Section 4
Health effects

Test Guideline No. 438

Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Isolated chicken eye 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 Isolated Chicken Eye (ICE) test method was evaluated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM), in 2006 and 2010 (1) (2) (3). In the original evaluation, the ICE was endorsed as a scientifically valid test method for use as a screening test to identify chemicals (substances and mixtures) inducing serious eye damage (Category 1) as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (1) (2) (4). A re-evaluation of the in vitro and in vivo dataset used in the validation study concluded that the ICE test method could also be used to identify chemicals not requiring classification for eye irritation and serious eye damage as defined by the UN GHS which led to the revised version of TG 438 adopted in 2013 (4) (5). Since then, the Decision Criteria used to identify chemicals not requiring classification according to the UN GHS Classification System, has been revised based on the latest acceptance standards (5) (6) (7) (8). Furthermore, histopathology has been shown to be a useful additional endpoint to identify UN GHS Category 1 non-extreme pH (2 < Ph < 11.5) detergents and surfactants (9) (10). This Test Guideline (adopted in 2009 and updated in 2013 and in 2018) includes the latest recommended uses and limitations of the ICE test method based on these evaluations.

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 for different chemical classes. It is therefore recommended to make use of alternative testing strategies such as those described in TG 467 and 492B to address the required ranges of irritation potential. Strategic combinations of 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 (7)(11). The Top-Down 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 (7)(11). The ICE 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 11 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 ICE test method is recommended as an initial step within a testing strategy such as the Top-Down approach recommended within the OECD GD 263 (7) to identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 without further testing (4). The ICE 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 (No Category) (4), and may therefore be used as an initial step within a Bottom-Up testing strategy approach (OECD GD 263 (7). However, a chemical that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage with the ICE test method would require additional information to establish a definitive classification. Choice of the most appropriate test method(s) and use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approach on Testing and Assessment for Serious Eye Damage and Eye irritation (7). Furthermore, the appropriate regulatory authorities should be consulted before using the ICE test method in a Bottom-Up approach for classification schemes other than the UN GHS.

3. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical as measured by its ability to induce or not induce toxicity in the enucleated eyes of chicken. Toxic effects to the cornea are measured by (i) a qualitative assessment of opacity, (ii) a qualitative assessment of damage to epithelium based on application of fluorescein to the eye (fluorescein retention), (iii) a quantitative measurement of increased thickness (swelling), and (iv) a qualitative evaluation of macroscopic morphological damage to the surface of the treated eyes. The corneal opacity, swelling, and damage assessments following exposure to a test chemical are assessed individually and then combined to derive an Eye Irritancy Classification. Furthermore, histopathological observations may also be used as an additional endpoint to potentially improve the prediction of UN GHS Category 1 non-extreme pH ($2 < pH < 11.5$) detergents and surfactants (see paragraphs 8 and 56).

4. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. This Test Guideline is based on the protocol suggested in the OECD Guidance Document 160 (12), which was originally adopted in 2011 and further updated in 2017 and 2018. The protocol is based on information obtained from published protocols (13) (14) (15) (16) (17).

6. A wide range of chemicals has been tested in the evaluation underlying this Test Guideline and the overall database currently amounts to 184 test chemicals including 75 substances and 109 mixtures (5). The Test Guideline is applicable to solids, liquids, emulsions and gels. The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Gases and aerosols have not been assessed yet in a validation study.

7. The ICE test method can be used to identify chemicals inducing serious eye damage, i.e., chemicals to be classified as UN GHS Category 1 (4). When used for this purpose, the identified limitations for the ICE test method are based on the high false positive rates for alcohols and the high false negative rates for solids and surfactants (1) (3) (18). Moreover, test chemicals inducing persistent non severe effects in vivo may also risk underprediction (22). However, false negative rates in this context (UN GHS Category 1 identified as not being UN GHS Category 1) 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. Furthermore, histopathology was found to be a useful additional endpoint to decrease the false negative rates when used to identify UN GHS Category 1 non-extreme pH (2 < pH < 11.5) detergents shown to induce mainly persistent non severe effects in vivo (and surfactants (9) (10) (19). Regarding solids, it should be noted that these may lead to variable and extreme exposure conditions in the in vivo Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (20). Investigators could consider using this test method for all types of chemicals, whereby a positive result should be accepted as indicative of serious eye damage, i.e., UN GHS Category 1 classification without further testing. However, positive results obtained with alcohols should be interpreted cautiously due to risk of over-prediction.

8. When used to identify chemicals inducing serious eye damage (UN GHS Category 1), the ICE test method (without use of histopathology) was found to have an overall accuracy of 83% (142/172), a false positive rate of 7% (9/127) and a false negative rate of 47% (21/45) when compared to in vivo rabbit eye test method data classified according to the UN GHS classification system (4) (5). When histopathology is considered as an additional endpoint to identify UN GHS Category 1 non-extreme pH (2 < pH < 11.5) detergents and surfactants, the false negative rate of the ICE test method and its accuracy are improved (from 64% to 27% false negatives (n=22) and from 53% to 77% accuracy (n=30)), whilst an acceptable false positive rate is maintained (from 0% to 12.5% false positives (n=8)) (10).

9. The ICE test method can also be used to identify chemicals that do not require classification for eye irritation or serious eye damage under the UN GHS classification system (4). The test method can be used for all types of chemicals, whereby a negative result could be accepted for not classifying a chemical for eye irritation and serious eye damage. However, on the basis of one result from the validation database, anti-fouling organic solvent-containing paints may be under-predicted (5).

10. When used to identify chemicals that do not require classification for eye irritation and serious eye damage, the ICE test method has an overall accuracy of 88% (161/184), a false positive rate of 24% (20/83), and a false negative rate of 3% (3/101), when compared to in vivo rabbit eye test method data classified according to the UN GHS (4) (5). When test chemicals within certain classes (i.e., anti-fouling organic solvent containing paints) are excluded from the database, the accuracy of the ICE test method is 88% (159/181), the false positive rate 24% (20/83), and the false negative rate of 2% (2/99) for the UN GHS classification system (4).

11. The ICE 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 UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassified as UN GHS Category 2, 2A or 2B. For this purpose, further information and if needed, additional testing with another suitable method may be required.

12. All procedures with chicken eyes should follow applicable geographical regulations and the test facility's procedures for handling of human or animal-derived materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended (21).

13. Whilst the ICE test method does not directly address conjunctival and iridial injuries as evaluated in the rabbit ocular irritancy test method, it addresses corneal effects which are the major

driver of classification in vivo when considering the UN GHS Classification. In this respect, it should be noted that effects on the iris are of lesser importance for classification of chemicals according to UN GHS (8) (22). Also, although the reversibility of corneal lesions cannot be evaluated per se in the ICE test method, it has been shown that histopathological observations can help in identifying test chemicals causing irreversible effects not linked with initial high level injury such as those caused by non-extreme pH (2 < pH < 11.5) detergents (9). Finally, the ICE test method does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

14. This Test Guideline will be updated periodically as new information and data are considered. For example, further histopathology data may become available for test chemicals other than non-extreme pH detergents and surfactants. To evaluate this possibility, users are encouraged to preserve eyes and prepare histopathology specimens that can be used to develop a database and decision criteria that may further improve the accuracy of this test method. The OECD has developed Guidance Document 160 to be considered when using the ICE and BCOP in vitro ocular toxicity test methods, which includes detailed procedures on the collection and processing of histopathology specimens for evaluation (12).

DEMONSTRATION OF PROFICIENCY

15. For any laboratory initially establishing the standard ICE test method, the proficiency chemicals provided in Annex 2 should be used. A laboratory can use these chemicals to demonstrate their technical competence in performing the standard ICE test method prior to submitting ICE data for regulatory hazard classification purposes. For any laboratory willing to establish ICE histopathology for the regulatory hazard classification of non-extreme pH detergents and surfactants, the ICE Atlas and recommendations provided within the revised OECD GD 160 should be used (12). Consolidated training, transferability and proficiency appraisal are recommended to ensure harmonized, consistent and reproducible histopathological observations. Furthermore, an internal pathology peer review should be conducted in accordance with current recommendations (23) and according to the OECD advisory document n. 16 on GLP requirements for peer review of histopathology (24), and as described in paragraph 50. Such peer review process allows to verify and improve the accuracy and quality of pathology diagnoses and interpretations. Finally, the proficiency chemicals provided in Annex 3 should be used for a laboratory to demonstrate technical competence in scoring the ICE histopathology effects, prior to submitting ICE histopathology data for the regulatory hazard classification of non-extreme pH detergents and surfactants.

PRINCIPLE OF THE TEST

16. The ICE test method is an organotypic model that provides short-term maintenance of the chicken eye in vitro. In this test method, damage by the test chemical is assessed by determination of corneal swelling, opacity, and fluorescein retention. Furthermore, histopathology can be used to increase the sensitivity of the method for identifying UN GHS Category 1 non-extreme pH (2 < pH < 11.5) detergents and surfactants (10). Whilst measurement of corneal swelling provides for a quantitative assessment, corneal opacity, fluorescein retention and histopathological changes each involve a qualitative assessment. Each measurement is either converted into a quantitative score used to assign an ICE Class (I to IV), or assigned a qualitative categorization that is used to

assign an in vitro ocular hazard classification, either as UN GHS Category 1 or as UN GHS No Category (see Decision Criteria). However, no prediction can be made for chemicals not identified as UN GHS Category 1 or as UN GHS No Category with the ICE test method (see paragraph 11); in these cases, the “No prediction can be made” result of the ICE test would require additional information for classification purposes [see (7) for guidance].

Source and Age of Chicken Eyes

17. Historically, eyes collected from slaughterhouse chickens killed for human consumption have been used for this assay, eliminating the need for laboratory animals. Only the eyes of healthy animals considered suitable for entry into the human food chain are used.

18. Although a controlled study to evaluate the optimum chicken age has not been conducted, the age and weight of the chickens used historically in this test method are that of spring chickens traditionally processed by a poultry slaughterhouse (i.e., approximately 7 weeks old, 1.5 - 2.5 kg).

Collection and Transport of Eyes to the Laboratory

19. Heads should be removed immediately after humane stunning of the chickens and incision of the neck for bleeding. Humane stunning methods include electrical stunning and controlled atmosphere stunning, as long as it can be shown not to adversely impact the quality of the chicken eyes (see paragraph 21). A local source of chickens close to the laboratory should be located so that their heads can be transferred from the slaughterhouse to the laboratory quickly enough to minimize deterioration and/or bacterial contamination. The time interval between collection of the chicken heads and placing the eyes in the superfusion chamber following enucleation should be minimized (typically within two hours) to assure meeting assay acceptance criteria. All eyes used in the assay should be from the same group of eyes collected on a specific day.

20. Since eyes are dissected in the laboratory, the intact heads are transported from the slaughterhouse at ambient temperature (typically between 18^{o}C and 25^{o}C) in plastic boxes humidified with tissues moistened with isotonic saline.

Selection Criteria and Number of Eyes Used in the ICE

21. Eyes that have high baseline fluorescein staining (i.e., > 0.5) or corneal opacity score (i.e., > 0.5) after they are enucleated are rejected.

22. Each treatment group and concurrent positive control consists of at least three eyes. The negative control group or the solvent control (if using a solvent other than saline) consists of at least one eye.

23. In the case of solid materials leading to a GHS No Category outcome, a second run of three eyes is recommended to confirm or discard the negative outcome.

PROCEDURE

Preparation of the Eyes

24. The eyelids are carefully excised, taking care not to damage the cornea. Corneal integrity is quickly assessed with a drop of 2% (w/v) sodium fluorescein applied to the corneal surface for a few seconds, and then rinsed with isotonic saline. Fluorescein-treated eyes are then examined with a slit-lamp microscope to ensure that the cornea is undamaged (i.e., fluorescein retention and corneal opacity scores ≤ 0.5).

25. If undamaged, the eye is further dissected from the skull, taking care not to damage the cornea. The eyeball is pulled from the orbit by holding the nictitating membrane firmly with surgical forceps, and the eye muscles are cut with a bent, blunt-tipped scissor. It is important to avoid causing corneal damage due to excessive pressure (i.e., compression artefacts).

26. When the eye is removed from the orbit, a visible portion of the optic nerve should be left attached. Once removed from the orbit, the eye is placed on an absorbent pad and the nictitating membrane and other connective tissue are cut away.

27. The enucleated eye is mounted in a clamp (stainless steel or suitable alternative) with the cornea positioned vertically, and avoiding too much pressure on the eye by the clamp (due to the relatively firm sclera of the chicken eye-ball, only slight pressure is needed to fix the eye properly). The clamp is then transferred to a chamber of the superfusion apparatus (25). The clamps should be positioned in the superfusion apparatus such that the entire cornea is supplied with the isotonic saline drip (3-4 drops per minute or 0.1 to 0.15 mL/min). The chambers of the superfusion apparatus should be temperature controlled at 32 ± 1.5°C. Annex 4 provides a diagram of a typical superfusion apparatus and the eye clamps, which can be obtained commercially or constructed. The apparatus can be modified to meet the needs of an individual laboratory (e.g., to accommodate a different number of eyes).

28. After being placed in the superfusion apparatus, the eyes are again examined with a slit-lamp microscope (e.g., Haag-Streit BP900) to ensure that they have not been damaged during the dissection procedure. Corneal thickness should also be measured at this time at the corneal apex using the depth measuring device on the slit-lamp microscope. Eyes with; (i), a fluorescein retention score of > 0.5; (ii) corneal opacity > 0.5; or, (iii), any additional signs of damage should be replaced. For eyes that are not rejected based on any of these criteria, individual eyes with a corneal thickness deviating more than 10% from the mean value for all eyes are to be rejected. For the Haag-Streit slit lamp BP900 fitted with depth-measuring device no. 1, the slit-width setting should be 9½ equalling 0.095 mm. Alternatively the slit-lamp BQ900 from Haag-Streit may be used as long as it can be mounted with the depth measuring device and a slit width of 0.095 can be applied (see also paragraph 53). Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different.

29. Once all eyes have been examined and approved, the eyes are incubated for approximately 45 to 60 minutes to equilibrate them to the test system prior to dosing. Following the equilibration period, a zero reference measurement is recorded for corneal thickness and opacity to serve as a baseline (i.e., time = 0). The fluorescein score determined at dissection is used as the baseline measurement for that endpoint.

Application of the Test Chemical

30. Immediately following the zero reference measurements, the eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test chemical is applied to the cornea.

31. Liquid test chemicals are typically tested undiluted, but may be diluted if deemed necessary (e.g., as part of the study design). The preferred solvent for dilution of test chemicals is physiological (isotonic) saline. However, alternative solvents may also be used under controlled conditions, but the appropriateness of solvents other than physiological saline should be demonstrated.

32. Liquid test chemicals are applied to the cornea such that the entire surface of the cornea is evenly covered with the test chemical; the standard volume is 0.03 mL.

33. If possible, solid test chemicals should be ground as finely as possible in a mortar and pestle, or comparable grinding tool. The powder is applied to the cornea such that the surface is uniformly covered with the test chemical; the standard amount is 0.03 g.

34. The test chemical (liquid or solid) is applied for 10 seconds and then rinsed from the eye with isotonic saline (approximately 20 mL) at ambient temperature. The eye (in its holder) is subsequently returned to the superfusion apparatus in the original upright position. In case of need, additional rinsing may be used after the 10-sec application and at subsequent time points (e.g., upon discovery of residues of test chemical on the cornea). In general the amount of saline additionally used for rinsing is not critical, but the observation of adherence of chemical to the cornea is important.

Control Chemicals

35. Concurrent negative or solvent/vehicle controls and positive controls should be included in each experiment.

36. When testing liquids at 100% or solids, physiological (isotonic) saline is used as the concurrent negative control in the ICE test method to detect non-specific changes in the test system, and to ensure that the assay conditions do not inappropriately result in an irritant response.

37. When testing diluted liquids, a concurrent solvent/vehicle control group is included in the test method to detect non-specific changes in the test system, and to ensure that the assay conditions do not inappropriately result in an irritant response. As stated in paragraph 31, only a solvent/vehicle that has been demonstrated to have no adverse effects on the test system can be used.

38. A known ocular irritant is included as a concurrent positive control in each experiment to verify that an appropriate response is induced. As the ICE test method is being used in this Test Guideline to identify chemicals inducing serious eye damage, the positive control should be a reference chemical inducing responses that fulfil the criteria for classification as UN GHS Category 1 in this test method. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of the severe response should not be excessive. Sufficient in vitro data for the positive control should be generated such that a statistically defined acceptable range for the positive control can be calculated. If adequate historical ICE test method data are not available for a particular positive control, studies may need to be conducted to

provide this information.

39. Examples of positive controls for liquid test chemicals are 10% acetic acid or 5% benzalkonium chloride, while examples of positive controls for solid test chemicals are sodium hydroxide or imidazole.

40. Benchmark chemicals are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses.

Endpoints Measured

41. Treated corneas are evaluated prior to treatment and at 30, 75, 120, 180, and 240 minutes (±5 minutes) after the post-treatment rinse. These time points provide an adequate number of measurements over the four-hour observation period, while leaving sufficient time between measurements for the requisite observations to be made for all eyes.

42. The endpoints evaluated are corneal opacity, swelling, fluorescein retention, and morphological effects (e.g., pitting or loosening of the epithelium). All of the endpoints, with the exception of fluorescein retention (which is determined only prior to treatment and 30 minutes after test chemical exposure) are determined at each of the above time points.

43. Photographs are advisable to document corneal opacity, fluorescein retention, morphological effects and, if conducted, histopathology.

44. After the final examination at four hours, users are encouraged to preserve eyes in an appropriate fixative (e.g., neutral buffered formalin) for possible histopathological examination in particular for non-extreme pH (2 < pH < 11.5) detergents and surfactants (see paragraphs 7, 14 and 56). If histopathology is conducted, eyes should be fixed, trimmed, embedded in paraffin wax, sectioned and stained according to the procedures described for the collection and processing of histopathology specimens within the OECD GD 160 (12).

45. Corneal swelling is determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope. It is expressed as a percentage and is calculated from corneal thickness measurements according to the following formula:

$$\left(\frac{corneal\ thickness\ at\ time\ t \quad - \quad corneal\ thickness\ at\ time = 0}{corneal\ thickness\ at\ time = 0} \right) \quad \times \quad 100$$

46. The mean percentage of corneal swelling for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal swelling, as observed at any time point, an ICE Class is assigned for each test chemical (see paragraph 53).

47. Corneal opacity is evaluated by using the area of the cornea that is most densely opacified for scoring according to the observations described in Table 1. The mean corneal opacity value for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal opacity, as observed at any time point, an ICE class is assigned for each test chemical (see paragraph 53).

Table 1. Corneal opacity scores

Score	Observation
0	No opacity
0.5	Very faint opacity
1	Scattered or diffuse areas; details of the iris are clearly visible
2	Easily discernible translucent area; details of the iris are slightly obscured
3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

48. Fluorescein retention is evaluated at the 30 minute observation time point only according to the scores shown in Table 2. The mean fluorescein retention value of all test eyes is then calculated for the 30-minute observation time point, and used to assign an ICE class for each test chemical (see paragraph 53).

Table 2. Fluorescein retention scores

Score	Observation
0	No fluorescein retention
0.5	Very minor single cell staining
1	Single cell staining scattered throughout the treated area of the cornea
2	Focal or confluent dense single cell staining
3	Confluent large areas of the cornea retaining fluorescein

49. Morphological effects include “pitting” of corneal epithelium, “loosening” of epithelium, “roughening” of the corneal surface and “sticking” of the test chemical to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the interpretation of the investigator.

50. If histopathology is conducted, the semi-quantitative scoring system described in Table 3 should be used. It is critical to distinguish, for example regarding epithelial vacuolation effects, the treatment-related effects from histopathological artefacts and/or background morphology. For this purpose the Atlas presented in Annex II of the OECD GD 160 should be carefully consulted (12). Furthermore, original slides (rather than photomicrographs) need to be used as some effects require a three-dimensional evaluation of the tissues. Only effects that are observed should be scored. No assumptions should be made (e.g., if the top layer of the epithelium is missing it will not be possible to score for vacuolation in that layer). Furthermore, effects/changes close to the limbus should be scored if the tissue architecture was preserved. However, effects/changes occurring within the limbus should not be scored due to effects not linked to the chemical exposure. An internal pathology peer review system should be conducted in accordance with current recommendations (23) and according to the OECD advisory document n. 16 on GLP requirements for peer review of histopathology (24). In this process, a pathologist (with expertise on the tissues

to be evaluated)peer-reviews a number of slides and pathology data (e.g., 1 out of 3 eyes) to assist the study pathologist in refining pathology diagnoses and interpretations. Such peer review process allows to verify and improve the accuracy and quality of pathology diagnoses and interpretations. Finally, consolidated training, transferability and proficiency appraisal are recommended to ensure consistent histopathological observations.

Table 3. Semi-quantitative histopathological scoring system used for isolated chicken eyes that were fixed, trimmed, embedded in paraffin wax, sectioned and stained

Parameter	Observation	Score	Description*
Epithelium: erosion	Very slight	½	Few single cells up to the entire single superficial layer
	Slight	1	Up to 3 layers are gone
	Moderate	2	Up to 50 % of the epithelial layer is gone*
	Severe	3	Epithelial layer is gone up to the basement membrane
Epithelium: vacuolation ***Separately scored for the top, mid, and lower parts of the epithelium*****	Very slight	½	Single to few scattered cells
	Slight	1	Groups of vacuolated cells or single string of cells with small vacuoles
	Moderate	2	Up to 50% of the epithelium consists of vacuolated cells*
	Severe	3	50 – 100% of the epithelium consists of vacuolated cells
Epithelium:necrosis***	Normal	-	< 10 necrotic cells†
	Very slight	½	10 – 20 necrotic cells†
	Slight	1	20 – 40 necrotic cells†
	Moderate	2	Many necrotic cells but < 50% of the epithelial layer
	Severe	3	50 – 100% of the epithelial layer is necrotic.
Stroma: pyknotic nuclei ††† ***In top or bottom region***	Normal	-	< 5 pyknotic nuclei
	Slight	1	5-10 pyknotic nuclei
	Moderate	2	> 10 pyknotic nuclei
Stromal disorder of fibres †††	Present	P	Irregular appearance of the fibres.
Endothelium:necrosis	Present	P	The endothelium consists of only one layer, so a grade is not relevant

Notes: Annex II of the OECD GD 160 (12) displays an Atlas with typical photomicrographs of untreated as well as treated Isolated Chicken Eyes illustrating the various possible histopathological effects described above.

*Over the entire cornea except in case of test chemicals (e.g. some solid chemicals) causing localized effects despite of the homogenous application of the test chemical as required within the OECD TG 438. In this case the evaluation should be based on the localized effects at the site(s) of exposure.

**Top, mid and lower parts represent equal one third parts of the epithelial layer each. If the top layer is missing, the mid layer does not become the 'new' top layer, but is still the mid layer (see Annex II of the OECD GD 160 for more details (12)).

***Only necrosis of attached cells/tissues.
† Necrotic cells are counted across the entire length of the cornea (there is no need for a specific fixed length to report cell counts because the entire length of the cornea is consistent on each slide as there is almost no variation in the size of the chicken eyes used and in the size of the samples evaluated microscopically). The scoring system uses absolute cell counts from 'normal' to 'slight', versus a percentage for 'moderate' and 'severe'. This is due to the way the evaluation is performed by the examiner: necrotic cells are seen as individual items. If there are more, they are usually scattered. Therefore the examiner counts them to get an impression of the amount of necrosis. This is in contrast to erosion, for which the first effect the examiner notices is that a part of the epithelium is missing, so it makes sense to use an estimated percentage of loss.
†† The ICE test method already includes a precise measurement of the thickness of the cornea using a slit lamp microscope. Therefore, swelling of the stroma is not separately scored during the subsequent histopathological evaluation.
††† The stromal effects that are scored consist of (1) pyknotic nuclei, which originate from the scoring system used by Maurer (2001) based on his observations in corneas of rabbits after *in vivo* exposure (described as keratocyte loss/necrosis), and of (2) disorder of fibres. Regarding (1), the presence of pyknotic nuclei is observed only occasionally and the development of pyknotic nuclei is proposed to be dependent on the depth of injury and/or the inflammation process of the cornea *(in vivo)*. Furthermore, due to the elongated form of the stromal fibroblasts, normal nuclei could be misleadingly considered as pyknotic nuclei depending on the section orientation of cells . Regarding (2), the observation and scoring of disorder of fibres may be difficult because the stromal fibres already show a "natural" disorder. The processing of the cornea for microscopy can also contribute to an artificial disorder of stromal fibres. In both cases (pyknotic nuclei and disorder of fibres), these observations coincide with severe corneal effects already observed by the slit-lamp microscope observations, and with effects observed in the mid and/or lower epithelial layer.

52. The OECD TG 438 requires test chemicals to be homogenously distributed on the surface of the treated eyes. Based on such exposure, test chemicals usually cause homogenous effects in the cornea of the isolated chicken eyes, and the mean of histopathological effects over the entire slide should be scored. However, some test chemicals may cause focal or multifocal effects confined to certain spots despite their homogenous application (e.g., as for some solid test chemicals). If (multi)focal effects are observed during the performance of the ICE test method, the histopathologist should be informed and the histopathological scoring should be conducted based on the localized adverse effects observed where exposure to the test chemical occurred. Furthermore, if doubts remain (e.g. a discrepancy between the ICE results and the histopathological observations is noticed), additional slices may be prepared on other parts of the cornea to ensure the localized effects are present in the observed section.

DATA AND REPORTING

Data Evaluation

53. Results from corneal opacity, swelling and fluorescein retention should be evaluated separately to generate an ICE class for each endpoint. The ICE classes for each endpoint are then combined to predict the In Vitro Classification of each test chemical. Similarly, histopathology evaluation, if applicable, should be conducted separately and considered according to paragraphs 55 and 56.

Decision Criteria

54. Once each endpoint has been evaluated, ICE classes can be assigned based on a predetermined range. Interpretation of corneal swelling (Table 4), opacity (Table 5), and fluorescein retention (Table 6) using four ICE classes is done according to the scales shown below. It is important to note that the corneal swelling scores shown in Table 4 are only applicable if thickness is measured with a Haag-Streit BP900 slit-lamp microscope (or alternatively a Haag-Streit BQ900 slit-lamp microscope) with depth-measuring device no. 1 and slit-width setting at 9½, equalling

0.095 mm. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different.

Table 4. ICE classification criteria for corneal swelling

Mean Corneal Swelling (%)*	ICE Class
0 to 5	I
>5 to 12	II
>12 to 18 (>75 min after treatment)	II
>12 to 18 (≥75 min after treatment)	III
>18 to 26	III
>26 to 32 (>75 min after treatment)	III
>26 to 32 (≥75 min after treatment)	IV
>32	IV

Note: Highest mean score observed at any time point.

Table 5. ICE classification criteria for opacity.

Maximum Mean Opacity Score*	ICE Class
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-4.0	IV

Note: *Maximum mean score observed at any time point (based on opacity scores as defined in Table 1). *Based on scores as defined in Table 2.

Table 6. ICE classification criteria for mean fluorescein retention.

Mean Fluorescein Retention Score at 30 minutes post-treatment*	ICE Class
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-3.0	IV

Note: Based on scores as defined in Table 2.

55. The in vitro classification for a test chemical is assessed by reading the UN GHS classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention as described in Table 7.

Table 7. Overall in vitro classifications

UN GHS Classification	Combinations of the 3 Endpoints
No Category	3 x I
	2 x I, 1 x II
	2 x II, 1 x I
No prediction can be made	Other combinations
Category 1	3 x IV
	2 x IV, 1 x III
	2 x IV, 1 x II*
	2 x IV, 1 x I*
	Corneal opacity = 3 at 30 min (in at least 2 eyes)
	Corneal opacity = 4 at any time point (in at least 2 eyes)
	Severe loosening of the epithelium (in at least 1 eye)

Note: Combinations less likely to occur.

56. If histopathology is used for non-extreme pH (2 < pH < 11.5) detergents and surfactants, the decision criteria shown in Table 8 should be used. In addition, in case stromal pyknotic nuclei scores ≥ slight (score 1) in at least 2 out of 3 eyes are observed; or any endothelium effects are observed in at least 2 out of 3 eyes, such effects should be noted as observations to give indication on the severity of effects.

Table 8. Histopathology decision criteria to be used in addition to the standard validated ICE test method for the identification of UN GHS Category 1 non-extreme pH (2<pH<11.5) detergents and surfactants

Tissue layer	Effects triggering eye serious damage (GHS Category 1) identification
Epithelium	- erosion = moderate (score 2) in at least 2 out of 3 eyes
	- and/or, any vacuolation (= very slight, score ½) observed in the mid and/or lower parts in at least 2 out of 3 eyes
	- or, if erosion = moderate (score 2) in 1 out of 3 eyes + vacuolation = very slight in mid and/or low part (score ½) is observed in at least another eye out of the 3 eyes
	- and/or, necrosis = moderate (score 2) observed in at least 2 out of 3 eyes

57. Furthermore, the prediction model shown in table 9 should be used. The ICE histopathology criteria and the prediction model described in Tables 8 and 9, respectively are applicable only to identify UN GHS Category 1 non-extreme pH (2 < pH < 11.5) detergents and surfactants.

Table 9. Prediction model for identification of non-extreme pH (2<pH<11.5) detergents and surfactants based on ICE histopathology evaluations

Standard ICE	ICE histopathology criteria described in Table 8	UN GHS Classification
No prediction can be made	Criteria met	UN GHS Category 1
	Criteria not met	No prediction can be made

Study Acceptance Criteria

58. A test is considered acceptable if the concurrent negative or vehicle/solvent controls and the concurrent positive controls are identified as GHS Non-Classified and GHS Category 1, respectively.

Test Report

59. The test report should include the following information, if 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 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;
- Treatment of the test/control chemical prior to testing, if applicable (e.g. warming, grinding);
- Storage conditions and stability to the extent available;

Information Concerning the Sponsor and the Test Facility

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

- Identification on the source of the eyes (e.g., the facility from which they were collected);

Test Method Conditions

- Description of test system used;
- Slit-lamp microscope and pachymeter used (e.g., model) and the instrument settings used;
- Reference to historical negative and positive control results and, if applicable, historical data demonstrating acceptable concurrent benchmark control ranges;
- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time (e.g., periodic testing of proficiency chemicals)).
- The procedure used for tissues fixation in case histopathology is performed.

Eyes Collection and Preparation

- Age and weight of the donor animal and if available, other specific characteristics of the animals from which the eyes were collected (e.g. sex, strain);
- Storage and transport conditions of eyes (e.g., date and time of eye collection, time interval between collection of chicken heads and placing the enucleated eyes in superfusion chamber);
- Preparation & mounting of the eyes including statements regarding their quality, temperature of eye chambers, and criteria for selection of eyes used for testing.

Test Procedure

- Number of replicates used;
- Identity of the negative and positive controls used (if applicable, also the solvent and benchmark controls);
- Test chemical dose, application and exposure time used;
- Observation time points (pre- and post- treatment);
- Description of evaluation and decision criteria used including for histopathology if applicable;
- Peer-review system used for histopathological observations, if applicable;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.
- Furthermore, if not included in the e.g. standard operating procedure (SOP), when available, the following information shall be included:
- Description of consolidated training and transferability;
- Fixative, dehydration and clarifying agents, and protocols used;
- Embedding material, infiltration solvents, and concentrations used;
- Thickness of tissue sections;
- Stain (in report) and the associated staining protocol used;
- Information on instruments used;

Results

- Tabulation of corneal swelling, opacity and fluorescein retention scores obtained for each individual eye and at each observation time point, including the mean scores at each observation time of all tested eyes;
- Description of any morphological effects observed;
- The highest mean corneal swelling, opacity and fluorescein retention scores observed (from any time point), and its relating ICE class.;
- Tabulation of histopathological semi-quantitative scoring observations and derived conclusions if applicable;
- If applicable, indication of use of localized effects for histopathological scoring;
- Description of any other effects observed;
- The derived in vitro GHS classification;
- If appropriate, photographs of the treated and control eyes
- If applicable, optional digital images or digital slide scans of the histopathology specimens;

Discussion of the Results.

Conclusion.

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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.

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.

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).

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

Corneal opacity: Measurement of the extent of opaqueness of the cornea following exposure to a test chemical. Increased corneal opacity is indicative of damage to the cornea.

Corneal swelling: An objective measurement in the ICE test of the extent of distension of the cornea following exposure to a test chemical. It is expressed as a percentage and is calculated from baseline (pre-dose) corneal thickness measurements and the thickness recorded at regular intervals after exposure to the test material in the ICE test. The degree of corneal swelling is indicative of damage to the cornea.

Detergents: a mixture (excluding dilutions of single surfactant) containing one or more surfactants at a final concentration of > 3%, intended for washing and cleaning processes. Detergents may be in any form (liquid, powder, paste, bar, cake, moulded piece, shape, etc.) and marketed for or used in household, or institutional or industrial purposes.

Eye Irritation: Production of changes in the eye following the application of 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" (4).

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.

Fluorescein retention: A subjective measurement in the ICE test of the extent of fluorescein sodium that is retained by epithelial cells in the cornea following exposure to a test chemical. The degree of fluorescein retention is indicative of damage to the corneal epithelium.

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.

IATA: Integrated Approach on Testing and Assessment.

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

Mixture: A mixture or a solution composed of two or more substances in which they do not react (4).

Negative control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

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

Positive control: A replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the severe response should not be excessive.

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.

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

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical 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" (4).

Slit-lamp microscope: An instrument used to directly examine the eye under the magnification of a binocular microscope by creating a stereoscopic, erect image. In the ICE test method, this instrument is used to view the anterior structures of the chicken eye as well as to objectively measure corneal thickness with a depth-measuring device attachment.

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.

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 (4).

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).

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.

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 (4).

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 test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose.

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 2: PROFICIENCY CHEMICALS FOR THE ICE 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 13 chemicals recommended in Table 10. The ICE outcomes provided represent examples of the range of responses observed during the evaluation studies and that may be expected (5)(18).These chemicals were selected to represent the range of responses for eye hazards based on results from the in vivo rabbit eye test (TG 405) and the UN GHS classification system (i.e., UN GHS Categories 1, 2A, 2B, or No Category) (4)(26). Other selection criteria were, to the extent possible that these chemicals produced reproducible results in the ICE test method, are commercially available and have high quality in vivo reference data available. Reference data are available in the SSD (5). 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 evaluation and validation of the ICE test method could be used (5) (18). Such deviations should however be justified.

Table 10. Recommended chemicals for demonstrating technical proficiency with ICE

Chemical	CASRN	Chemical Class[1]	Physical Form	*In Vivo* UN GHS Classification[2]	ICE UN GHS Classification[3,4]
Benzalkonium chloride (10%)	8001-54-5	Onium compound	Liquid	Category 1	Category 1
Chlorhexidine	55-56-1	Amine, amidine	Solid	Category 1	Category 1
Sodium hydroxide (10%)	1310-73-2	Alkali	Liquid	Category 1	Category 1
Imidazole	288-32-4	Heterocyclic	Solid	Category 1	Category 1
Trichloroacetic acid (30%)	76-03-9	Carboxylic acid	Liquid	Category 1	Category 1
2,6-Dichlorobenz-oyl chloride	4659-45-4	Acyl halide	Liquid	Category 2A	No predictions can be made [4]
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2A[5]	No predictions can be made [4]
Sodium hydroxide (1%)	1310-73-2	Alkali	Liquid	Category 2B	No predictions can be made [4]
Dimethyl sulfoxide	67-68-5	Organic sulphur compound	Liquid	No Category	No Category
Ethyl trimethyl acetate	3938-95-2	Ester	Liquid	No Category	No Category
Methylcyclo-pentane	96-37-7	Hydrocarbon (cyclic)	Liquid	No Category	No Category
n-Hexane	110-54-3	Hydrocarbon (acyclic)	Liquid	No Category	No Category
Triacetin	102-76-1	Lipid	Liquid	No Category	No Category

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; ICE: Isolated Chicken Eye test; n.a.: not available; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals (4).

[1]Chemical classes were assigned to each chemical using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http//www.nlm.nih.gov/mesh)

[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS (4)(26).

[3]Based on results in ICE as described in table 7.

[4] Combination of ICE scores other than the ones described in table 6 for the identification of GHS no-category and GHS Category 1 (see table 7)

[5] Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, *i.e.* 1 out of 3 vs. 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from conjunctiva redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a conjunctiva redness score of 1 (at day 7) that fully recovered at day 10.

ANNEX 3: PROFICIENCY CHEMICALS FOR THE ICE HISTOPATHOLOGY TO BE USED IN ADDITION TO THE STANDARD ICE TEST METHOD FOR THE LIMITED APPLICABILITY DOMAIN OF NON-EXTREME PH (2 < PH < 11.5) DETERGENTS AND SURFACTANTS

Prior to routine use of ICE histopathology in addition to the standard ICE test method for the limited use domain of non-extreme pH (2 < pH < 11.5) detergents and surfactants, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the 6 chemicals recommended in Table 11. These chemicals were selected to represent the range of responses for eye hazards based on results from the in vivo rabbit eye test (TG 405) and the UN GHS classification system (i.e., UN GHS Categories 1, 2, or No Category) (4)(26). Other selection criteria were, to the extent possible that these chemicals produced reproducible results in the ICE histopathology, are commercially available and have high quality in vivo data available. 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 evaluation of the ICE histopathology could be used (10). Such deviations should however be justified.

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; NPCM:

Table 11. Recommended chemicals for demonstrating technical proficiency with ICE histopathology

Chemical	CASRN	Surfactant type	Physical Form	*In Vivo* Classification[1]	Standard ICE UN GHS classification[2]	ICE Histopathology UN GHS classification [3]
Benzalkonium chloride (5%)	8001-54-5	Cationic	Liquid	Category 1	Category 1	Category 1 (erosion) in 3 out of 3 laboratories
Benzensulphonylchloride	98-09-9	Anionic	Liquid	Category 1	Category 1	Category 1 (necrosis and vacuolation) in 3 out of 3 laboratories
Cetylpiridinium bromide (10%)	140-72-7	Cationic	Liquid	Category 1	No predictions can be made	Category 1 (vacuolation) in 3 out of 3 laboratories
Cetylpiridinium bromide (1%)	140-72-7	Cationic	Liquid	Category 2A	No predictions can be made	No predictions can be made in 3 out of 3 laboratories
N-Lauroyl sarcosine Na salt (10%)	137-16-6	Anionic	Liquid	Category 2A	No predictions can be made	No predictions can be made in 3 out of 3 laboratories
Cetylpiridinium bromide (0.1%)	140-72-7	Cationic	Liquid	No Category	No predictions can be made	No predictions can be made in 3 out of 3 laboratories

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; NPCM: No Prediction Can Be Made

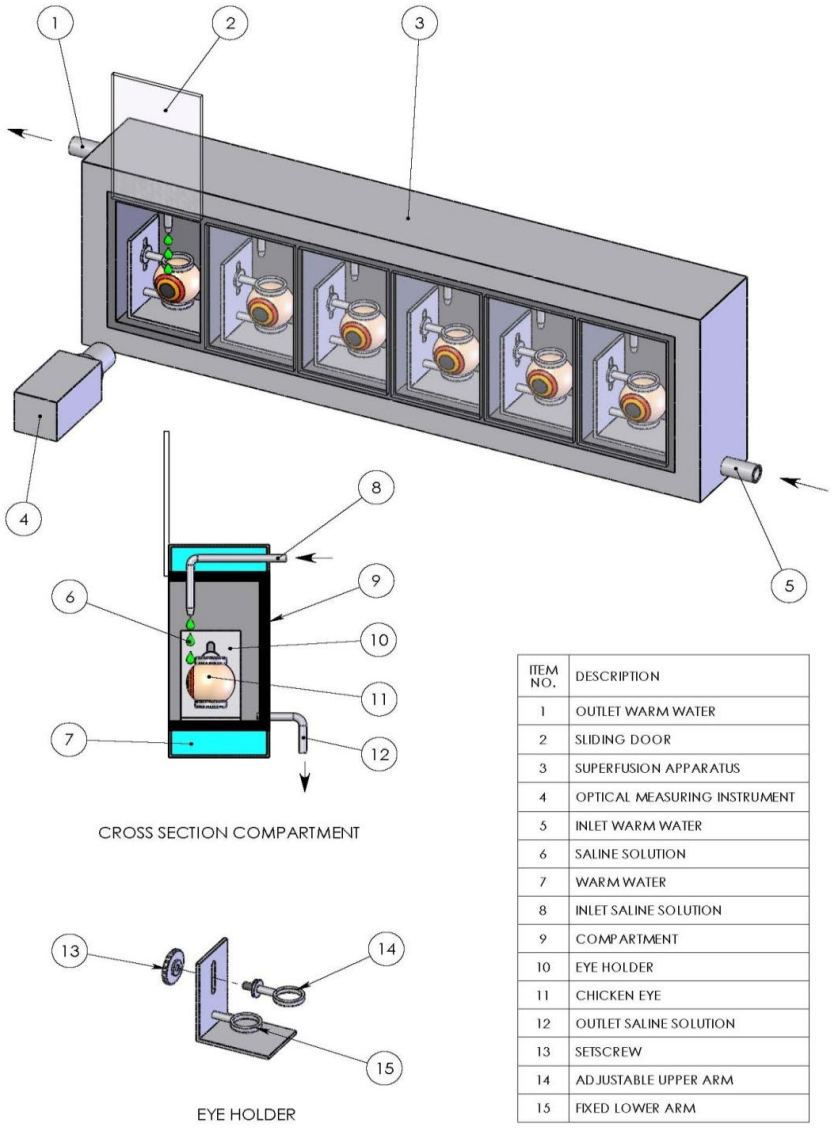
1Based on results from the in vivo rabbit eye test (OECD TG 405) and using the UN GHS (4)(26).

2Based on results in ICE as described in table 7.

3Based on ICE histopathology criteria as described in tables 8 and 9 and within the revised OECD GD 160 (12).

ANNEX 4

Figure 1. Diagrams of the ice superfusion apparatus and eye clamps



ITEM NO.	DESCRIPTION
1	OUTLET WARM WATER
2	SLIDING DOOR
3	SUPERFUSION APPARATUS
4	OPTICAL MEASURING INSTRUMENT
5	INLET WARM WATER
6	SALINE SOLUTION
7	WARM WATER
8	INLET SALINE SOLUTION
9	COMPARTMENT
10	EYE HOLDER
11	CHICKEN EYE
12	OUTLET SALINE SOLUTION
13	SETSCREW
14	ADJUSTABLE UPPER ARM
15	FIXED LOWER ARM

Note: See (25) for additional generic descriptions of the superfusion apparatus and eye clamp.