulation of the OD_{405}, Net OD_{405} and IDE scores obtained for each individual test chemical dose;
- Results of applicability criteria checks for the test chemicals: i.e. excluded result or a prompt/flag for retesting
- Results from re-testing, if applicable
- Description of any other effects observed at the end of the procedure e.g. membrane intactness, condensation on plate cover indicating evaporation, volume reduction; coloration
- The Maximal Qualified Score, and its predicted *in vitro* UN GHS Category;

Discussion of the Results

Conclusion

Appendix A - Literature

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Appendix A - Annex 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of "relevance." The term is often used interchangeably with "concordance", to mean the proportion of correct outcomes of a test method (7).

Activator: Solution employed to initiate formation of the ordered macromolecular matrix when the protein has been rehydrated.

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

Blanking buffer: A control solution used to account for the background contribution of the test chemical to the OD_{405} readings in the test system.

Bottom-Up Approach: step-wise approach used for a chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome) (4) (5).

Calibrators: Four defined irritant solutions (Cal 0, 1, 2 and 3) having well characterized IDE scores in the Ocular Irritection® test method. The calibrators are used to derive a standard curve with which the results of the test method are compared to, and ensure optimal performance.

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

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" (11).

False negative rate: The proportion of all positive chemicals falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative chemicals that are falsely identified by a test method as positive. It is one indicator of test method performance.

Foam test: employed to determine whether the unknown substance should be tested utilizing surfactant or non-surfactant application procedure (10).

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.

Hydrating Solution: Solution employed to rehydrate the reagent powder and facilitate formation of the ordered protein matrix.

IATA: Integrated Approach on Testing and Assessment -A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

Inhibition check solution: An irritating substance known to quickly react with the macromolecular reagent and produce evident turbidity, which can be employed to verify the functionality of macromolecular reagent when the OD readings of qualified test chemical doses/concentrations are less than Calibrator 2. Application of the inhibition check solution verifies that the macromolecular reagent in those wells is still able to produce evident turbidity (e.g., > OD Calibrator 2) and identifies inaccurate low OD reading (or inaccurate non-irritant) results when the turbidity is less than OD Calibrator 2.

Irreversible effects on the eye: see "Serious eye damage" and "UN GHS Category 1".

Irritection Draize Equivalent (IDE) Score: A numerical score derived from the optical density measurement of the Ocular Irritection® test method for a tested dose/concentration when compared to the curve obtained with the calibrators.

Maximal Qualified Score (MQS): Represents the highest IDE score obtained from the different tested doses/concentrations of a test chemical. Ranging from 0 to 51 it is used to predict the irritation potential of the test chemical.

Membrane discs: A semi-permeable membrane that facilitates controlled delivery of the test chemical into the protein reagent.

Mixture: A mixture or a solution composed of two or more substances in which they do not react (11).

Net Optical Density Check: Provides a measure of the net optical density by measuring the OD of the activated protein reagent and subtracting the OD of the activated blanking buffer. The Net OD ($OD_{reagent} - OD_{blank} = OD_{Net}$) should be > - 0.015.

Not Classified: Test chemicals that are not classified for eye irritation (UN GHS Category 2) or serious damage to eye (UN GHS Category 1). The term is interchangeable with “UN GHS No Category”.

Quality Control chemicals: Two defined irritant solutions (QC1 and QC2) with well-characterized IDE scores that lie within the lower (7.2-20.8) and mid-upper range (23.6-35.6) of the Ocular Irritection® test method. The quality control check verifies that the method is functioning properly and can correctly detect eye irritation potency in the lower and mid/upper IDE ranges.

Reagent Powder: Consists of a mixture of proteins, glycoproteins, carbohydrates, lipids and low molecular weight components. When hydrated, the reagent powder forms a solution containing an ordered macromolecular matrix. Proteins in this solution undergo changes in conformation when exposed to an irritant test chemical.

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 (7).

Reversible effects on the Eye: see "Eye Irritation" and "UN GHS Category 2".

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (7).

Serious eye damage: 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"" (11)

Solvent/vehicle control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical-treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (7).

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 (11).

Surfactants: Also called surface-active agent, this is a substance and/or its dilution (in an appropriate solvent/vehicle), which consists of one or more hydrophilic and one or more hydrophobic groups, that is capable of reducing the surface tension of a liquid and of forming spreading or adsorption monolayers at the water-air interface, and/or of forming emulsions and/or microemulsions and/or micelles, and/or of adsorption at water-solid interfaces.

Top-Down Approach: step-wise approach used for a chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome) (4) (5).

Test chemical: Chemical (substance or mixture) assessed in the test method.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight-of-evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the

irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made (4) (5).

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (11).

UN GHS Category 1: see "Serious damage to eyes" and/or "Irreversible effects on the eye".

UN GHS Category 2: see "Eye Irritation" and/or "Reversible effects to the eye".

UN No Category: Test chemicals that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B). Interchangeable with “Not classified”.

Validated Reference Method(s) (VRM(s)): one (or more) test method(s) that was(were) used to develop the related official Test Guidelines and Performance Standards (PS). The VRM(s) is(are) considered the reference test method(s) to compare new proposed similar or modified test methods in the framework of a PS-based validation study.

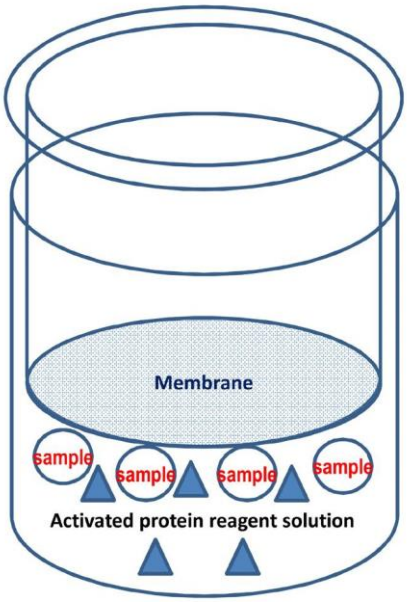
Weight-of-evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a chemical.

Version 19 December 2023

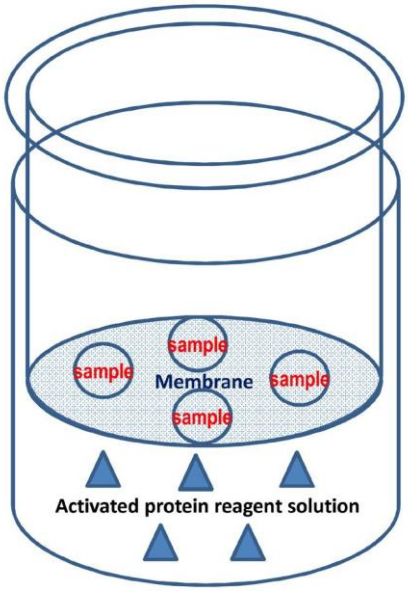
Appendix A - Annex 2a

Illustration to paragraph 15

Application of test materials



For surfactant & Non-*surfactant Waxy Solid*



For Non-*surfactant* (except waxy solid)

Appendix A - Annex 2b

Details of IDE score determination by the software (cf. to paragraph 18)

After incubation, the raw OD_{405} readings for the test chemicals and controls are collected by the spectrophotometer (as described in paragraph 18) and IDE scores are calculated by the integrated software using the following formulas:

Equation 1: When $OD_{QC1,2}$ or Net OD_X < $OD_{Cal\ 1}$, then:

IDE = ($OD_{QC1,2}$ or Net OD_X / $OD_{Cal\ 1}$) X 12.5

Equation 2: When $OD_{Cal\ 1}$ < $OD_{QC1,2}$ or Net OD_X < $OD_{Cal\ 2}$, then:

IDE= [($OD_{QC1,2}$ or Net OD_X - $OD_{Cal\ 1}$) / ($OD_{Cal\ 2}$ - $OD_{Cal\ 1}$)] X 17.5 + 12.5

Equation 3: When $OD_{Cal\ 2}$ < $OD_{QC1,\ 2}$ or Net OD_X < $OD_{Cal\ 3}$, then:

IDE= [($OD_{QC1,2}$ or Net OD_X - $OD_{Cal\ 2}$) / ($OD_{Cal\ 3}$ - $OD_{Cal\ 2}$)] X 21.0 + 30

When Test Chemical Net ODχ is > $OD_{Cal\ 3}$, the IDE Score cannot be calculated by linear extrapolation because there is no greater calibrator value.

Net OD_X for Test Chemical = Reagent ODχ –Blanking buffer ODχ, accounts for the potential background reading from the test chemical

Where:
x is the dose or concentration of test chemical.
Reagent OD represents the Test Chemical reading in the well containing reagent solution
Blank OD represents reading in the well containing the test chemical in blanking buffer.

$OD_{QC1,2}$ and $OD_{Cal0,\ 1,23}$ represent the OD reading for Calibrators (Cal) and Quality Control (QC) chemicals in the wells containing the reagent solution. Those control chemicals are known not to contribute to the background readings at 405nm.

Appendix A - Annex 3

Proficiency chemicals for the OI® *In vitro* Macromolecular Test Method

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the 12 chemicals recommended in Table 1. The Ocular Irritection® *in vitro* macromolecular test method outcomes provided represent examples of the results observed during its validation study (8). As recommended by OECD GD 34[2], the selection includes, to the extent possible, chemicals that: (i) cover the full range of *in vivo* serious eye damage/eye irritation responses based on the UN GHS classification system (i.e., Categories 1, 2A, 2B or No Category); (ii) are based on quality results obtained by the reference *in vivo* rabbit eye test (OECD TG 405) (3) (10); (iii) cover different physical states; (iv) cover a broad range of the chemical classes and organic functional groups, representative of those used in the validation study (8); (v) cover the range of *in vitro* responses based on high quality Ocular Irritection® data (0 to 51 MQS); (vi) produced correct and reproducible predictions in the VRM; (vii) are commercially available; and (viii) 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 Ocular Irritection® *in vitro* macromolecular test method or listed as a reference chemical within the Performance Standards (OECD, 2019) could be used (6)(8). Such deviations should however be justified.

[2] OECD Guidance Document 34 - *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (OECD GD 34).

Table 1: Recommended chemicals for demonstrating technical proficiency with the Ocular Irritection® *in vitro* macromolecular test method.

Chemical name	CASRN	*In vivo* UN GHS	Physical state	pH [A]	MQS range/ n= runs Average (SD)	VRM Prediction (7)
2-methylresorcinol	608-25-3	Category 1	Solid	5.8	>51/ n=9 n.a (n.a)	Cat. 1
4-tert-butylcatechol	98-29-3	Category 1	Solid	5.5	>51/ n=9 n.a. (n.a)	Cat. 1
Benzalkonium chloride (5%)	63449-41-2	Category 1	Liquid	6.5	49.5/ n=1 n.a. (n.a)	Cat. 1[B]
Promethazine hydrochloride	58-33-3	Category 1	Solid	4.5	>51/ n=9 n.a. (n.a)	Cat. 1
Ammonium nitrate	6484-52-2	Category 2A	Solid	4.8	14.1-27.3/ n=12 20.2 (3.0)	NPCM
Cetylpyridinium bromide (1%)	140-72-7	Category 2A	Liquid	4.7	15/ n=1 n.a. (n.a)	NPCM [B]
Methyl acetate	79-20-9	Category 2A	Liquid	6.8	15.0-21.1/ n=12 18.6 (1.5)	NPCM
Sodium benzoate	532-32-1	Category 2A	Solid	8.2	7.4-20/ n=9 15.4 (2.5)	NPCM
1,5-dibromopentane	111-24-0	No category	Liquid	5.7	6.7-10.3/ n=9 8.6(1.0)	No Cat.
Cetyl pyridinium bromide 0.1%	140-72-7	No category	Liquid	7.1	4-12.5/ n=10 6.8 (1.5)	No Cat.
Myristyl myristate	3234-85-3	No category	Solid	6.3	2.7-6.5/ n=9 4.6 (1.3)	No Cat.
Potassium tetrafluoroborate	14075-53-7	No category	Solid	4.5	6.8-19.2/ n=11 9.9 (2.1)	No Cat.

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Cat.: category; n.a.: not available; NPCM: No Prediction Can be Made; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (11).

[A] The pH values are rounded to one decimal point, and values were obtained from the original sources as indicated in (10).

[B] Test chemicals having limited data in within- and between- laboratory reproducibility but included as representing relevant chemistries and/or outcome.

Appendix B: OptiSafe Eye Irritation Test™ method for Identifying Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

1. The OptiSafe Eye Irritation Test™ (OS) method consists of a proprietary macromolecular test matrix. The method measures the extent of damage of the macromolecular matrix upon exposure to a test chemical and this is used to predict the toxicity of the tested chemical to the eye. The OS test method has procedures that increase the sensitivity, specificity, and accuracy and expand the application domain (1 – 10). These include tear levels of ascorbic acid that lower the false-positive rate (FPR) (3, 4, 16 – 10), a pretest for solubility and specific physiochemical handling procedures (PCHPs) for materials with different solubilities to reduce the false-negative rate (FNR) (1), a chemical buffering pretest and specific PCHPs for materials with different buffering capacities to reduce the FNR and expand the application domain (1), and a PCHP for surfactants that reduces the FNR for nonionic surfactants (9, 10). At this time, the applicability domain of this Test Guideline for the OS test method only includes soluble test chemicals that do not foam and have little or no buffering capacity and are therefore within the OS Membrane Assay (MA) PCHP application domain (as determined from the pretest). The test method developer is conducting additional testing on chemicals that have physiochemical properties that are not MA..

2. The OS assay is an independently validated macromolecular test assay to identify chemicals that do not require classification for eye irritation or serious eye damage as defined by the UN GHS (UN GHS No Category). No Performance Standards are available to facilitate the validation of new or modified *in vitro* macromolecular test methods similar to OS in accordance with the principles of Guidance Document No. 34 (11).

3. The term "test chemical" is used in this Test Guideline to refer to what is tested and is not related to the applicability of the *in vitro* macromolecular test method to the testing of substances and/or mixtures. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

4. The in chemico macromolecular test method OS underwent an independent validation study in 2018 and additional studies and an update from 2019 to 2022 (1 – 10). In the independent validation study in 2018, three laboratories (lead laboratory and two other laboratories) evaluated the performance of the OS test method in two different coded phases: transferability and application domain. After the validation study, the OS test method underwent additional studies that led to an update in the test matrix formulation with the addition of ascorbic acid. This Test Guideline covers the updated version of the OS test method that contains ascorbic acid, updates to the pretest, and a unified prediction model (8, 9). For the updated method validation studies, 147 chemicals were tested in triplicate. Of the 147 chemicals, the pretests indicated that 71 chemicals (including 59

liquids and 12 solids) are within the OS MA PCHP currently covered by this TG. (9). These chemicals cover a broad spectrum of functional groups and were categorized as 12 UN GHS Category 1, 20 UN GHS Category 2, and 39 UN GHS NC.

5. Specific limitations have been identified for test chemicals that are considered "criteria not met" (CNM). The OS test method cannot provide results for "criteria not met" (CNM) chemicals. CNM occurs when the photometric range of the spectrophotometer has been exceeded and there is an inverse dose-response curve below the irritant cut-off value (8, 9). CNM can result from extremely high positive results, assay interference resulting from high concentrations of metal oxides or very intensely colored materials. Zinc oxide and high concentrations of metal oxides found in eye shadows may result in CNM or high positive results; sunscreens and eye shadows containing zinc oxide are typically outside of the application domain. CNM must be retested with another type of nonanimal eye irritation test. Gases and aerosols have not yet been assessed in a validation study and are therefore outside of the applicability domain.

6. The OS method is applicable to solid and liquid chemicals (substances and mixtures) that do not foam and have little or no buffering capacity that are within the MA PCHP application domain. Liquids may be viscous or nonviscous. The OS cores for identifying test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS NC) are given in Table 4.

7. Performance of the OS test method was evaluated using a majority prediction approach of individual predictions from each result for each test chemical used to validate and expand the application of the original version. When used to identify chemicals within the MA PCHP application domain that do not require classification for eye irritation and serious eye damage, the OS test method was found to have an overall accuracy of 90.0% (63/70), a sensitivity of 100% (31/31), and a specificity of 82.1% (32/39), calculated based on a majority prediction approach compared to in vivo rabbit eye test method data classified according to UN GHS (12).

8. The OS test method is currently not recommended for the identification of test chemicals that induce serious eye damage, i.e., chemicals to be classified as UN GHS Category 1, due to the considerable number of in vivo UN GHS Category 2 chemicalsover classified as UN GHS Category 1. In addition, OS is not recommended for identification of test chemicals to be classified as irritating to eyes (i.e., UN GHS Category 2 or Category 2A) or test chemicals that should be classified as mildly irritating to eyes (UN GHS Category 2B). For this purpose, further information and/or testing with other test methods will be required for classification purposes according to the IATA guidance document (13).

PRINCIPLE OF THE TEST

9. The OS test method consists of a proprietary macromolecular test matrix that is used to quantify the potential of an unknown test chemical to cause eye irritation or eye damage

(1, 4). In this acellular test method, the extent of measured macromolecular damage is used to predict the toxicity of the test chemical. The method measures the extent of damage or fixation of macromolecules (1–10). To conduct the test, solubility, pH, and foaming of the test chemical are first determined using a standardized protocol that defines the "MA applicability domain". The MA applicability domain includes non-foaming chemicals with minimal or no buffering. Test chemicals are added to “ocular discs” to control the delivery of the chemical to be tested as it enters the reagent mixture (1, 4). Results are read using a spectrophotometer, and the OD (at 400 nm) and pH values are compared with quality controls (QCs) and a standard curve to calculate the OS score. The OS score is then applied to a prediction model to classify the material tested.

DEMONSTRATION OF PROFICIENCY

10. For any laboratory establishing the OS test method, the proficiency chemicals provided in Appendix B – Annex 2 should be used. A laboratory should consider using these proficiency chemicals to demonstrate their technical competence in performing the macromolecular test method prior to submitting results for regulatory hazard classification purposes.

PROCEDURE

11. The protocol for the OS test method is available and should be employed when implementing and using the test method in a laboratory (8, 9, 14). At this time, only the OS MA physiochemical handling procedure is currently covered by this Test Guideline. The following paragraphs describe the main components and procedures of the *in vitro* macromolecular test method based on the OS protocol.

Characterisation of the test chemical

12. For the OS test method, specific physiochemical properties (solubility, buffering capacity, and foaming) of the test chemical are measured during the pretest step. Based on these physiochemical properties, there are minor changes in the protocol that improve sensitivity and accuracy (6 – 9). The solubility of a test chemical is characterized by measuring the optical density of a 10% solution of the test chemical in blanking buffer (BB), provided in the kit and also observing if the test chemical floats to the top (“F”), aggregates in the middle (“A”), or sinks (“S”) to the bottom of the tube. The buffering capacity of a test chemical is characterized by measuring the pH of a 10% solution (in active agent, AA) and calculating the buffering score. The foaming of a test chemical is characterized by vortexing a 10% solution (in BB) and measuring the foaming column. The objectively measured physiochemical properties are then used to assign minor physiochemical handling steps. Table 1 relates all of these possible pretest outcomes with the specific PCHP for each of the outcomes. As shown in Table 1, only the MA PCHP is included in this test guideline. .

Table 1. OptiSafe Eye Irritation Test™ Pretest Outcomes

PCHP	Pretest Outcome				Procedure Modification	Regulatory Status
	Physical State	Foaming	Solubility	Buffering Capacity		
*MA	L, S	N	Y	Little	No modifications	Included in this guideline
H	L, S	N	Y	Extreme	Buffering capacity test	Pending
HMA	L, S	N	Y	Mid	pH of standards adjusted to match test chemical	Pending
CiS	S (sinks)	N	N	Little	Longer incubation (42 hours)	Pending
CiF	S (floats or aggregates)	N	N	Little	Longer incubation (42 hours); no membrane discs	Pending
SA	L, S	Y	Y	Little	Test chemical diluted; no membrane discs, capillary movement test	Pending

*Currently MA is the only PCHP covered within this test guideline. PCHP = Physiochemical Handling Procedures; OptiSafe Eye Irritation Test method pretest procedure includes: foaming test, solubility test, and buffering capacity test; L = Liquid; S = Solid; N = No; Y = Yes; Pending = Pending additional studies and regulatory acceptance.

Reagent preparation and activation

13. As the basis of the OS test method, a macromolecular matrix is prepared by thawing the frozen AA tubes provided within the kit in a water bath (26°C–28°C) for 40 minutes (50 minutes maximum). While the reagent is thawing, reconstitute the pH adjust solution (provided with the kit) with 10 mL of deionized water. After thawing, the pH should fall within pre-established ranges (7.8–8.1). The pH of the active agent (test matrix reagent) solution should be adjusted to exactly 6.36 using the pH adjust solution. Aliquots of the active agent (test matrix reagent) solution are transferred to a 24-well plate.

Application of Test Chemicals

14. For the OS test method, a titration of five doses are applied to the macromolecular test matrix. After test chemical application, there is an incubation at 30.6 °C–31.3 °C. After incubation, changes in the test matrix OD are measured using a spectrophotometer. The resulting OD values

are used to calculate OS scores, and each OS score is applied to the prediction model to classify the test material (discussed below). For the main procedure, liquid test chemicals are applied to the ocular discs at a series of doses of 25 µL, 50 µL, 75 µL, 100 µL, and 125 µL, while solid test chemicals are applied to the ocular discs at a series of doses of 50 mg, 100 mg, 150 mg, 200 mg, and 250 mg. Solid test chemicals should be applied to the ocular discs before starting the assay. Liquid test chemicals should be applied while the disc is on the active agent (test matrix reagent). Once in the 24-well plate with the active agent (test matrix reagent), the assay is incubated for 18–19 hours. After incubation, the discs are removed and the OD of the test reagent is read using a spectrophotometer. The resulting OD values are calculated based on a standard dose response curve and applied to the prediction model.

Control Chemicals

15. The OS test kit includes three standard chemicals and two QC chemicals (QC1 = 20% glycerol; CASRN 56-81-5 and QC2 = ethanol; CASRN 64-17-5). The standard chemicals cover a defined range of OD responses that are used to calculate the OS score. The two QC chemicals have defined ranges of OS scores associated with their irritation potential, which fall close to the prediction model cut-off values. The OS score for QC1 should fall below 4.0 and the acceptable OD400 range is 0.175 to 0.275. The OS score for QC2 should be above 15.0 and the acceptable OD400 range is 0.300 to 0.875.

DATA AND REPORTING

OS Score Determination

16. Table 2 shows how to calculate the OS score. The measured values (MV) are the sample ODs (at each dose) minus the blank and minus the standard 0 OD. The CSV is the standard with an OD closest to the sample OD. The designated value (DV) is the value assigned to the closest standard: standard IV (DV = 8.0) and standard III (DV = 12.5). To calculate the score, the MV, CSV and DV are applied to the Equation shown below:

Table 2. OptiSafe Eye Irritation Test™ Score Calculation

OptiSafe Eye Irritation Test Score = (MV / CSV) × DV
MV (Measured Value) = Sample OD – Blank OD – Mean Standard 0 OD **CSV** (Closest Standard Value) = Average Standard OD – Mean Standard 0 OD **DV** (Designated Value) = The multiplier value corresponding to the standard.

Acceptance Criteria

17. The test sample is considered positive if any one of the OS scores exceeds the prediction model cut-off value. Negatives are expected to have a flat dose response [no more than a 35% decrease from highest to lowest dose (raw OD values)]. For test samples with a highest OS score less than or equal to 15 and have more than a 35% decrease from the highest to lowest dose, the test sample is considered "CNM" and is outside of the application domain and therefore cannot be evaluated

by the OptiSafe Eye Irritation Test method. Test samples with an OS score that are greater than 15 and with a 35% or more decrease from highest to lowest dose are predicted to be NPCM.

18. The three standard chemicals and two QC chemicals should fall within pre-established ranges shown in Table 3 below. In addition, the OS score for QC1 should fall below 4.0 and the S score for QC2 should be above 15.0.

Table 3. Acceptance criteria for standard chemicals and quality control chemicals in the OptiSafe Eye Irritation Test™ method

	Acceptance OD_{400} range
Standard 0	0.100–0.290
Standard IV	> Standard 0, < Standard III
Standard III	0.300–0.600
QC 1	0.175–0.275
QC 2	0.300–0.875

Quality Assurance Checklist

1. For a quality-assured study, a second individual should be present at each critical phase, which includes dosing, reading the OD_{400} results, and entering data on the data capture sheet. The second individual should repeat all calculations, verify that results comply with this quality assurance checklist and sign or initial the data capture sheets and any final report.
2. Complete documentation should be verified on the pretest and main test data capture sheets.
3. The QC1 score must be less than 4.0; the QC2 score must be greater than 15.0.
4. The highest OS score should be confirmed to have been used to make the irritancy prediction for the substance being tested.
5. The dose-response curve should be evaluated and the results confirmed to comply with the dose-response criteria.
6. The incubator temperature at the start and end of incubation should be confirmed to have been recorded on the data capture sheet and that it is within 30.6 °C–31.3 °C.
7. If three or more blanks (BB) exceed 2.000, the result is CNM.
8. If four or five test wells (AA) are greater than the upper range of the spectrophotometer (“>3.000”), the result is CNM.
9. The OS test method kit should be confirmed to have been used within its expiration period; the lot number and expiration date should be recorded on the data sheet.

Interpretation of Results and Prediction Model

19. The highest OS score calculated is then used to predict the ocular hazard potential of the test chemical according to the UN GHS classification system (Table 4).

Table 4. OptiSafe Eye Irritation Test™ GHS Prediction Model

OS Score	Predicted UN GHS Classification*
≤ 15	No Category (NC)
> 15	NPCM

GHS = Globally Harmonized System of Classification and Labeling of Chemicals; NPCM = No prediction can be made.
*If the test chemical result is "Criteria Not Met (CNM)", this result cannot be used to conclude a predicted UN GHS classification.

Test report

Test and Control Chemicals

- Chemical identification, such as CAS registry number(s), structural formula, and/or other identifiers, to the extent this information is available;
- Purity and composition of the test substance or mixture (in percentage(s) by weight), to the extent this information is available;
- Physiochemical properties, such as physical state, pH, stability, solubility relevant to the conduct of the study, as described in the protocol;
- pH of the test chemcial, measured during the pretest step, as described in the protocol;
- Outcome of the foaming "surfactant" pretest;
- Treatment of the test material prior to testing, if applicable (e.g., warming, grinding); and
- Storage conditions and stability of the test material, to the extent available.

Information Concerning the Sponsor and Test Facility

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

Test Method Conditions

- Description of the test system used (paragraph 14 or equivalent); and
- The procedure used to ensure the performance (i.e., accuracy and reliability) of the test method over time (e.g., historical OS scores for the QC1 and QC2 chemicals and/or periodic testing of proficiency chemicals).

Test Procedure

- Number of test doses (five doses are used for OS) and concentration;
- Identity of benchmark controls (QC1 = 20% Glycerol; CASRN 56-81-5 and QC2 = Ethanol; CASRN 64-17-5);
- Test chemical dose, application, and exposure time used (see specific PCHPs); and
- Description of any modifications to the test procedure, if applicable.

Results

- OD_{400} measurements and OS scores for the test chemical;
- OD_{400} measurements and OS scores for standards and QCs;
- Calculations
- Outcome of the acceptance criteria;
- Results from retesting, if applicable;
- Description of any deviations or any other effects observed during the procedure; and
- The final highest OS score and the predicted UN GHS classification.

Discussion of the Results

Conclusion

Appendix B - Literature

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Appendix B – Annex 1

DEFINITIONS

Accuracy: Number of correctly predicted test chemicals divided by the total number of test chemicals; [(TP + TN) / (TP + TN + FP + FN)].

Active Agent (AA): The macromolecular matrix of the OptiSafe Eye Irritation Test (OS) test system.

Completely Insoluble – Floats (CiF) Assay: Physiochemical handling procedure (PCHP) for solid test chemicals in which the test chemical floats during the pretest step (Completely Insoluble Check).

Completely Insoluble – Sinks (CiS) Assay: Physiochemical handling procedure (PCHP) for solid test chemicals in which the test chemical sinks during the pretest step (Completely Insoluble Check).

Criteria Not Met (CNM): A test chemical is considered CNM if three or more blanks (BB) exceed 2.000 and/or if four or five test wells (AA) are greater than the upper range of the spectrophotometer (“>3.000”). The CNM result cannot be used to conclude a predicted UN GHS classification.

False Negative Rate (FNR): Number of in vivo positive test chemicals predicted to be negative divided by the total number in vivo positive chemicals; [(FN) / (FN + TP)].

False Positive Rate (FPR): Number of in vivo negative test chemicals predicted to be positive divided by the total number of in vivo negative chemicals; [(FP) / (FP + TN)].

H Assay: OS assay with extreme adjustments for pH; H > 100. This assay does not differentiate GHS Category 2 and Category 1.

HMA Assay: Membrane Assay (MA) with moderate adjustments for pH; H < 100 but > 5_A or > 2_B.

Membrane Assay (MA): Main OS assay.

Physiochemical Handling Procedures (PCHPs): Standardized assay adjustment protocols for test chemicals with the following physiochemical properties; extreme pH, insolubility, and foaming (surfactant).

Sensitivity: Number of correctly predicted positive in vivo chemicals divided by the total number of correctly and incorrectly predicted positive in vivo chemicals; [(TP) / (TP + FN)].

Specificity: Number of correctly predicted negative in vivo chemicals divided by the total number of correctly and incorrectly predicted negative in vivo chemicals; [(TN) / (TN + FP)].

Surfactant Assay (SA): Assay for test chemicals identified as surfactants during the pretest step (Foaming Check).

UN GHS: United Nations Globally Harmonized System of Classification and Labeling of chemicals.

Appendix B – Annex 2

Proficiency chemicals for the OptiSafe Eye Irritation Test Method ™

Prior to the routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the proficiency chemicals in Table 1.

Table 1: Recommended chemicals for demonstrating technical proficiency with the OptiSafe Eye Irritation Test Method

Chemical name	CASRN	Supplier	Catalog Number	Purity (%)	in vivo GHS category	Physical State	PCHP	OS Irritation Score, AVG ± SD	OS Pred.
Methylpentynol	77-75-8	SiAl	137561	98.0	1	L	MA	76.2 ± 7.1	NPCM
Cyclohexanol	108-93-0	SiAl	105899	99.0	1	L	MA	66.9 ± 4.9	NPCM
Methylthioglycolate	2365-48-2	SiAl	108995	95.0	1	L	MA	82.7 ± 6.5	NPCM
Promethazine hydrochloride	58-33-3	SiAl	P4651	n/a	1	S	MA	152.9 ± 19.1	NPCM
n-Octanol	111-87-5	SiAl	297887	≥99.0	2A	L	MA	18.9 ± 0.9	NPCM
Ammonium nitrate	6484-52-2	SiAl	A3795	n/a	2A	S	MA	20.7 ± 0.7	NPCM
2-Methyl-1-pentanol	105-30-6	SiAl	214019	99.0	2B	L	MA	33.2 ± 3.5	NPCM
Methyl acetate	79-20-9	SiAl	45999	99.8	2A	L	MA	23.9 ± 2.5	NPCM
Triethylene glycol	112-27-6	SiAl	95126	≥99.0	NC	L	MA	5.1 ± 1.7	No Cat.
n,n-Dimethylguanidine sulfate	598-65-2	SiAl	276669	97.0	NC	S	MA	12.5 ± 1.0	No Cat.
2,4-Pentanediol	625-69-4	SiAl	156019	98.0	NC	L	MA	10.2 ± 0.7	No Cat.
1-Bromo-4-chlorobutane	6940-78-9	SiAl	B60800	99.0	NC	L	MA	4.7 ± 2.1	No Cat.