In vitro Macromolecular Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

INTRODUCTION

1. The *in vitro* macromolecular test method Ocular Irritection (OI®) is a biochemical *in vitro* method that can be used to identify the potential of chemicals (substances and mixtures) to induce serious eye damage / eye irritation.

2. It is currently generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to fully replace the *in vivo* Draize eye test to predict across the full range of mechanistic aspects of irritation for different chemical classes. However, strategic combinations of alternative test methods within a (tiered) testing strategy and/or Integrated Approaches to Testing and Assessment (IATA) may be able to replace the Draize eye test (2)(3) 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 (2)(3).

3. The *in vitro* macromolecular test method is an *in vitro* test method that can be used, under certain circumstances and with specific limitations as described in paragraphs 7 to 12, 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 *in vitro* macromolecular test method is recommended as an initial step of a Top-Down testing strategy approach as described within the OECD Guidance Document (GD) 263 (2) to positively identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 (1) without further testing. However, in case a negative result is obtained with a first in vitro test method in the Top-Down approach, the use of additional suitable in vitro test method should be considered based on the WoE analysis as outlined in the OECD GD 263 (2). The *in vitro* macromolecular 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) (1), and may therefore be used as an initial step within a Bottom-Up testing strategy approach (OECD GD 263) (2). However, a chemical that is not predicted as causing serious eye damage i.e. is predicted as not needing classification for eye irritation/serious eye damage with the *in vitro* macromolecular test method by the Bottom-up approach would require additional information to establish a definitive UN GHS classification. The choice of the most appropriate test method(s) 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 (2).

4. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical using the *in vitro* macromolecular test method. Corneal opacity is described as the most important driver for classification of eye hazard (4). 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 (3). The *in vitro* macromolecular 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 and mimics the highly ordered structure of the transparent cornea (5, 6). 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 and iridal injuries as evaluated in the rabbit ocular irritancy test method. However, being an acellular biochemical test system, the macromolecular assay does not address the cytotoxicity aspect of ocular toxicity.

5. One commercially available test method is included in this Test Guideline, namely the Ocular Irritection® assay referred to as the Validated Reference Method (VRM). The assay has been considered scientifically valid 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). Performance Standards (7) 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 (8), 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.

6. 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 1a.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

7. The *in vitro* macromolecular test method Ocular Irritection® underwent an independent validation study between 2009 and 2012 (9), followed by an independent peer-review by EURL-ECVAM Scientific Advisory Committee (ESAC) in 2016 (10). Additional assessment of supplemental data 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 as recommended by ESAC. A total of 88 test chemicals, including 13 mixtures and 75 substances, were assessed during the validation study. They covered a broad spectrum of functional groups distributed as 20 UN GHS Cat. 1, 25 UN GHS Cat. 2 and 43 UN GHS No Category test chemicals and including 25 solids, 56 liquids and 7 viscous test chemicals. The Test Guideline is applicable to solid and liquid chemicals whose

10% solution/dispersion 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.

8. Specific limitations have been identified from in-house data with earlier versions of the macromolecular test assay or validation study (10) for some chemicals that fall within the applicability domain as defined within paragraph 7 (e.g. intensely coloured chemicals, chemicals which caused salting-out precipitation, high concentrations of some surfactants, and highly volatile chemicals), that either interfere with the OD_{405} readings or the proper functioning of the macromolecular matrix. However, the OI® test assay includes a set of acceptance criteria (paragraphs 22-23) within the integrated software that allow continuing identification of such limitations. This inbuilt capability of the macromolecular OI® test assay to identify potential miss-predictions for unknown test chemicals without a-priory knowledge of the physicochemical challenge/limitation that a particular test chemical may pose within the test system is a unique characteristic and advantage of this in vitro assay.

9. The Test Guideline is applicable to substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful, or acceptable for the intended regulatory purpose.

10. 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 74% (65/88), a specificity of 81% (55/68), a sensitivity of 50% (10/20), a false positive rate of 19% (13/68) and a false negative rate of 50% (10/20) as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (1) (9) with the latter bearing their own uncertainties as summarized elsewhere (2). 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 (2).

11. When used to identify chemicals that do not require classification for eye irritation and serious eye damage, the *in vitro* macromolecular test method was found to have an overall accuracy of 76% (67/88), a sensitivity of 93% (42/45) and a specificity of 58% (25/43) as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (1) (9) with the latter bearing their own uncertainties as summarized elsewhere (2). When used for this purpose, the *in vitro* macromolecular test showed a false positive rate of 42% (18/43), and a false negative rate of 7% (3/45) as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (1) (9). Analysis of the mispredicted chemicals in the context of identification of chemicals not requiring classification identified potential limitation for the *in vitro* macromolecular test method when testing chemicals with certain organic functional groups as defined by the OECD QSAR toolbox. Namely 4 out of 5 chemicals

having *cycloalkene* and *3 out of 3 chemicals having carboxamide* (3/3)organic functional groups were overpredicted while 1 out of the one test chemical having the *acrylate*iorganic functional group was underpredicted as No Cat compared to its Cat 1 classification based on the in vivo data. Excluding the test chemicals having these three functional groups, enhances the performance of the *in vitro* macromolecular test method for identification of chemicals not requiring classification to to overall accuracy of 81% (64/79), sensitivity of 98% (40/41), specificity of 63% (24/38), false positive rate of 37% (14/38) and low false negative rate of 2% (1/41), as compared to *in vivo* rabbit eye test method data classified according to the UN GHS (1) (9). It is noted that under the considerations of the IATA outlined in the OECD GD 263 (2), chemicals containing 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 and skin sensitisation 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 (2)). 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 (2).

12. The *in vitro* macromolecular test method is not recommended for the identification of test chemicals irritating or mildly irritating to eyes (i.e., UN GHS Category 2, Category 2A or Category 2B). This is because the validation study found a considerable number of in vivo UN GHS Category 1 chemicals were underclassified as UN GHS Category 2, 2A or 2B with the macromolecular test method and, considerable number of in vivo UN GHS No Category chemicals were overclassified as UN GHS Category 2, 2A or 2B in the macromolecular test method, using the prediction model specified in Table 2 below. For this purpose, further information and/or testing with other test methods will be required for classification purposes according to the IATA guidance document (2).

PRINCIPLE OF THE TEST

13. 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 19). 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 22).

DEMONSTRATION OF PROFICIENCY

14. For any laboratory establishing the *in vitro* macromolecular test method, the proficiency chemicals provided in Annex 2 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

15. Ocular Irritection® is the only *in vitro* macromolecular test method currently covered by this Test Guideline. The protocol for this test method is available and should be employed when implementing and using the test method in a laboratory (11). 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

16. 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 procedure for pH measurement for chemicals with different degree of solubility is described in the test protocol (11). In addition, for test chemicals for which surfactant properties have not been clearly identified, the foam test is performed as described in the protocol (11) to determine the appropriate test chemical application procedure described in paragraph 16. Briefly, the foam test evaluates the proportion and the persistence of the foam layer created after 10 seconds of vortexing of the 10% solution of the test chemical (11).

Reagent preparation and activation

17. 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 20-25^{o}C). 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 (i.e. 6.4-6.7 in the case of Ocular Irritection®) 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

18. 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 Annex 1b). For solids, non-surfactants or unknown test chemicals characterized as not having surfactant-like properties based on the foam test described in paragraph 14 and in the test protocol (11), 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 discs 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 (11), 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. Waxy solid (pieces) test chemicals are applied undiluted also directly to the reagent solution and covered by the membrane disc.

19. 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 (11)), 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

20. 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 Annex 1a for definitions). The calibrating chemicals include four chemicals with UN GHS classification (1) ranging from No Category to Category 1and cover a defined range of OD responses (Table1) which are used to derive the standard curve for Irritection Draize Equivalent (IDE) Score determination (described in paragraph 19 and Annex1b). 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 score determination

21. Following incubation, controls and test samples 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 (11). The IDE scores for the QCs and test chemicals are calculated by the software following the formulas outlined in Annex1b:

DATA AND REPORTING

Study Acceptance Criteria

22. Qualified results in the VRM Ocular Irritection® are determined by the software which automatically performs for the following qualification checks:

§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

23. The following additional checks are performed and prompted by the software for the test data to be accepted for interpretation and determination of MQS (see paragraph 22) for a test chemical.

§. Net Optical Density Check: The Net OD_X for a test sample should be greater than the pre-established value (i.e. > -0.015). When a test sample Net OD_X 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_X for a test sample 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_X 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 sample 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 (8).

Interpretation of Results and Prediction Model

24. 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 (1). 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 10) and were therefore under-predicted with the macromolecular test assy. In addition, considerable number of in vivo UN GHS No Category showed MQS within this interval i.e. were over-predicted (paragraph 11). .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.

Test report

25. 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 (8)
- 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;
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Discussion of the Results

Conclusion

LITTERATURE

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ANNEX 1a

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 (8).

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.

Blank qualification: The blank OD for each sample is checked to fall in an appropriate range (i.e., pre-established minimum and maximum blank OD), and for flatness (i.e., OD variability between two consecutive doses/concentrations, and between the highest and lowest doses/concentrations in a group of 3 doses/concentrations).

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).

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 following the application of a test chemical to the anterior surface of the eye, which are fully reversible, within 21 days of application. Interchangeable with "Reversible effects on the Eye" and with "UN GHS Category 2" (1).

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 (8).

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.

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 (1).

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 (8).

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 (8).

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with “Irreversible effects on the eye” and with “UN GHS Category 1” (1)

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 (8).

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).

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) (2) (3).

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 (2) (3).

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 (1).

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”.

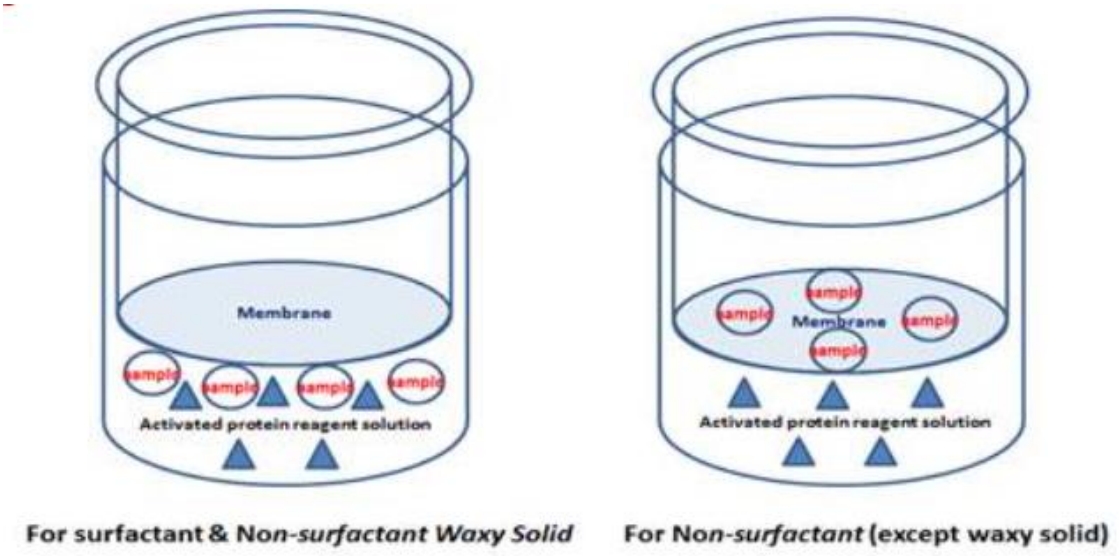
Validated Reference Method(s) (VRM(s)): one (or more) test method(s) that was(were) used to develop the related official Test Guidelines and Performance Standards (PS). The VRM(s) is(are) considered the reference test method(s) to compare new proposed similar or modified test methods in the framework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

ANNEX 1b

Illustration to paragraph 18

Application of test materials



Details of IDE score determination related to paragraph 21

First, the raw OD readings from each well are obtained. Next, the OD readings for Cal 1, 2, 3, and QC1, 2 and samples are obtained by subtracting the OD reading of Cal 0 from their raw OD readings. The Blank OD is the reading in the sample well containing blanking buffer. Then the Net OD_X is calculated according to the following equation, where $_X$ is the dose or concentration of test sample:

Net OD_X = (Sample OD_X – Blank OD_X)

Following Net OD_X determination, IDE scores are calculated according to the following equations:

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 Sample Net ODχ is > Cal 3, the IDE Score cannot be calculated by linear extrapolation because there is no greater calibrator value.

ANNEX 2

PROFICIENCY CHEMICALS FOR THE *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 (9). As recommended by OECD GD 34[1], 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) (4) (11); (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 (9); (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, 20XX) could be used (7)(9). Such deviations should however be justified.

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=6 n.a (n.a)	Cat. 1
4-tert-butylcatechol	98-29-3	Category 1	Solid	5.5	>51/ n=6 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[c]
Promethazine hydrochloride	58-33-3	Category 1	Solid	4.5	>51/ n=6 n.a. (n.a)	Cat. 1
Ammonium nitrate	6484-52-2	Category 2A	Solid	4.8	14.1-27.3/ n=9 20.2 (3.0)	NPCM
Cetylpyridinium bromide (1%)	140-72-7	Category 2A	Liquid	4.7	15/ n=1 n.a. (n.a)	NPCM [c]
Methyl acetate	79-20-9	Category 2A	Liquid	6.8	15.0-21.1/ n=9 18.6 (1.5)	NPCM
Sodium benzoate	532-32-1	Category 2A	Solid	8.2	7.4-20/ n=6 15.4 (2.5)	NPCM
1,5-dibromopentane	111-24-0	No category	Liquid	5.7	6.7-9.3/ n=6 8.6(1.0)	No Cat.
Cetyl pyridinium bromide 0.1%	140-72-7	No category	Liquid	7.1	4-12.5/ n=7 6.8 (1.5)	No Cat.
Myristyl myristate	3234-85-3	No category	Solid	6.3	2.7-6.4/ n=6 4.6 (1.3)	No Cat.

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

Chemical name	CASRN	*In vivo* UN GHS	Physical state	pH [A]	MQS range/ n= runs Average (SD)	VRM Prediction (7)
Potassium tetrafluoroborate	14075-53-7	No category	Solid	4.5	6.8-19.2/ n=8 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 (1).

[A] The pH values are rounded to one decimal point, and values were obtained from the original sources as indicated in (7).

[B] The organic functional groups were characterized using the OECD QSAR toolbox (version 2.3) as described in (7).

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