

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 Center for the Validation of Alternative Methods (JaCVAM), in 2006 and 2010 (1) (2) (3). In the first evaluation, the ICE was endorsed as a scientific 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). In the second evaluation, the ICE test method was evaluated for use as a screening test to identify chemicals not classified for eye irritation or serious eye damage as defined by UN GHS (3) (4). The results from the validation study and the peer review panel recommendations maintained the original recommendation for using the ICE for classification of chemicals inducing serious eye damage (UN GHS Category 1), as the available database remained unchanged since the original ICCVAM validation. At this stage, no further recommendations for an expansion of the ICE applicability domain to also include other categories were suggested. A re-evaluation of the *in vitro* and *in vivo* dataset used in the validation study was made with the focus of evaluating the usefulness of the ICE to identify chemicals not requiring classification for eye irritation or serious eye damage. . This re-evaluation concluded that the ICE test method can also be used to identify chemicals not requiring classification for eye irritation and serious eye damage as defined by the UN GHS (4) (5). This Test Guideline (adopted in 2009 and updated in 2013) includes the recommended uses and limitations of the ICE test method based on its evaluations. . The main differences between the original 2009 version and the 2013 updated version include, but are not limited to, the use of the ICE test method to identify chemicals not requiring classification according to the UN GHS Classification System, an update to the test report elements, an update of Annex I on definitions, and an update to Annex II on the proficiency chemicals.

2. 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 8 to 10 for eye hazard classification and labelling of chemicals. While it is not considered valid as a complete replacement for the *in vivo* rabbit eye test, the ICE test method is recommended as an initial step within a Top-Down approach (6) to identify chemicals inducing serious eye damage, i.e., classified as UN GHS Category 1 (4).

3. 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, NC) (4), and may therefore be used as an initial step within a Bottom-Up testing strategy approach (6).

4. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test substance as measured by its ability to induce or not toxicity in an enucleated chicken eye. 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. The corneal opacity, swelling, and damage assessments following exposure to a test substance are assessed individually and then combined to derive an Eye Irritancy Classification.

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2 5. Definitions are provided in Annex I.

3 INITIAL CONSIDERATIONS AND LIMITATIONS

4 6. This Test Guideline is based on the protocol suggested in the OECD Guidance Document 160
5 (7), which was developed following the ICCVAM international validation study (1) (3) (8), with
6 contributions from the European Centre for the Validation of Alternative Methods, the Japanese Center for
7 the Validation of Alternative Methods, and TNO Quality of Life Department of Toxicology and Applied
8 Pharmacology (Netherlands). The protocol is based on information obtained from published protocols, as
9 well as the current protocol used by TNO (9) (10) (11) (12) (13).

10 7. A wide range of chemicals has been tested in the validation underlying this Test Guideline and
11 the empirical database of the validation study amounted to 151 chemicals including 68 substances and
12 83 mixtures (1) (3) (5). The Test Guideline is applicable to solids, liquids, emulsions and gels. The
13 liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Gases and aerosols
14 have not been assessed yet in a validation study.

15 8. The ICE test method can be used to identify chemicals inducing serious eye damage, i.e.,
16 classified as UN GHS Category 1 (4). When used for this purpose, the identified limitations for the ICE
17 test method are based on the high false positive rates for alcohols and the high false negative rate for
18 solids as surfactants (1) (3) (8). However, false negative rates in this context (UN GHS Category 1
19 identified as not being UN GHS Category 1) are not critical since all test substances that come out
20 negative would be subsequently tested with other adequately validated *in vitro* test(s), or as a last option in
21 rabbits, depending on regulatory requirements, using a sequential testing strategy such as recommended in
22 Test Guideline 405 (14). It should be noted that solids may lead to variable and extreme exposure
23 conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true
24 irritation potential (15). Investigators could consider using this test method for all types of chemicals,
25 whereby a positive result should be accepted as indicative of serious eye damage, i.e., UN GHS Category
26 1 classification. However, positive results obtained with alcohols should be interpreted cautiously due to
27 risk of over-prediction.

28 9. When used to identify chemicals inducing serious eye damage (UN GHS Category 1), the ICE
29 test method has an overall accuracy of 86% (119/139), a false positive rate of 6% (7/112) and a false
30 negative rate of 48% (13/27) when compared to *in vivo* rabbit eye test method data classified according to
31 the UN GHS classification system (4) (5). When test substances within certain chemical (*i.e.*, alcohols and
32 surfactants) or physical (*i.e.*, solids) classes are excluded from the database, the accuracy of the ICE test
33 method is of 94% (76/81), the false positive rate of 4% (3/74), and the false negative rate of 29% (2/7) for
34 the UN GHS classification system (4) (5).

35 10. The ICE test method can also be used to identify chemicals that do not require classification for
36 eye irritation or serious eye damage under the UN GHS classification system (4). This test method can be
37 used for all types of chemicals, whereby a negative result could be accepted for not classifying a chemical
38 for eye irritation and serious eye damage. However, on the basis of one result from the validation
39 database, anti-fouling organic solvent-containing paints may risk under-prediction (5).
40 When used to identify chemicals that do not require classification for eye irritation and serious eye
41 damage, the ICE test method has an overall accuracy of 83% (125/151), a false positive rate of 32%
42 (25/78), and a false negative rate of 1% (1/73), when compared to *in vivo* rabbit eye test method data
43 classified according to the UN GHS (4) (5). When test substances within certain classes (*i.e.*, anti-fouling
44 organic solvent containing paints) are excluded from the database, the accuracy of the ICE test method
45 is of 83% (123/148), a false positive rate of 32% (25/77), and a false negative rate of 0% (0/71) for the
46 UN GHS classification system (4) (5).

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2 11. The ICE test method is not recommended for the identification of test substances that should be
3 classified as irritating to eyes (*i.e.*, UN GHS Category 2 or Category 2A) or test substances that should be
4 classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS
5 Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category
6 chemicals overclassified as UN GHS Category 2, 2A or 2B. For this purpose, further testing with another
7 suitable method may be required.

8 12. All procedures with chicken eyes should follow the test facility's applicable regulations and
9 procedures for handling of human or animal-derived materials, which include, but are not limited to,
10 tissues and tissue fluids. Universal laboratory precautions are recommended (16).

11 13. Whilst the ICE test method does not consider conjunctival and iridal injuries as evaluated in the
12 rabbit ocular irritancy test method, it addresses corneal effects which are the major driver of classification
13 *in vivo* when considering the UN GHS Classification. Also, although the reversibility of corneal lesions
14 cannot be evaluated *per se* in the ICE test method, it has been proposed, based on rabbit eye studies, that an
15 assessment of the initial depth of corneal injury may be used to identify some types of irreversible effects
16 (17). In particular, further scientific knowledge is required to understand how irreversible effects not linked
17 with initial high level injury occur. Finally, the ICE test method does not allow for an assessment of the
18 potential for systemic toxicity associated with ocular exposure.

19 14. This Test Guideline will be updated periodically as new information and data are considered. For
20 example, histopathology may be potentially useful when a more complete characterization of corneal
21 damage is needed. To evaluate this possibility, users are encouraged to preserve eyes and prepare
22 histopathology specimens that can be used to develop a database and decision criteria that may further
23 improve the accuracy of this test method. The OECD has developed a Guidance Document on the use of *in*
24 *vitro* ocular toxicity test methods, which includes detailed procedures on the collection of histopathology
25 specimens and information on where to submit specimens and/or histopathology data (7).

26 15. For any laboratory initially establishing this assay, the proficiency chemicals provided in Annex
27 II should be used. A laboratory can use these chemicals to demonstrate their technical competence in
28 performing the ICE test method prior to submitting ICE data for regulatory hazard classification purposes.

29 **PRINCIPLE OF THE TEST**

30 16. The ICE test method is an organotypic model that provides short-term maintenance of the
31 chicken eye *in vitro*. In this test method, damage by the test substance is assessed by determination of
32 corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative
33 assessment, analysis of corneal swelling provides for a quantitative assessment. Each measurement is
34 either converted into a quantitative score used to calculate an overall Irritation Index, or assigned a
35 qualitative categorization that is used to assign an *in vitro* ocular corrosivity and severe irritancy
36 classification. Either of these outcomes can then be used to predict the *in vivo* ocular corrosivity and severe
37 irritation potential of a test substance (see Decision Criteria).

38 ***Source and Age of Chicken Eyes***

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

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

4 ***Collection and Transport of Eyes to the Laboratory***

5 19. Heads should be removed immediately after sedation of the chickens, usually by electric shock,
6 and incision of the neck for bleeding. A local source of chickens close to the laboratory should be located
7 so that their heads can be transferred from the slaughterhouse to the laboratory quickly enough to minimize
8 deterioration and/or bacterial contamination. The time interval between collection of the chicken heads and
9 placing the eyes in the superfusion chamber following enucleation should be minimized (typically within
10 two hours) to assure meeting assay acceptance criteria. All eyes used in the assay should be from the same
11 group of eyes collected on a specific day.

12 20. Because eyes are dissected in the laboratory, the intact heads are transported from the
13 slaughterhouse at ambient temperature (typically between 18°C and 25°C) in plastic boxes humidified with
14 tissues moistened with isotonic saline.

15 ***Selection Criteria for Eyes Used in the ICE***

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

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

20 **PROCEDURE**

21 ***Preparation of the Eyes***

22 23. The eyelids are carefully excised, taking care not to damage the cornea. Corneal integrity is
23 quickly assessed with a drop of 2% (w/v) sodium fluorescein applied to the corneal surface for a few
24 seconds, and then rinsed with isotonic saline. Fluorescein-treated eyes are then examined with a slit-lamp
25 microscope to ensure that the cornea is undamaged (*i.e.*, fluorescein retention and corneal opacity scores ≤
26 0.5).

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

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

34 26. The enucleated eye is mounted in a stainless steel clamp with the cornea positioned vertically.
35 The clamp is then transferred to a chamber of the superfusion apparatus (18). The clamps should be
36 positioned in the superfusion apparatus such that the entire cornea is supplied with the isotonic saline drip
37 (3-4 drops per minute or 0.1 to 0.15 mL/min). The chambers of the superfusion apparatus should be
38 temperature controlled at 32 ± 1.5°C. Annex III provides a diagram of a typical superfusion apparatus and

1 the eye clamps, which can be obtained commercially or constructed. The apparatus can be modified to
2 meet the needs of an individual laboratory (*e.g.*, to accommodate a different number of eyes).

3 27. After being placed in the superfusion apparatus, the eyes are again examined with a slit-lamp
4 microscope to ensure that they have not been damaged during the dissection procedure. Corneal thickness
5 should also be measured at this time at the corneal apex using the depth measuring device on the slit-lamp
6 microscope. Eyes with; (i), a fluorescein retention score of > 0.5; (ii) corneal opacity > 0.5; or, (iii), any
7 additional signs of damage should be replaced. For eyes that are not rejected based on any of these criteria,
8 individual eyes with a corneal thickness deviating more than 10% from the mean value for all eyes are to
9 be rejected. Users should be aware that slit-lamp microscopes could yield different corneal thickness
10 measurements if the slit-width setting is different. The slit-width should be set at 0.095 mm.

11 28. Once all eyes have been examined and approved, the eyes are incubated for approximately 45 to
12 60 minutes to equilibrate them to the test system prior to dosing. Following the equilibration period, a zero
13 reference measurement is recorded for corneal thickness and opacity to serve as a baseline (*i.e.*, time = 0).
14 The fluorescein score determined at dissection is used as the baseline measurement for that endpoint.

15 ***Application of the Test Substance***

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

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

22 31. Liquid test substances are applied to the cornea such that the entire surface of the cornea is
23 evenly covered with the test substance; the standard volume is 0.03 mL.

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

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

33 ***Control Substances***

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

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

39 36. When testing diluted liquids, a concurrent solvent/vehicle control group is included in the test
40 method to detect non-specific changes in the test system, and to ensure that the assay conditions do not

1 inappropriately result in an irritant response. As stated in paragraph 25, only a solvent/vehicle that has been
2 demonstrated to have no adverse effects on the test system can be used.

3 37. A known ocular irritant is included as a concurrent positive control in each experiment to verify
4 that an appropriate response is induced. As the ICE assay is being used in this Test Guideline to identify
5 corrosive or severe irritants, the positive control should be a reference substance that induces a severe
6 response in this test method. However, to ensure that variability in the positive control response across
7 time can be assessed, the magnitude of the severe response should not be excessive. Sufficient *in vitro* data
8 for the positive control should be generated such that a statistically defined acceptable range for the
9 positive control can be calculated. If adequate historical ICE test method data are not available for a
10 particular positive control, studies may need to be conducted to provide this information.

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

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

16 ***Endpoints Measured***

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

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

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

27 43. After the final examination at four hours, users are encouraged to preserve eyes in an appropriate
28 fixative (*e.g.*, neutral buffered formalin) for possible histopathological examination (see paragraph 13 and
29 (7) for details).

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

$$33 \left(\frac{\text{corneal thickness at time } t - \text{corneal thickness at time } = 0}{\text{corneal thickness at time } = 0} \right) \times 100$$

34 45. The mean percentage of corneal swelling for all test eyes is calculated for all observation time
35 points. Based on the highest mean score for corneal swelling, as observed at any time point, an overall
36 category score is then given for each test substance.

37 46. Corneal opacity is calculated by using the area of the cornea that is most densely opacified for
38 scoring. The mean corneal opacity value for all test eyes is calculated for all observation time points. Based

1 on the highest mean score for corneal opacity, as observed at any time point, an overall category score is
2 then given for each test substance (Table 1).

3 Table 1. Corneal opacity scores.

<u>Score</u>	<u>Observation</u>
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

4

5 47. The mean fluorescein retention value for all test eyes is calculated for the 30-minute observation
6 time point only, which is used for the overall category score given for each test substance (Table 2).

7 Table 2. Fluorescein retention scores.

<u>Score</u>	<u>Observation</u>
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

8 48. Morphological effects include “pitting” of corneal epithelial cells, “loosening” of epithelium,
9 “roughening” of the corneal surface and “sticking” of the test substance to the cornea. These findings can
10 vary in severity and may occur simultaneously. The classification of these findings is subjective according
11 to the interpretation of the investigator.

12 **DATA AND REPORTING**

13 *Data Evaluation*

14 49. Results from corneal opacity, swelling, and fluorescein retention should be evaluated separately
15 to generate an ICE class for each endpoint. The ICE classes for each endpoint are then combined to
16 generate an Irritancy Classification for each test substance.

1 *Decision Criteria*

2 50. Once each endpoint has been evaluated, ICE classes can be assigned based on a predetermined
 3 range. Interpretation of corneal swelling (Table 3), opacity (Table 4), and fluorescein retention (Table 5)
 4 using four ICE classes is done according to the following scales:

5 Table 3. 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

6 ^{*}Corneal swelling scores only applicable if thickness is measured with a
 7 Haag-Streit BP900 slit-lamp microscope with depth-measuring device no. I and
 8 slit-width setting at 9½, equalling 0.095 mm. Users should be aware that slit-
 9 lamp microscopes could yield different corneal thickness measurements if the
 10 slit-width setting is different.

11 Table 4. ICE classification criteria for opacity.

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

12 ^{*}See Table 1.

13 Table 5. 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

14 ^{*}See Table 2.

1 51. The *in vitro* classification for a test substance is assessed by reading the GHS classification that
 2 corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and
 3 fluorescein retention as described in Table 6.

4 Table 6. Overall *in vitro* classifications.

<u>UN GHS Classification</u>	<u>Combinations of the 3 Endpoints</u>
No Category	3 x I
	2 x I, 1 x II
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)

5 *Combinations less likely to occur.

6 ***Study Acceptance Criteria***

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

9 ***Test Report***

10 53. The test report should include the following information, if relevant to the conduct of the study:

11 *Test and Control Substances*

12 Chemical name(s) such as the structural name used by the Chemical Abstracts Service
 13 (CAS), followed by other names, if known;

14 The CAS Registry Number (RN), if known;

15 Purity and composition of the test/control substance or preparation (in percentage(s) by
 16 weight), to the extent this information is available;

17 Physicochemical properties such as physical state, volatility, pH, stability, chemical class
 18 water solubility relevant to the conduct of the study;

19 Treatment of the test/control substances prior to testing, if applicable (*e.g.*, warming,
 20 grinding);

21 Stability, if known;

1 *Information Concerning the Sponsor and the Test Facility*

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

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

4 *Test Method Conditions*

5 Description of test system used;

6 Slit-lamp microscope used (*e.g.*, model) and instrument settings for the slit-lamp
7 microscope used;

8 Reference to historical negative and positive control results and, if applicable, historical
9 data demonstrating acceptable concurrent benchmark control ranges;

10 The procedure used to ensure the integrity (*i.e.*, accuracy and reliability) of the test method
11 over time (*e.g.*, periodic testing of proficiency chemicals)).

12 *Eyes Collection and Preparation*

13 Age and weight of the donor animal and if available, other specific characteristics of the
14 animals from which the eyes were collected (*e.g.*, sex, strain);

15 Storage and transport conditions of eyes (*e.g.*, date and time of eye collection, time interval
16 between collection of chicken heads and placing the enucleated eyes in superfusion
17 chamber);

18 Preparation & mounting of the eyes including statements regarding their quality,
19 temperature of eye chambers, and criteria for selection of eyes used for testing

20 *Test Procedure*

21

22 Number of replicates used;

23 Identity of the negative and positive controls used (if applicable, also the solvent and
24 benchmark controls);

25 Test substance dose, application and exposure time used;

26 Observation time points (pre- and post- treatment);

27 Description of evaluation and decision criteria used;

28 Description of study acceptance criteria used;

29 Description of any modifications of the test procedure;

30 *Results*

31 Tabulation of corneal swelling, opacity and fluorescein retention scores obtained for each
32 individual eye and at each observation time point, including the mean scores at each
33 observation time of all tested eyes;

34 The highest mean corneal swelling, opacity and fluorescein retention scores observed
35 (from any time point), and its relating ICE class.

36 Description of any other effects observed;

- 1 The derived *in vitro* GHS classification;
- 2 If appropriate, photographs of the eye;

3
4 *Discussion of the Results*

5 *Conclusion*

6

1 LITERATURE

- 2 (1) ICCVAM (2007). Test Method Evaluation Report - *In Vitro Ocular Toxicity* Test Methods for
3 Identifying Ocular Severe Irritants and Corrosives. Interagency Coordinating Committee on the Validation
4 of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for
5 the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No.: 07-4517.
6 Available: [http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm]
- 7 (2) ESAC (2007). Statement on the conclusion of the ICCVAM retrospective study on organotypic *in vitro*
8 assays as screening tests to identify potential ocular corrosives and severe eye irritants. Available:
9 [<http://ecvam.jrc.it/index.htm>].
- 10 (3) ICCVAM (2010). ICCVAM Test Method Evaluation Report – Current Status of In Vitro Test Methods
11 for Identifying Mild/Moderate Ocular Irritants: The Isolated Chicken Eye (ICE) Test Method. Interagency
12 Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National
13 Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
14 (NICEATM). NIH Publication No.: 10-7553A. Available:
15 [<http://iccvam.niehs.nih.gov/methods/ocutox/MildMod-TMER.htm>]
- 16 (4) United nations (UN) (2011). Globally Harmonized System of Classification and Labelling of
17 Chemicals (GHS), Fourth revised edition, UN New York and Geneva, 2011. Available at:
18 [http://www.unece.org/trans/danger/publi/ghs/ghs_rev04/04files_e.html]
- 19 (5) Revised Streamlined Summary Document for TG 438 (To be prepared).
- 20 (6) Scott L, Eskes C, Hoffman S, Adriaens E, Alepee N, Bufo M, Clothier R, Facchini D, Faller C, Guest
21 R, Hamernik K, Harbell J, Hartung T, Kamp H, Le Varlet B, Meloni M, Mcnamee P, Osborn R, Pape W,
22 Pfannenbecker U, Prinsen M, Seaman C, Spielmann H, Stokes W, Trouba K, Vassallo M, Van den Berghe
23 C, Van Goethem F, Vinardell P, Zuang V (2010). A proposed Eye Irritation Testing Strategy to Reduce
24 and Replace *in vivo* Studies Using Bottom-up and Top-down Approaches. *Toxicology In Vitro* 24, 1-9.
- 25 (7) OECD (2011) Guidance Document on “The Bovine Corneal Opacity and Permeability (BCOP) and
26 Isolated Chicken Eye (ICE) Test Methods: Collection of Tissues for Histological Evaluation and
27 Collection of Data on Non-Severe Irritants. Series on Testing and Assessment no. 160.
28 ENV/JM/MONO(2011)45, 63 pp.
- 29 (8) ICCVAM. (2006). Background review document: Current Status of *In Vitro* Test Methods for
30 Identifying Ocular Corrosives and Severe Irritants: Isolated Chicken Eye Test Method. NIH Publication
31 No.: 06-4513. Research Triangle Park: National Toxicology Program. Available at:
32 [http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_brd_ice.htm]
33
- 34 (9) Prinsen, M.K. and Koëter, B.W.M. (1993). Justification of the enucleated eye test with eyes of
35 slaughterhouse animals as an alternative to the Draize eye irritation test with rabbits. *Fd. Chem. Toxicol.*
36 31:69-76.
37
- 38 (10) DB-ALM (INVITTOX) (2009). Protocol 80: Chicken enucleated eye test (CEET) / Isolated Chicken
39 Eye Test, 13pp. Available: [<http://ecvam-dbalm.jrc.ec.europa.eu/>].
40
- 41 (11) Balls, M., Botham, P.A., Bruner, L.H. and Spielmann H. (1995). The EC/HO international validation
42 study on alternatives to the Draize eye irritation test. *Toxicol. In Vitro* 9:871-929.
- 43 (12) Prinsen, M.K. (1996). The chicken enucleated eye test (CEET): A practical (pre)screen for the
44 assessment of eye irritation/corrosion potential of test materials. *Food Chem. Toxicol.* 34:291-296.

- 1 (13) Chamberlain, M., Gad, S.C., Gautheron, P. and Prinsen, M.K. (1997). IRAG Working Group I:
2 Organotypic models for the assessment/prediction of ocular irritation. *Food Chem. Toxicol.* 35:23-37.
- 3 (14) OECD (2012). Test Guideline 405. OECD Guideline for Testing of Chemicals. Acute eye
4 irritation/corrosion, 19 pp. Available at:
5 [http://www.oecd.org/document/40/0,2340,en_2649_34377_37051368_1_1_1_1,00.html]
- 6 (15) Prinsen, M.K. (2006). The Draize Eye Test and *in vitro* alternatives; a left-handed marriage?
7 *Toxicology in Vitro* 20,78-81.
- 8 (16) Siegel, J.D., Rhinehart, E., Jackson, M., Chiarello, L., and the Healthcare Infection Control Practices
9 Advisory Committee (2007). Guideline for Isolation Precautions: Preventing Transmission of Infectious
10 Agents in Healthcare Settings. Available: [<http://www.cdc.gov/ncidod/dhqp/pdf/isolation2007.pdf>].
- 11 (17) Maurer, J.K., Parker, R.D. and Jester J.V. (2002). Extent of corneal injury as the mechanistic basis for
12 ocular irritation: key findings and recommendations for the development of alternative assays. *Reg. Tox.*
13 *Pharmacol.* 36:106-117.
- 14
15 (18) Burton, A.B.G., M. York and R.S. Lawrence (1981). The *in vitro* assessment of severe irritants. *Fd.*
16 *Cosmet.- Toxicol.-* 19, 471-480.
- 17

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

Chemical: see test substance.

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 substance. 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 substance. 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.

Eye Irritation: Production of changes in the eye following the application of test substance 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 substances falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative substances 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 substance. 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.

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 substance-treated samples and other control samples to determine whether the solvent interacts with the test system.

Not Classified: Substances 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 substance 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 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" (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 substance-treated and other control samples to establish the baseline response for the samples treated with the test substance 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).

Surfactant: Also called surface-active agent, this is a substance, such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

Surfactant-containing mixture: In the context of this Test Guideline, it is a mixture containing one or more surfactants at a final concentration of >5%.

Test substance: Chemical (substance or mixture) assessed in the test method. Chemical and Test substance are interchangeable terms.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test substance 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 substance can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test substance 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: Substances 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 substance.

ANNEX II**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 substances recommended in Table 1. These substances 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)(14). Other selection criteria were that substances are commercially available, there are high quality *in vivo* reference data available, and there are high quality data from the ICE *in vitro* method. Reference data are available in the SSD (5) and in the ICCVAM Background Review Documents for the ICE test method (8).

Table 1: Recommended substances for demonstrating technical proficiency with ICE

Chemical	CASRN	Chemical Class ¹	Physical Form	<i>In Vivo</i> Classification ²	<i>In Vitro</i> Classification ³
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid	Category 1	Category 1
Chlorhexidine	55-56-1	Amine, Amidine	Solid	Category 1	Category 1
Dibenzoyl-L-tartaric acid	2743-38-6	Carboxylic acid, Ester	Solid	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-Dichlorobenzoyl chloride	4659-45-4	Acyl halide	Liquid	Category 2A	No predictions can be made
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2A ⁴	No predictions can be made
Ethyl-2-methylacetoacetate	609-14-3	Ketone, Ester	Liquid	Category 2B	No predictions can be made
Dimethyl sulfoxide	67-68-5	Organic sulphur compound	Liquid	No Category	No Category
Glycerol	56-81-5	Alcohol	Liquid	No Category	No Category (borderline)
Methylcyclopentane	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	Not classified	No Category

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

¹Chemical classes were assigned to each test substance 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>)

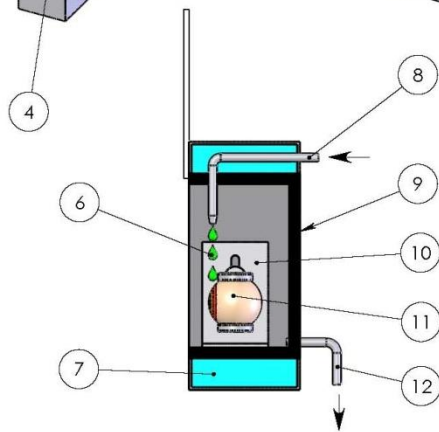
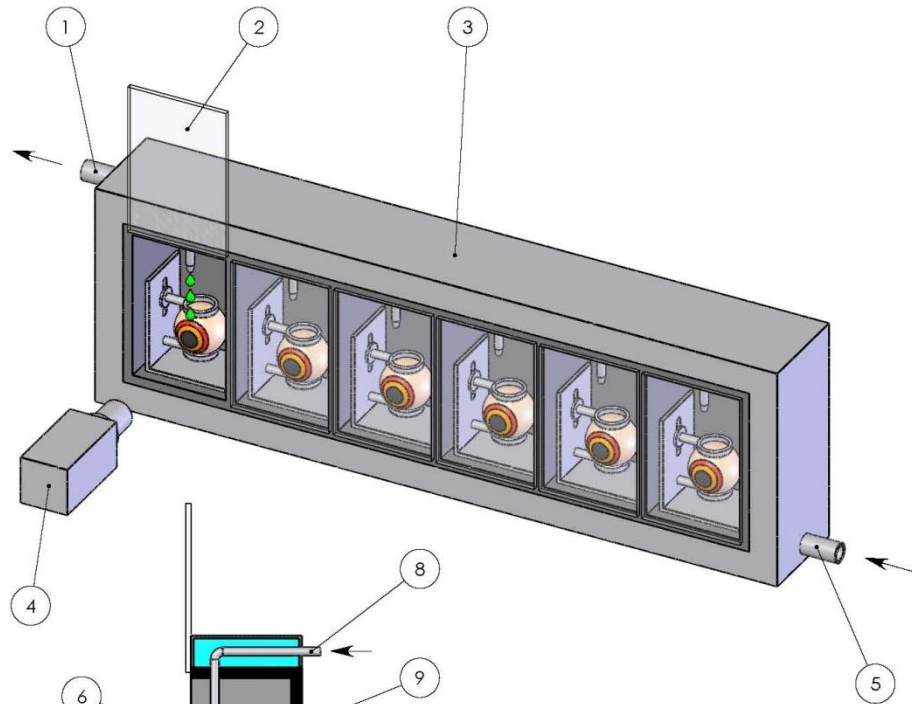
²Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS (4)(14).

³Based on results in ICE.

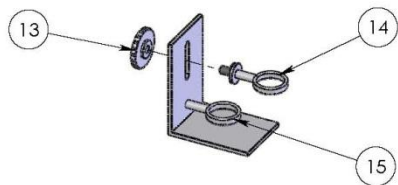
⁴ Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, *i.e.* 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart 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 III: DIAGRAMS OF THE ICE SUPERFUSION APPARATUS AND EYE CLAMPS

(See Burton et al. (18) for additional generic descriptions of the superfusion apparatus and eye clamp)



CROSS SECTION COMPARTMENT



EYE HOLDER

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