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# GUIDANCE DOCUMENT ON THE COLLECTION OF EYE TISSUES FOR HISTOLOGICAL EVALUATION AND COLLECTION OF DATA

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# GUIDANCE DOCUMENT ON THE COLLECTION OF EYE TISSUES FOR HISTOLOGICAL EVALUATION AND COLLECTION OF DATA

**(Second Edition)**

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

**Environment Directorate**
**ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT**
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# FOREWORD

This Guidance Document (GD) was originally developed in 2011 to (i) promote the use of histopathological evaluation as an additional endpoint for ocular toxicity testing; and (ii) provide specific guidance on using the TG 437 (BCOP) and TG 438 (ICE) for the purpose of expanding their respective databases towards optimising their use for identifying all hazard categories, including the complete recommended decision criteria for both test methods.

The second edition, dated 2018, reflects increased knowledge on the use of histopathology especially with the ICE test method including: (i) the recommendation for having an internal peer-review process when evaluating histopathological effects, (ii) the use of semi-quantitative scoring systems for e.g. the ICE histopathology, and (iii) inclusion of an Atlas describing typical ICE histopathological effects. It was further updated in 2018 to be aligned with the revised TG 438.

The Second Edition of the Guidance Document was approved by the Working Group of the National Coordinators in 2018. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 30 June 2018.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

# INTRODUCTION

1. This Guidance Document (GD) accompanies the OECD Test Guideline (TG) 437 on the bovine corneal opacity and permeability (BCOP) test method (OECD 2017a) and TG 438 on the isolated chicken eye (ICE) test method (OECD 2018a). It provides users with guidelines for collecting histopathology data for in vitro and/or in vivo ocular safety test methods. The primary purposes of this GD are: i) to promote the collection of histopathological data; (ii) to provide guidance on performing histopathological evaluations; (iii) to support further understanding of the usefulness and limitations of histopathology as an additional endpoint to improve the accuracy of in vitro ocular safety test methods; iv) to provide comprehensive protocols on the BCOP and ICE test methods to promote harmonization of approaches; and v) for those test chemicals (i.e. substances and mixtures) that are tested as a last resort, in vivo, to provide standard procedures for enucleating, fixing, and processing eyes from the in vivo rabbit eye studies for histopathological evaluation. Note that for a full evaluation of eye hazard effects after acute exposure, the Guidance Document on Integrated Approaches for Testing Assessment (IATA) should be considered (OECD, 2017b). In particular, the IATA approach includes the use of recommended testing strategies based on in vitro test methods and on other information sources before considering testing in living animals (see paragraph 5).

2. Histopathological evaluation may be useful for (i) assessment of the histological damage of chemical classes or formulations that are not well characterized in the before-mentioned test methods; (ii) assisting with determination of a mode of action; (iii) assisting with determination of the likelihood of delayed effects; (iv) evaluation of the depth of injury, which has been proposed as a measure of reversibility or irreversibility (Maurer et al. 2002); (v) further characterization of the severity or scope of the damage as needed (Harbell et al. 2006) (ICCVAM 2010b) (Maurer et al. 2002); (vi) assisting with discrimination of cases where the response falls along the borderline between two categories based on the test method decision criteria. Therefore, users are encouraged to preserve tissues for histopathological evaluation.

3. Histopathological evaluation may also be used to support the development of other in vitro ocular safety test methods (e.g. Isolated Rabbit Eye test method (ICCVAM 2010a), Porcine Corneal Opacity and Permeability Assay (Van den Berghe et al. 2005), and 3-dimensional human corneal tissue constructs (Carrier et al. 2009) including the Reconstructed human cornea-like Epithelium test methods (OECD TG 492, 2018b). Furthermore, in cases where an in vivo rabbit eye test is still needed as a last resort, histopathological evaluation may be used, when relevant, as an additional endpoint to more thoroughly evaluate the type and extent of ocular damage produced, as well as to provide a reference against which to compare effects produced in vitro. These additional data may help in the development of more accurate, mechanism-based in vitro alternatives to the rabbit eye test. Although the in vivo eye irritation study in rabbits seems to offer the possibility of performing histopathology of the treated eye in order to provide additional information on the inflammation process, in normal practice it will not be relevant. After all, in the standard in vivo rabbit eye irritation test, the rabbits may be sacrificed at the end of the observation period at which point the eye effects may have reversed. In the event that rabbits have to be sacrificed prematurely because of the severe nature of the eye effects, or in the event of persistence of effects in the cornea at the end of the observation period, sampling of the eyes for histopathology may be useful e.g. for better mechanistic understanding.

4. This GD describes the general procedures for the collection, preservation, and preparation of in vitro and in vivo ocular tissues for use in performing histopathological evaluations. Based on the latest progress on the use of histopathology for the ICE test method, it provides guidance in performing ICE histopathological evaluations including the recommendation of having an in-house peer-review system, the use of a semi-quantitative scoring system to assess histopathological effects, and the use of an Atlas describing typical histopathological effects. In particular, the use of histopathology has been accepted as an additional parameter to improve the identification of GHS Category 1 chemicals within the ICE method (TG 438) for the specific applicability domain of non-extreme pH ($2 < pH < 11.5$) detergents and surfactants (OECD, 2018a; OECD, 2018c; Cazelle et al., 2014). A semi-quantitative scoring system and decision criteria have been developed for the ICE histopathology for non-extreme pH detergents and surfactants (see paragraphs 39, 62 and 63). Prior to use of this ICE semi-quantitative histopathological scoring system and/or decision criteria with other test method(s) and/or applicability domain(s), its/their adequacy to the new test method(s) and/or applicability domain(s) should be demonstrated first. Finally, in the case of the BCOP or of the in vivo test method, if differences exist regarding the collection, preservation, preparation, assessment and interpretation of the corneas or in vivo eyes, laboratories that routinely perform histopathological evaluations of ocular tissue can employ their existing procedures. When additional information becomes available, this GD will be updated accordingly.

5. It is currently generally accepted that, in the foreseeable future, no single in vitro eye irritation test will be able to replace the in vivo Draize eye test to predict across the full range of irritation for different chemical classes. The IATA for Serious Eye Damage and Eye Irritation describes several modules which group information sources and analysis tools, and provides guidance on (i) how to integrate and use existing testing and non-testing data for the assessment of eye hazard effects and (ii) proposes an approach when further testing is needed (OECD, 2017b). In particular, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test (OECD, 2017b). For example, 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 (Scott et al., 2010; OECD, 2017b). As described in TG 437 and 438, BCOP and ICE data are accepted for the hazard classification and labelling of test chemicals inducing serious eye damage (i.e., UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (i.e., UN GHS No Category) (OECD 2017a) (OECD 2018a). As a consequence these assays may be used to initiate the Top-Down and the Bottom-Up approaches at the same time, so that the two tiers of the strategy recommended in the OECD GD 263 (OECD, 2017b) could be covered with one single in vitro assay, provided the test chemical fits the applicability domain and does not fall within the limitations of the test method for each tier. However, since the BCOP has a high overprediction rate for the test chemicals that do not require classification for eye hazard (69%), it should not be the first choice to initiate a Bottom-Up approach (OECD, 2017a). Furthermore, appropriate regulatory authorities should be consulted before using these assays in a Bottom-Up approach under other classification schemes than the UN GHS. Finally, even if none of these predictions are obtained, BCOP or ICE data can still be useful, within an IATA approach in conjunction with other testing and/or non-testing data, to further evaluate in a weight of evidence approach the potential eye hazard of the test chemical including moderate and mild irritants (i.e., UN GHS Category 2/2A and 2B)

(OECD, 2017b). This GD provides further insights on the decision criteria and protocols of these two assays including the use of histopathology that can be reported in parallel with other data available.

6. Definitions are provided in Annex 1.

# HISTOPATHOLOGICAL EVALUATION IN OCULAR SAFETY TEST METHODS

## 1.1. Background

7. With the exception of some research projects (Cuellar et al. 2003) (Kadar et al. 2001) (Maurer et al. 2002), few in vivo eye irritation studies include histopathological evaluation. The lack of such data has impeded the identification of relevant histopathology endpoint(s) that can be used in in vivo eye irritation/corrosivity testing, and its use to develop in vitro ocular safety test methods. While this GD provides examples on the evaluation and interpretation of histopathological data, it is important to recognize that the markers of injury in isolated eyes or corneas are different from those observed in eyes treated in vivo. For example, ex vivo test methods are devoid of an intact inflammatory response. However, the depth of injury in isolated corneas, as determined by histopathological evaluation, has been proposed to predict the degree and duration of the injury (Maurer et al. 2002).

8. To facilitate consideration of histopathological evaluation as a useful endpoint for in vitro and in vivo ocular safety testing, users are encouraged to submit data and histopathological specimens generated according to this GD to international validation organizations (i.e. the US National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods [US-NICEATM], the EU European Union Reference Laboratory for Alternatives to Animal Testing [EURL-ECVAM], or the Japanese Center for the Validation of Alternative Methods [JaCVAM]).

## 1.2. Source of Tissue for Histopathological Evaluation

9. The source of tissue to be considered for histopathological evaluation includes whole eyes or isolated portions of the anterior segment (e.g. cornea), obtained after completion of an in vitro or in vivo ocular safety test method. All information related to the type and treatment of a particular tissue sample should be included in the Test Report.

10. All procedures using animal eyes should follow applicable geographical regulations and the test facility's procedures for handling animal-derived materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory safety precautions are recommended (Siegel et al. 2007).

## 1.3. Sample Identification

11. Each sample should be assigned a unique identifier that will allow it to be traced back to the study from which it was obtained (Billings and Grizzle 2008) (Harbell et al. 2006) (ICCVAM 2010b).

## 1.4. Tissue Preparation

12. In the case of the in vitro Isolated Chicken Eye test method, treated eyes are collected after the final examination i.e., four hours after treatment, of the test method described within the OECD TG 438 (OECD, 2018a). All three eyes treated with a test chemical, as obtained from the standard ICE test method (OECD, 2018a), are used for

histopathological evaluation. Histopathology is considered appropriate for the overall assessment of effects in conjunction with the standard ICE endpoints. Eyes can be incised almost completely in half with a scalpel just behind the level of the lens and through the vitreous body, leaving a part of the posterior tissue still attached where eyes can be held (that will later be discarded) to ensure that the cornea is not damaged during manipulation by dropping on a surface, whilst at the same time allowing optimal penetration of the fixation agent (see paragraphs 15 to 19).

13. In the case of the in vitro Bovine Corneal Opacity and Permeability test method, after completion of the fluorescein permeability endpoint sampling as described within OECD TG 437 (2017a), remaining fluorescein and medium are removed from the corneal holders, the holders are carefully disassembled, and the corneas are carefully removed and transferred to individually labelled tissue cassettes. The corneas are placed endothelial surface down onto a histology sponge to protect the endothelium. The cassettes are placed in labelled containers filled with 10% neutral buffered formalin and fixed at room temperature for a minimum of 24 hours.

14. Corneas to be used for histopathological evaluation following in vivo studies according to the OECD TG 405, conducted as last resort within the framework of the IATA for Serious Eye Damage and Eye Irritation (OECD, 2017c), are kept moist with drops of physiological saline (pre-warmed from 31 to 32°C) applied throughout the dissection process. Scientists with expertise in performing the dissection have provided details of the procedure (Jones P, Guest R, personal communications) (ICCVAM 2006a). The nictitating membrane is deflected away using forceps and the conjunctivae are cut using angled forceps and curved scissors. The eyeball is removed by applying gentle pressure with fingers above and below the orbit. The remaining conjunctival tissue, the orbital muscles and the optic nerve (leaving approximately a 5-10 mm section to prevent loss of intraocular pressure) are removed and the eyeball is lifted from the orbit. Any tissue adhering to the globe is then removed by careful dissection, and the eyeball is gently rinsed with a stream of physiological saline to remove any adherent debris.

## 1.5. Tissue Preservation

15. Tissue fixatives prevent autolysis by inactivating autolytic enzymes that are released post-mortem (Banks 1993). Fixation also hardens the tissue thereby allowing thin sections to be cut without inducing mechanical artefacts (e.g. compression of the tissue). Factors that affect tissue fixation include time and temperature during incubation, the volume of the fixative relative to tissue size, the physicochemical properties of the fixative, and the concentration of the fixative (Banks 1993) (Grizzle et al. 2008). To prevent the tissues from drying out, which would induce substantial artefacts, they should remain immersed in fixative before processing and embedding.

16. Tissues should be placed in prelabelled containers filled with fixative. Most histology protocols recommend a fixative volume at least 5- to 10-fold greater than the size of the tissue (Billings and Grizzle 2008) (Kiernan 1990) (Samuelson 2007), although Banks (1993) recommends up to a 30-fold fixative-to-tissue size ratio. In the case of the ICE test methods, eyes (incised or not) are placed in a container with the fixation agent (e.g., approximately 20 mL of e.g. 10% formalin (see paragraph 18) for at least 24 hours). In the case of the BCOP test method, bovine corneas are placed into 10% neutral buffered formalin (10% NBF) at a rate of approximately 20 corneas per 300 mL.

17. All tissues should be completely immersed in the fixative. Smaller tissues may be placed into cassettes; however, for consistency in sectioning, care should be taken to orient them so that the epithelial (anterior) surface faces the top of the cassette (Harbell et al. 2006) (ICCVAM 2010b).

18. The depth of penetration of most fixatives is directly proportional to the square root of the duration of fixation (t) dependent on the coefficient of diffusibility (k) of the fixative, which averages to 1 for typically used fixatives. Fixation time thus translates to the square of the distance the fixative should penetrate. At a rate of 1 mm/hour, the time of fixation for a 10-mm sphere in neutral buffered formalin (NBF) will be (5)2 or 25 hours of fixation (Grizzle et al., 2008). Therefore, tissues are typically fixed for at least 24 hours at room temperature. However, the reported range for fixation is 4 to 48 hours (Kimura et al. 1995) (Kjellström et al. 2006), and some protocols perform fixation at 4□C (Kjellström et al. 1996) (Maaijwee et al. 2006).

19. The fixatives most commonly used for ocular tissues are 10% NBF and Davidson's (Bancroft and Cook, 1994) (Spencer and Bancroft, 2008). However, for the ICE test method, neutral aqueous phosphate buffered 4% solution of formaldehyde (i.e., 10% formalin) has been generally used for incised eyes (Prinsen, 2011), and Davidson's fixative has been suggested in case whole eyes are used due to the rapid penetration into the deeper tissues by the alcoholic component of the fixative (Latendresse et al. 2002). For the isolated corneas used in the BCOP test method, extensive experience indicates that fewer artefacts are induced following fixation with 10% NBF than with Davidson's fixative (Raabe H, personal communication). Other fixatives that have been used for ocular tissues include 4% glutaraldehyde (Chen et al. 2008), a mixture of 2.5% glutaraldehyde and 2% formaldehyde (Kimura et al. 1995) (Zhang and Rao 2005), and 4% paraformaldehyde (Kjellström et al. 2006) (Maaijwee et al. 2006).

## 1.6. Post-fixation Tissue Trimming

20. Prior to initiating the tissue-processing step, it may be necessary to trim the fixed tissues to ensure that they are adequately dehydrated and infiltrated with paraffin wax. Any post-fixation trimming should be done using a sharp scalpel, scissors, and/or razor blades to minimize tissue artefacts. In the case of the ICE test method, the fixed eye is trimmed with scissors in such a way that a thin piece containing the entire cornea and the adjacent sclera are embedded in the paraffin wax.

## 1.7. Tissue Processing and Embedding

21. Ocular tissues contain approximately 75% water (Banks 1993) and should be thoroughly dehydrated prior to embedding. This is most commonly achieved by immersing the fixed tissue in a graded alcohol series such as ethanol from 60%-70%, 90%-95%, and 100% (Rosa and Green 2008) (Spencer and Bancroft 2008). Lower concentrations, such as 30% ethanol, are recommended for delicate tissue (Spencer and Bancroft 2008). Other water-miscible solvents have also been used successfully (e.g. n-butanol, dioxane, isopropanol, propanol, tetrahydrofuran, and tetrahydrofurfuryl alcohol (Banks 1993) (Fischer et al. 2008) (Kiernan 1990) (Pantcheva et al. 2007). In the case of the ICE test method an ethanol series of 50%, 70%, 80%, 96%, 100% is generally used.

22. Because alcohols are not miscible with the paraffin wax used for embedding, a substance that is miscible with ethanol and paraffin wax in the absence of water should be used for intermediate clearing. This step also increases the transparency of the resulting tissue section (i.e. "tissue clearing" (Samuelson 2007) (Spencer and Bancroft 2008)). Xylene is the most common clearing agent used, although others have been used, including benzene, chloroform, n-butanol, n-butyl acetate, amyl acetate, ligroin, petroleum solvents (mainly hexanes), toluene, and trichloroethane, or terpenes such as cedarwood oil, limonene, and terpineol (Banks 1993) (Fischer et al. 2008) (Kiernan 1990) (Pantcheva et al. 2007). Many of these solvents may be toxic or potentially carcinogenic, so it is important to consult the Safety Data Sheets to determine proper handling conditions prior to use.

23. Because of the damage and resulting morphological artefacts produced by elevated temperatures (i.e. heating), tissues should ideally be dehydrated and cleared at room to moderate temperature. For example, in the case of the ICE test method, isolated eyes are usually dehydrated at 40oC.

24. Ocular tissue is typically embedded in paraffin wax, a polycrystalline mixture of solid hydrocarbons (Barequet et al. 2007) (Cerven et al. (1996) (Chen et al. 2008) (Harbell et al. 2006) (ICCVAM 2010b) (Maaijwee et al. 2006). Plastic materials such as glycol methacrylate have also been used to embed corneal or globe tissue of the rabbit (Kimura et al. 1995). Plastic embedding has some advantages over paraffin embedding for corneal disc preparations (e.g. no heat exposure, reduced distortion) (Lee 2002).

25. When processing only the isolated cornea (i.e. when using the BCOP test method or other isolated corneal models), following infiltration with liquid paraffin, the cornea should be bisected so that both halves can be embedded in the same block.

26. Processed tissues should be embedded so as to maintain the appropriate orientation in the hardened tissue block once the paraffin cools. For example, in case of need for measuring the corneal thickness due to e.g. swelling, true corneal cross-sections (i.e. anterior to posterior) are usually desired to permit an accurate measurement of the effects caused by the test chemical relative to the negative control (although this is not applicable to the ICE test method for which corneal swelling is measured prior to histopathology using a slit-lamp microscope). In any case, the tissue should be embedded in the block on its edge in the correct orientation to permit relevant sections to be made according to the evaluations sought.

27. A routine schedule for processing in vivo eyes with a tissue processor is provided by Barequet et al. (2007). Enucleated globes that are initially fixed overnight in 10% NBF are dehydrated in 4% phenol/70% alcohol for 1 hr each. Phenol is added to soften the sclera and lens. The eyes are then incubated in two separate stations of 95% alcohol (1 hr each), followed by two separate stations of 100% alcohol (1.5 hr each). Tissue-clearing steps include incubations in 50% alcohol/50% xylene for 2 hr, followed by two separate stations of 100% xylene (2 hr/each). Tissue is then infiltrated with liquid paraffin in two separate 2-hr incubations. This schedule may require modification depending on the manufacturer's specifications and the type of tissue processor used as e.g. described above.

## 1.8. Tissue Sectioning and Slide Preparation

28. Once embedded, the tissue is usually sectioned using a microtome with a sharpened steel blade. Depending on the type of microtome used, the thickness of microtome sections for tissue is generally 3-8 µm (Banks 1993) (Fischer et al. 2008) (Samuelson 2007) (Spencer and Bancroft 2008) (Lee 2002). In the case of the ICE and BCOP test methods, longitudinal serial slides are generally sectioned at 4-5 µm, prepared from the central area of the cornea and further processed with the staining. The microtome should be placed on a stable surface composed of a dense material that will minimize vibrations (e.g. a marble desktop). Vibrations can cause substantial tissue artefacts (Harbell et al. 2006) (ICCVAM 2010b) (Spencer and Bancroft 2008).

29. For embedded globes or corneas that have been bisected, tissue sections from each half of the bisected globe containing adequate corneal tissue or the bisected cornea itself are cut and placed on a slide for staining (i.e. a series of tissue sections in which the trailing edge of one section adheres to the trailing edge of the next section are usually floated on warm water to reduce wrinkles when they are mounted on glass slides) (Banks 1993) (Harbell et al. 2006) (Kiernan 1990). In the case of the ICE test method, usually one section per eye is prepared whereas in the case of the BCOP test method (for which the cornea is bisected), two sections are usually prepared from each cornea. It is important to remove tissue from the water before it expands and causes artefactual spaces between tissues, cells, and extracellular fibres (Samuelson 2007) (Spencer and Bancroft 2008). While there is no standardized length of time for allowing the sections to float, they are typically allowed to expand to approximately the same dimensions as the block face from which they were cut for comparison purposes.

30. Poly-L-lysine-coated glass microscope slides are often used to ensure that the tissue sections adhere to the microscope slide throughout the staining procedures. Alternatively, gelatine can be added to the water bath (Spencer and Bancroft 2008).

31. Sharp knife blades should always be used; dull blades can cause microtome artefacts such as compression lines, knife marks or tears, and/or uneven thickness of the tissue section (Samuelson 2007) (Spencer and Bancroft 2008).

## 1.9. Staining of the Tissues

32. For routine histopathological evaluations, tissues are most commonly stained with hematoxylin and eosin (H&E) (Gamble 2008) (Fischer et al. 2008). Additional information on staining and other aspects of histopathological evaluation are available in the histology manuals edited by Bancroft and Cook (1994) or Bancroft and Gamble (2008).

33. In the case of the ICE test method it is advised to follow the guidance given in the manual AFIP Laboratory Methods in Histotechnology (Prophet et al., 1992) using the Periodic Acid-Schiff (PAS) staining as described previously (Prinsen et al., 2011). Staining histological slides alternatively with H&E (haematoxylin and eosin) is also possible. However, a better visibility of the basement membrane can be obtained when PAS is used. Apart from the effect on the visibility of the basement membrane, both stainings are suitable for histopathological evaluation of all relevant endpoints in the ICE and BCOP test methods. The differences in appearance of both types of staining are illustrated in Annex II.

## 1.10. Evaluation of Quality and Acceptability of the Corneal Sections

34. Tissues from animals/samples treated with test chemical should be processed together with positive and negative control tissues. Concurrent negative control tissues (or, if applicable, tissues treated with the solvent control) may be used to determine acceptability of the other slides in a group. They may also be used to evaluate the quality of the stain, artefacts, tissue architecture, and tissue thickness (Harbell et al. 2006) (ICCVAM 2010b). Concurrent positive control data allows to confirm that the test has been conducted adequately and tissues react in an appropriate way. Furthermore, the existing positive control data from a testing laboratory may be used to develop a database for ocular damage produced by severe irritants that shall be used to assess the observed effects of the tested chemicals. Benchmark controls could be used to identify potential mechanisms of action based on the type of injury produced by a given chemical or product class (e.g. oxidizer, surfactant). Furthermore benchmark chemicals having similar physical chemical properties as the tested chemical (e.g. similar colour, state of aggregation, viscosity etc) might help to evaluate actual adverse effects of a test chemical more accurately. Hence, the use of appropriate benchmark reference chemicals might be important and should be assessed on a case-by-case basis.

35. Before using histopathology for regulatory purposes, it is recommended that laboratories develop an in-house bandwidth of morphological effects based on the negative controls, as well as a range of induced histopathological changes such as illustrated in Annex II.

# DATA AND REPORTING

## 1.11. Evaluation of Slides

36. The prepared slides should be maintained for archival purposes. Furthermore, if feasible, digital slide scans of all tissue sections might be prepared as an additional option for archival purposes. In the case of the ICE test method, three eyes per test chemical and one section per eye is considered sufficient. In the case of the BCOP test method, also three corneas are used for each test chemical, but two sections are usually prepared from each cornea (see paragraph 29).

37. All histopathological evaluations should be performed by personnel trained to identify the relevant morphological changes in treated corneas or eyes. Original slides should preferably be used for assessment.

38. When used for regulatory purposes, consolidated training, transferability and proficiency appraisal are recommended to ensure harmonized, consistent and reproducible histopathological observations. Original slides (rather than photomicrographs) need to be used as some effects require a three-dimensional evaluation of the tissues. Furthermore, an internal pathology peer review system is recommended especially when histopathology is needed for a risk assessment or classification and labelling decision, in accordance with current recommendations (Morton et al., 2010) and in accordance with the OECD Advisory document n. 16 on GLP requirements for peer review of histopathology (OECD, 2014). 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.

## 1.12. Scoring system

39. In the case of the ICE, a semi-quantitative scoring system has been developed to promote harmonized observations of tissue effects and enable comparison of effects caused by different test chemicals (Prinsen et al., 2011; see also Annex II). Table 1 shows the typical tissue effects and scores attributed to treated Isolated Chicken Eyes that were fixed, trimmed, embedded in paraffin wax, sectioned and stained.

**Table 1. 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** | Very slight | ½ | Single to few scattered cells |
| ***Separately scored for the top, mid, and lower parts of the epithelium*** ** | 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** ††; ††† | Normal | - | < 5 pyknotic nuclei |
| ***In top or bottom region*** | 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 |

*Note*: Annex II 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 for more details).

***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.

40. 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.

41. 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 is preserved. However, effects/changes occurring within the limbus should not be scored due to effects not linked to the chemical exposure.

42. It is critical to distinguish treatment-related effects from histopathological artefacts and/or background morphology, especially for vacuoles (see Annex II). For this purpose the Atlas presented in Annex II describes both types of effects. Furthermore consolidated training, transferability and proficiency appraisal are recommended to ensure consistent histopathological observations (see paragraph 38).

## 1.13. Test Report

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

### *Test Chemical and Control Substances*

- •Mono-constituent substance: chemical identification, such as IUPAC or Chemical Abstracts Service (CAS) name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- •Multi-constituent substance, UVCB and mixture: 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;
- •Purity, chemical identity of impurities as appropriate and practically feasible;
- •Physical state, volatility, pH, stability, chemical class, water solubility, and additional properties relevant to the conduct of the study, to the extent available;
- •Treatment 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, study director, and study pathologist;
- •Identification of the source of the eyes (e.g. the facility from which they were collected);
- •Storage and transport conditions of eyes (e.g. date and time of eye collection, time interval prior to initiating testing);
- •If available, specific characteristics of the animals from which the eyes were collected (e.g. age, sex, strain, weight of the donor animal).

### *Histology Report*

- •Unique sample identifier;
- •Type of tissue analyzed (e.g. cornea, whole eye);
- •Tissue species (e.g. bovine, rabbit);
- •Time of animal slaughter and/or eye collection and time of tissue fixation;
- •Number of tissues analyzed for each test chemical and control (e.g. n=3);
- •Peer-review system used if applicable;
- •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***

- •Optional digital images or digital slide scans, if feasible;
- •Detailed descriptions of all lesions and artefacts using a semi-quantitative scoring system or, if not available, standard histopathological terminology;
- •If applicable, indication of use of localized effects for histopathological scoring;
- •Description of the decision criteria used in the evaluation;
- •Individual specimen data tables and if applicable, summary tables.

## 1.14. Decision Criteria for All Ocular Hazard Categories

44. As described in TG 437 (OECD 2017a) and 438 (OECD 2018a), BCOP and ICE can be used, under certain circumstances and with specific limitations, to classify substances and mixtures for eye hazards. They are considered relevant information sources to be used within an IATA approach before considering testing in living animals (OECD, 2017b). In particular, while not considered valid as a stand-alone replacement for the in vivo rabbit eye test, both the ICE and BCOP test methods are accepted for the hazard classification and labelling of test chemicals inducing serious eye damage (i.e., UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (i.e., UN GHS No Category) (OECD 2017a) (OECD 2018a).

45. Within the context of the IATA for Serious Eye Damage and Eye Irritation, a substance or mixture that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage requires consideration of additional information sources such as additional testing (in vitro and/or in vivo as a last resort) to establish a definitive classification. Even if no predictions can be made on the classification based on the OECD TG 437 and 438, BCOP or ICE data can be useful within an IATA approach, in conjunction with other testing and/or non-testing data, to further evaluate eye hazard effects in a weight-of-evidence approach. Therefore, the following detailed decision criteria are provided to correspond to all current UN GHS hazard categories. These data can then be reported in parallel with the other data available.

## 1.15. The BCOP Test Method

46. A detailed protocol for BCOP is provided in Annex V. As described in OECD TG 437 (OECD 2017a), the mean opacity and permeability OD490 values for each treatment group are combined to calculate an in vitro irritancy score (IVIS) for each treatment group as follows: IVIS = mean opacity value + (15 x mean OD490 value).

47. A substance or mixture that induces an IVIS > 55 is predicted as inducing serious eye damage (UN GHS Category 1) and a substance or mixture that has an IVIS ≤ 3.0 is predicted to not require classification according to the UN GHS (No Category). The

recommended decision criteria for using BCOP to identify other hazard categories are provided in Table 2.

**Table 2. Overall BCOP classification criteria**

| UN GHS Classification (OECD TG 437) | *In Vitro* Prediction* (ICCVAM, 2010a) | IVIS Score Range |
|---|---|---|
| **No Category** | Not Classified | ≤ 3 |
| **No prediction can be made** | Mild | > 3; ≤ 25 |
| | Moderate | > 25; ≤ 55 |
| **Category 1** | Severe | > 55 |

*Note:*
* Adapted according to criteria according to OECD TG 437

48. The ability of the BCOP test method to identify all categories of ocular irritation potential, as defined by the EPA, EU, and GHS classification systems (EPA 2003a) (EU 2008) (UN 2015), was evaluated by ICCVAM (2010a). Based on the then available BCOP database (n=211 test chemicals),(ICCVAM 2006b), the overall correct classification ranged from 49% (91/187) to 55% (102/187) when evaluating the entire database, depending on the hazard classification system used. Based on these performance statistics, the BCOP test method is not considered valid as a complete replacement for the in vivo rabbit eye test.

49. Although not considered valid as a stand-alone replacement for the in vivo rabbit eye test, the BCOP test method falling within the OECD TG 437 can be used to identify UN GHS Category 1 chemicals and UN GHS No Category chemicals without further testing (UN, 2015). If no predictions can be made on the classification based on the OECD TG 437, the BCOP test data may still be useful within an IATA approach, in conjunction with other testing and/or non-testing data, to further evaluate eye hazard effects in a weight-of-evidence approach (OECD, 2017b). In addition, the detailed decision criteria as shown in Table 2 may be used to further evaluate the usefulness and limitations of the BCOP test method for identifying all categories of ocular irritation.

50. When such data are generated, the criteria described above may need to be modified in order to optimize the BCOP test method for identifying moderate and mild irritants (i.e. UN GHS Categories 2/2A and 2B). Furthermore, the concurrent testing of benchmark chemicals (as described in paragraph 34) or materials relevant in chemistry and formulation to the test chemical or material, and for which sufficient and adequate data on eye hazard classification exist, may provide further support for predicting the test chemical eye hazard potential in a Weight of Evidence approach.

## 1.16. The ICE Test Method

51. A detailed protocol for ICE is provided in Annex IV. As described in OECD TG 438 (OECD 2018a), 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 (see Table 8). Furthermore morphological effects are observed.

52. 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:

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

53. 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 (Table 3).

**Table 0. ICE classification criteria for corneal thickness**

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

54. The above mean corneal swelling scores 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. I 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. If another

slit-lamp microscope, depth-measuring device or settings are used, equivalence should be demonstrated and/or the appropriate range for classification shall be established.

55. Corneal opacity is evaluated by using the area of the cornea that is most densely opacified for scoring according to the observations described s shown in Table 4. 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 (Table 5).

**Table 4. ICE 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 |

**Table 5. 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 |

56. Fluorescein retention is evaluated at the 30 minute observation time point only according to the scores shown in Table 6. 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 (Table 7).

**Table 6. ICE 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 |

**Table 7. ICE classification criteria for mean fluorescein retention**

| Mean Fluorescein Retention Score at 30 minutes post-treatment | ICE Class |
|---|---|
| **0.00.5** | I |
| **0.61.5** | II |
| **1.62.5** | III |
| **2.63.0** | IV |

57. Morphological effects observed are also evaluated including "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.

58. 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 (see Table 8). The in vitro classification for a test chemical is assessed by reading the UN GHS classification that corresponds to the combination of ICE Classes obtained for corneal swelling, corneal opacity, and fluorescein retention as described in Table 8.

**Table 8. Overall ICE classification criteria**

| UN GHS Classification (OECD TG 438, 2018a) | *In vitro* Prediction (OECD GD 188, 2013c) | Combinations of Three Endpoints |
|---|---|---|
| **No Category** | Not Classified[2] | 3 x I |
| | | 2 x I, 1 x II |
| | | 2 x II, I x I |
| **No prediction can be made** | Mild[3] | 3 x II |
| | | 2 x II, 1 x III |
| | | 2xI, 1xIII** |
| | | 1 x I, 1 x II, 1 x III |
| | Moderate[4] | 3 x III |
| | | 2 x III, 1 x I |
| | | 2 x III, 1 x II |
| | | 2 x III, 1 x IV |
| | | 2 x I, 1 x IV** |
| | | 2 x II, 1 x IV** |
| | | 1xI, 1xII, 1xIV** |
| | | 1xI, 1xIII, 1xIV** |
| | | 1 x II, 1 x III, 1 x IV** |
| **Category 1** | Severe[5] | 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.

59. 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 (OECD GD 188, XXX).

60. 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 (OECD GD 188, XXX). 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.

61. To further evaluate the usefulness and limitations of the ICE test method for identifying all categories of ocular irritation it is recommended that the complete classification scheme of the ICE test method (see Table 8) be applied and that these data are reported in parallel with any other data available e.g. within the IATA context (OECD, 2017b). When such data are generated, the criteria described above may need to be modified in order to optimize the ICE for identifying moderate and mild irritants (i.e. UN GHS Categories 2/2A and 2B).

62. If histopathology is used for non-extreme pH ($2<pH<11.5$) detergents and surfactants, the decision criteria shown in Table 9 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 9. 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 detergents (2<pH<11.5) 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<br>- and/or, any vacuolation (= very slight, score ½) observed in the mid and/or lower parts in at least 2 out of 3 eyes<br>- 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<br>- and/or, necrosis = moderate (score 2) observed in at least 2 out of 3 eyes |

63. Furthermore, the prediction model shown in Table 10 should be used. It is important to note that, based on the dataset currently available, histopathology cannot be used in a stand-alone manner to identify UN GHS Cat. 2 and UN GHS No Cat. test chemicals. Furthermore, the ICE histopathology criteria and prediction model described in Tables 9 and 10, respectively, are applicable only to identify UN GHS Cat. 1 non-extreme pH (2<pH<11.5) detergents and surfactants.

**Table 10. 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 9 | UN GHS Classification |
|---|---|---|
| **No prediction can be made** | Criteria met | UN GHS Cat. 1 |
| | Criteria not met | No prediction can be made |

64. Overall, when histopathology is used as an additional endpoint to identify UN GHS Cat. 1 non-extreme pH (2 < pH < 11.5) detergents and surfactants, the false negative rate of the ICE test method and its accuracy (as compared to Draize in vivo data and LVET Cat. 1 data) 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)) (OECD, 2018c).

## 1.17. Study Acceptance Criteria

65. For the BCOP and ICE test methods, the study acceptance criteria are outlined in TG 437 (OECD 2017a) and 438 (OECD 2018a), respectively.

## 1.18. Test Report

66. For the BCOP and ICE test methods, the information to be included in the test report is outlined in TG 437 (OECD 2017a) and 438 (OECD 2018a), respectively.

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# 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 (OECD, 2005).

**Benchmark control**: A sample containing all components of a test system and treated with a known substance (i.e. the benchmark substance) to induce a known response. The sample is processed with test chemical-treated and other control samples to compare the response produced by the test chemical to the benchmark substance to allow for an assessment of the sensitivity of the test method to assess a specific chemical class or product class.

**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, functional and/or chemical or product class similarity to the chemical(s) being tested; (iii), known physical/chemical characteristics; (iv), supporting data on known effects; (v), known potency in the range of the desired response

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

**Bowman's layer**: The anterior lamina of the cornea located under the epithelial layer in some species (e.g. humans, avians, cetaceans) and above the corneal stroma (see Annex II).

**Chemical**: Means a substance or mixture.

**Clearing solvent**: Substance miscible with ethanol or any other dehydrating agent that is also miscible with an embedding agent such as paraffin wax. Infiltration of this solvent results in clearing of the tissue or in an increase in the transparency of the tissue.

**Concordance**: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of "relevance". The term is sometimes used interchangeably with "accuracy", and is defined as the proportion of all chemicals tested that are correctly classified as positive or negative. Concordance is highly dependent on the prevalence of positives in the types of chemicals being examined (OECD, 2005).

**Cornea**: The transparent part of the coat 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. Opacity can be evaluated subjectively as done in the Draize rabbit eye test, or objectively with an instrument such as an "opacitometer."

**Corneal permeability**: Quantitative measurement of damage to the corneal epithelium by a determination of the amount of sodium fluorescein dye that passes through all corneal cell layers.

**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.

**Corneoscleral button**: A cornea dissected from an enucleated eye that typically includes a rim of 2-3 mm of scleral tissue.

**Cutting**: Use of a microtome or other knife-bladed instrument to produce thin ribbons of tissue (e.g. 3 to 8 □M for tissue) that can be mounted on glass slides prior to staining.

**Davidson's Fixative**: A rapid tissue fixative that may be used in place of 10% neutral buffered formalin to reduce tissue shrinkage, particularly useful for large ocular tissues (e.g. enucleated whole globe eyes).

**Descemet's membrane**: The posterior lamina of the cornea that lies at the posterior end of the stroma and precedes the endothelial layer (see Annex II).

**Dehydration:** The process of removing the natural water content of the tissue using a series of increasing concentrations of a solvent such as ethanol that is miscible with water.

**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.

**Embedding:** Process of surrounding a pathological or histological specimen with a firm and sometimes hard medium such as paraffin, wax, celloidin, or a resin, to allow for cutting thin tissue sections for microscopic examination.

**Endothelium:** A single layer of flat, hexagonally arranged cells continuous with the irido-corneal angle of the anterior chamber of the eye. The endothelium actively maintains corneal transparency by regulation of fluid exchange with the aqueous humor (Samuelson 2007, see also Annex II).

**Epithelium:** The anterior epithelium covers the anterior corneal surface. It is composed of a thin basement membrane with columnar epithelial cells, followed by two or three layers of polyhedral wing cells, various layers of non-keratinized squamous cells (Samuelson 2007, see also Annex II).

**Eye irritation**: Defined in vivo as the production of change in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application (UN, 2015). Interchangeable with “reversible effects on the eye” and with UN GHS Category 2.

**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.

**Fixation:** The process of placing a tissue sample in 5 to 10 volumes of a substance known to stabilize the tissue from decomposition (e.g. 10% NBF or Davidson's fixative) as soon as possible after procurement and trimming. The time needed to infiltrate the tissue depends on the chemical characteristics of the fixative (e.g. □24 hr for NBF and no more than 24 hr for Davidson's fixative).

**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.

**Good Laboratory Practices (GLP):** Regulations promulgated by a number of countries and national regulatory bodies that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to regulatory authorities; the subject of the OECD Series on "Principles of Good Laboratory Practise and Compliance Monitoring".

**Hazard:** Inherent property of a substance or mixture having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that substance or mixture.

**Histopathology**: The science or study dealing with the cytologic and histological structure of abnormal or diseased tissue.

**Infiltration**: The passive diffusion of a dehydrating solvent, clearing solvent, or liquid embedding material into a fixed tissue sample.

**In Vitro Irritancy Score (IVIS):** An empirically-derived formula used in the BCOP assay whereby the mean opacity and mean permeability values for each treatment group are combined into a single in vitro score for each treatment group. The IVIS = mean opacity value + (15 x mean permeability value).

**Iris:** The contractile diaphragm perforated by the pupil and forming the coloured portion of the eye.

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

**Limbus:** Transition zone between the corneosclera and conjunctiva that houses the collecting vessels for aqueous humor outflow and stem cells for regeneration of epithelium in wound healing.

**Mixture:** Means a mixture or a solution composed of two or more substances in which they do not react (UN, 2015).

**Mono-constituent substance**: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

**Multi-constituent substance**: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

**Negative control:** An untreated sample containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the chemical or its solvent (if applicable) interacts with the test system.

**Neutral Buffered Formalin (10%):** 10% neutral buffered formalin is a tissue fixative composed of 37 to 40% formaldehyde solution in 0.1 M phosphate buffer, pH 7.4.

**Not classified:** Chemicals that are not classified for eye irritation (UN GHS Category 2, 2A or 2B) or serious damage to eye (UN GHS Category 1). Interchangeable with "UN GHS No Category". .

**Opacitometer:** An instrument used to measure "corneal opacity" by quantitatively evaluating light transmission through the cornea. The instrument has two compartments, each with its own light source and photocell. One compartment is used for the treated cornea, while the other is used to calibrate and zero the instrument. Light from a halogen lamp is sent through a control compartment (empty chamber without windows or liquid) to a photocell and compared to the light sent through the experimental compartment, which houses the chamber containing the cornea, to a photocell. The difference in light transmission from the photocells is compared and a numeric opacity value is presented on a digital display.

**Positive control**: A sample containing all components of a test system and treated with a substance known to induce a positive response in the test system. This sample is processed with the test chemical-treated samples and other control samples. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

**Relevance:** Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (OECD, 2005).

**Reliabilit**y: 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 (OECD, 2005).

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

**Sclera**: A portion of the fibrous layer forming the outer envelope of the eyeball, except for its anterior sixth, which is the cornea.

**Sensitivity**: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (OECD, 2005).

**Serious eye damage**: Defined in vivo as the 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 (UN, 2015). Interchangeable with "irreversible effects on the eye" and with UN GHS Category 1.

**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 interacts with the test system.

**Specificity**: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (OECD, 2005).

**Staining**: The addition of substances to tissue that has been processed, cut, and mounted on a glass slide that adds colour and permits visualization of the tissue of interest.

**Standard Operating Procedures (SOP):** Formal, written procedures that describe in detail how specific routine, and test-specific, laboratory operations should be performed. They are required by GLP.

**Stroma:** The framework of connective tissue and keratocytes that provides structure to the eye. The anterior portion of the stroma begins after Bowman's layer or the anterior lamina and ends with Descemet's membrane or the posterior lamina that precedes the endothelial cell layer.

**Substance:** Means 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 (UN, 2015).

**Surfactant:** 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 forming emulsions and/or microemulsions and/or micelles, and/or of adsorption of water-solid interfaces.

**Test:** An experimental system used to obtain information on the adverse effects of a substance. Used interchangeably with assay.

**Test chemical:** The term "test chemical" is used to refer to what is being tested including e.g., substances and mixtures.

**Test method:** A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with “test” and “assay.” See also “validated 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 procedure is performed until an unequivocal classification can be made.

**Tissue:** A collection of similar cells and the intercellular substances surrounding them. There are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue.

**Tissue processing:** The protocol followed for fixation, post-fixation trimming, dehydration, clearing, and embedding of tissue for use in histology.

**Top-Down Approach**: A step-wise approach used for a test 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).

**Trimming:** The process of removing non-critical, excess tissue before or after fixation by cutting with scissors or a scalpel to minimize a tissue sample to those sections that are needed for the evaluation.

**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 (UN, 2015).

**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”.

**UVCB:** Substances of unknown or variable composition, complex reaction products or biological materials.

**Valid test method**: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (OECD, 2005).

**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 (OECD, 2005).

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

# ANNEX II: ATLAS OF HISTOPATHOLOGICAL LESIONS OF ISOLATED CHICKEN EYES

(see separate Annex II on the OECD public site, Series on Testing and Assessment)

# ANNEX III: GUIDELINES FOR HISTOPATHOLOGICAL EVALUATION OF BOVINE CORNEAS AS AN ENDPOINT OF THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

(see separate Annex II on the OECD public site, Series on Testing and Assessment)

# ANNEX IV: DETAILED PROTOCOL FOR STUDIES USING THE ISOLATED CHICKEN EYE TEST METHOD

This protocol represents the technical procedures recommended by Triskelion, the developer of the Isolated Chicken Eye Test (Prinsen and Koeter 1993; DB-ALM 1994; Prinsen 1996). It was used as a basis for the validation studies undertaken on the Isolated Chicken Eye test, and of the test method evaluation conducted by the Interagency Coordinating Committee on the Validation of Alternative Methods (Balls et al. 1995; Chamberlain et al. 1997; ICCVAM 2010), in conjunction with the European Centre for the Validation of Alternative Methods and the Japanese Center for the Validation of Alternative Methods, which included an international independent scientific peer review of the validation status and scientific validity of the ICE test method.

## 1. Principle

The test chemical is applied onto the cornea of at least three eyes in one single dose, for an exposure period of 10 seconds. Prior to dosing, each test eye provides its own baseline values for the assessment of corneal effects. One untreated eye serves as a control of the experimental conditions. The reactions of the corneas are examined at regular intervals up to 4 hrs after treatment. Based on the mean scores for corneal swelling, corneal opacity, and fluorescein retention, an assessment of the eye irritation potential of the test chemical, ranging from non to severely irritating, can be made. Microscopically, the "depth-of-injury" may be assessed by examination and recording of the lesions of the cornea.

## 2. Experimental design

### *2.1 Requisites*

*Collection of heads:*

- Containers for transportation of the heads (for instance in plastic boxes with lids)
- Slaughter knife
- Proper clothing (as required by the slaughterhouse)
- Rubber boots or plastic coverall for shoes
- Surgical gloves and protective glove, if considered necessary

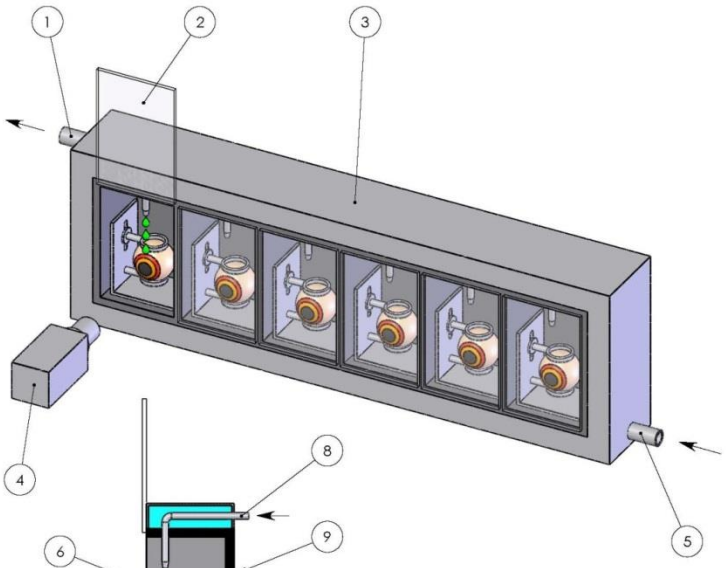
*Enucleation of eyes:*

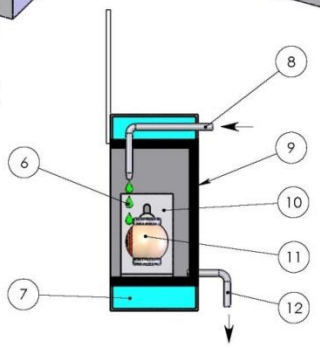
- Surgical forceps
- Anatomical forceps (bent)
- Scissors; at least one with blunt bent tips
- Underpads
- Eye clamps
- Fluorescein sodium 2% w/v (Minims® or equivalent)

- Physiological saline (also used during other phases of the study)

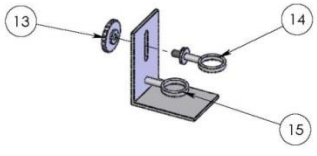
*For maintaining enucleated eyes:*

- superfusion apparatus



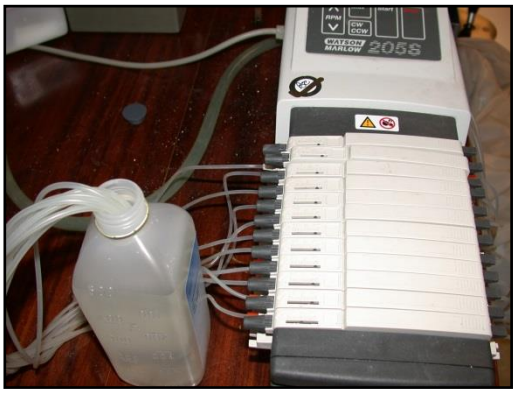

CROSS SECTION COMPARTMENT



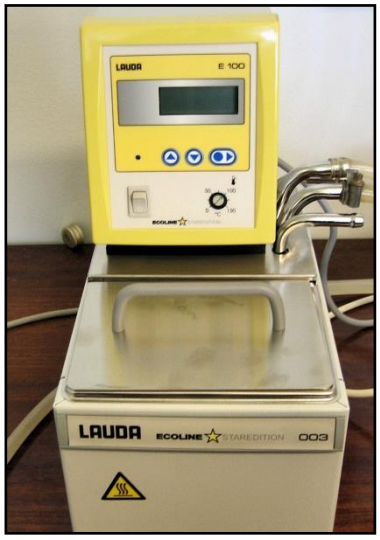

EYE HOLDER

| 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 |

- Peristaltic pump with the appropriate number of channels




- Waterbath with suitable range (for instance 30-60oC)




*Examination of eyes:*

- Slit-lamp microscope (slit-lamp BP 900, Haag-Streit AG, Liebefeld-Bern, Switzerland). 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.
- Depth Measuring Attachment no. I

*Administration of the test chemical:*

- Analytical balance
- Weighing paper
- Mortar and pestle or coffee grinder (for solids)
- Micropipette with positive displacement suitable for 30 µL delivery
- Test chemical(s)
- Repeater pipette (range 1-10 mL) with matching 500-600 mL flask

*Scoring of all effects at all time points:*

- Suitable scoring form.
- Scoring criteria see OECD TG 438

### *2.2 Settings and general directions*

**Superfusion apparatus**: No special settings are needed.

**Peristaltic pump:**

- set the speed at a rate that results in 3-4 drops of saline per minute.

*NOTE. Maximum rinsing is applied to rinse off the fluorescein application at the t = 30 min observation.*

**Temperature-controlled water pump:**

The water pump is connected to the superfusion apparatus by plastic tubing.

One tube is attached to the outlet of the water pump and to the inlet of the superfusion apparatus (right-hand bottom). Another tube is connected to the outlet of the superfusion apparatus (left-hand top) and returns to the water reservoir of the pump.

The temperature of 32 ± 1.5°C in the chambers of the superfusion apparatus is maintained by using the appropriate temperature setting which is established beforehand. Once determined this checking can be used for a longer period (an annual check is considered sufficient).

*NOTE. It is sufficient to once establish the temperature range of 32 ±1.5°C in the superfusion apparatus. Only if temperature settings are changed an additional check is performed.*

**Slit-lamp microscope (Haag-Streit 900 BP ):**

First check both ocular settings. Once the correct setting is determined it can be used for all tests.

-set the general magnification of the slit-lamp (10, 16 or 25) at 16 (see figure below).

For the "whole eye" observation, necessary for the assessment of corneal opacity and fluorescein retention, the settings are:

Opacity:

- set light intensity knob at ½ (see figure below).

| | | |
|---|---|---|
| slit length | full length | upper handle (1) of the light column: far left position |
| | | ribbed turn knob (2) at top of the light column: position 8 |
| slit width | fully open | position lower ribbed knob (3); position on indefinite |


Observe the cornea as follows:

- Focus the light beam on the surface of the cornea (saline film) by using the movement control knob (joystick) on the mounting table, until a sharp image of the corneal surface is obtained.
- Perform the observation.

Fluorescein:

- set light intensity knob at ½.

| | | |
|---|---|---|
| slit length | full length | upper handle (1) of the light column: on green symbol |
| | | ribbed turn knob (2) at top of the light column: position 8 |
| slit width | fully open | position lower ribbed knob (3); position on indefinite |


Observe the cornea as follows:

- Apply one drop of the fluorescein solution onto the cornea (eye remains in the chamber) and rinse with saline by increasing the saline drip to its maximum. Return the drip rate to normal after the observation.
- Focus the light beam on the surface the cornea (saline film) by using the movement control knob (joystick) on the mounting table, until a sharp image of the corneal surface is obtained.
- Perform the observation.

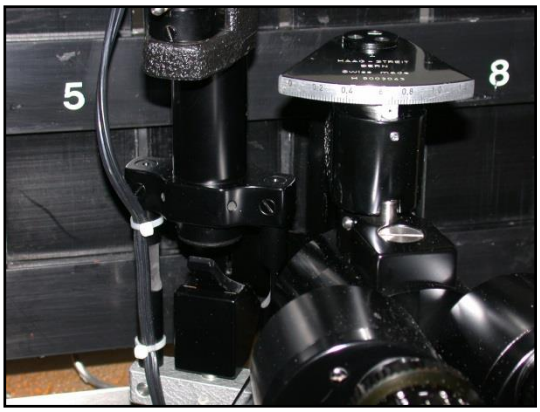
| | | |
|---|---|---|
| slit length | full length | upper handle (1) of the light column: on green symbol |
| | | ribbed turn knob (2) at top of the light column: position 8 |
| slit width | fully open | position lower ribbed knob (3) exactly in between 9 and 10 (9½) |

For the corneal thickness measurement, the settings are:

- set light intensity knob at 2 (see last figure of page 56).

To determine the thickness of the cornea the following directions are followed, which for the Haag-Streit slit-lamp microscope with the Depth Measuring Attachment no. I are:

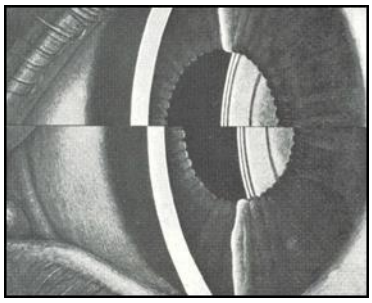
- Mount the device on the slit-lamp.
- Change the right ocular with the depth measuring ocular with the “I 16x” upwards.





- Assure that the light beam of the slit-lamp is passing the slit of the depth measuring device, otherwise adjust.
- Focus the light beam on the centre of the cornea.
- Determine the exact thickness of the cornea in instrument units by turning the dial on the device, while looking through the right ocular only, until the upper and lower half of the slit light beam are in the right position (see figure below).
- Read the value on the scale, once the correct position is reached.

*NOTE. The best results are obtained by first turning the halves completely apart and then turning them back to the correct position.*



**Micropipette (used to e.g. apply liquid test materials – see section 2.6):**

- No calibration is required since the 30 µl is considered an over-dose and the application is open.
- Put the syringe in place.
- Set the volume adjustment dial at 30 µl.
- Fill syringe and remove air.
- lace tip above the centre of the cornea of the eye which is placed in an open, plastic box or on a layer of tissue.
- Expel 30 µl onto the cornea by pressing thumb knob once until stop.

*NOTE. If the drop(s) of the test chemical bounces off or if the cornea is not properly exposed an additional volume may be applied to obtain an adequate exposure. The approximate volume applied is noted.*

**Seripettor (used for e.g. rinsing the eyes – see sections 2.4 and 2.6):**

- Fill the flask with physiological saline.
- Set the volume adjustment knob at ca 5 mL.
- Apply a 5 mL volume of saline by pulling the handle to its highest position and then by pushing it down to its lowest position at a rate of approximately 3-4 sec per 5 mL (gently).
- For rinsing after administration of the test chemical the above procedure is carried out 4 times (total of 20 mL).
- For powders, it is advised to apply the first 5 mL of saline as drops and not as a beam.
- If the upper-arm of the clamp does not fit the eye-ball entirely, make sure that additional rinsing is applied to the area on top of the upper-arm to remove any residue. This need not to be recorded separately because it does not concern the cornea.

### *2.3 Collection of chicken eyes*

On the day of testing, in the morning, chicken heads (normally chickens of ca 2 kg) are collected at a poultry slaughterhouse nearest to the laboratory (ideally within 45 min driving distance). If chickens of a different weight are used, data should be generated to demonstrate the validity of the use of chickens of a different weight.

The collection procedure is the following:

**At the slaughterhouse**

- Cut off the heads of the animals at the bleeding location (after sedation and incision of the neck for bleeding), and before they reach the hot water scrub station (next station of the process line).

*NOTE. The eyes should not be touched in any way, while cutting the head off!*

- Place each head in the container box.
- Immediately transport the boxes with the heads to the testing facility at ambient temperature (during winter the boxes should be in the driver's compartment of the car (not in the trunk). The arrival at the test facility, the name of the slaughterhouse and the approximate time of kill is noted in the log.

### *2.4 Enucleation of the eyes*

Carefully dissect the eyes within a maximum interval of 2 hours after kill.

- The eyes are enucleated and placed in the superfusion apparatus using the following procedure:
- Cut away the eye-lids as far as possible of one eye of the head.
- Apply one small drop of fluorescein onto the centre of the cornea
- Immediately rinse-off with ca 5 mL saline (ambient).
- Examine the cornea in situ with the microscope (see "whole eye" observation) for possible staining of damaged epithelial cells.

*NOTE. Only corneas without staining should be used (some minor single cell staining, maximum score 0.5 is still acceptable for testing). Corneal opacity or other abnormalities should also be absent before further dissection may be carried out, otherwise repeat the above steps with another eye.*

- Pull the eye ball from the orbit by holding the nictitating membrane with a surgical forceps, while cutting the eye muscles with bent scissors.

*NOTE. Avoid too much pressure, because corneal opacity may result.*

- Once removed from the orbit, place the eye on the underpad and cut away the nictitating membrane and other connective tissue around the cornea taking great care to avoid any contact with the cornea. In case of doubt, it is advisable to reassess corneas by means of fluorescein assessment.
- Clamp the eye in the holder with the cornea.

*NOTE. Again avoid too much pressure on the eye by the clamp. Because of the relatively firm sclera of the chicken eye-ball, only slight pressure is needed to fix the eye properly.*

- If there is any doubt that the cornea is still undamaged, again use fluorescein to detect possible damage.
- This fluorescein determination or the one before is 'taken as score t = 0 and noted on the recording form.
- Place the holder with the eye in one of the chambers of the superfusion apparatus.
- Supply the cornea with the saline drip (see settings).
- Repeat the above procedure until enough eyes are available for testing.
- Measure the corneal thickness of the eyes (see settings) and record the values on recording form at t = -45 min. Note the actual time in the time column and in the log .Optional: Note the expected times of the time points in the time column. The actual times are noted in the log when performed.
- Allow the eyes to acclimatize in the superfusion apparatus for 45-60 min.

### *2.5 Base-line assessments*

At the end of the acclimatization period usually 5 minutes before the start of the exposure (t=0), the base-line determinations for corneal swelling, corneal opacity, and fluorescein retention are made as follows:

- Observe each cornea for corneal opacity or other abnormalities.
- Measure the thickness of each cornea and record the values at t=0.
- Do not change the setting of the lower ribbed knob of 9½ until the t = 30 min thickness assessment has been completed.

*NOTE. The corneal thickness of the eyes should not have increased by more than 5-7% (or 3 instrument units). Slight thinning (up to 7%) may also be observed, but is considered normal when maintaining enucleated eyes. Corneal opacity should not be observed in any of the eyes. However, very faint opacity is acceptable, provided it is not observed in all of the eyes.*

### *2.6 Administration of the test chemical*

After the baseline assessments, the test chemical is applied in one single dose to each of the test eyes. The control eye is left untreated, but is otherwise similarly handled.

**Liquids**

Liquids are applied with a micropipette in a standard dosing-volume of 30 µL. Other volumes may be appropriate and, if so, noted on the recording form.

Pastes may be softened by means of a warm water (ca 70°C), collected with the micropipette and applied after cooling down to lukewarm temperature, unless other handling procedures are specified. This procedure is also applicable when dealing with highly viscous liquids, if they cannot be handled properly at room temperature. The procedure followed is noted on the recording form.

Optional: Prior or during testing, the hydrophobicity or hydrophilicity of the liquid test chemicals can be established. This can easily be checked during application on the cornea or done by putting some of the test chemical in a beaker with water and observing whether or not the test chemical mixes with the water. Such observation serves as general information on the nature of the test chemicals, which may explain in some cases, the pattern of effect observed on the cornea.

NOTE. Pipettes using air pressure should not be used, because such small volumes cannot be handled adequately (especially viscous liquids).Pipettes using direct pressure by means of a piston are recommended.

**Solids**

Solids, ground to a fine powder, if necessary, are applied by powdering the surface of the cornea with a standard amount of 30 mg placed on a weighing paper.

NOTE. In some cases with strongly hygroscopic powders (such as NaOH) or with very tough pastes, a spatula or the tip of the dosing syringe is needed to keep the test chemical in place on the cornea. Very tough pastes or dough may need to be applied as a moulded piece of material placed directly onto the cornea. In those cases the exact weight of the dose is not considered to be relevant. Priority is given to a good contact of the test chemical with the cornea.

The administration procedure for each eye is as follows:

**Test eyes**

- Take the first eye out of its chamber and place it on a layer of tissue or in a plastic box with the cornea facing upwards.
- Apply the test chemical onto the centre of the cornea, according to the directions already given in this paragraph. Record any unusual findings, such as sliding down of the test chemical as with hydrophobic test chemicals, or immediate corneal opacity. Also if distribution of the test chemical over the cornea is non-homogeneous this should be recorded.
- Leave the test chemical on the cornea for a period of 10 seconds, which starts after completion of the administration.
- Next, rinse the cornea thoroughly, but carefully with 20 mL of isotonic saline of ambient temperature from the seripettor (in 4 doses of 5 mL each). Record any

unusual findings, such as adherence of the test chemical to the cornea or precipitation of the test chemical on the cornea.

- Return the clamp and eye to its chamber
- Repeat this procedure for each test eye.

*NOTE. If remains of the test chemical are still present on the cornea (as observed by visual inspection) sufficient rinsing is applied to fully remove the test chemical. The approximate volume applied is noted. If remains cannot be removed by additional rinsing, this is also noted.*

**Control eye**

- Take the control eye out of its chamber and place it on a layer of tissue with the cornea facing upwards or in a plastic box.
- Rinse the cornea carefully with 20 mL of isotonic saline of ambient temperature.
- Return the clamp and eye to its chamber.

### *2.7 Observation and assessment of corneal effects*

The control eye and test eyes are examined, using the criteria and scoring system given in OECD TG 438 at approximately 30, 75, 120, 180, and 240 minutes (± 10 min for all time points except 30 minutes) after treatment. All examinations are carried out with the slit-lamp microscope.

**30-min interval:**

- First observe each eye for corneal thickness (using the setting of t = 0 min) and record the findings.
- observe each eye for corneal opacity.
- If some test chemical is observed to remain on the cornea, try to remove it by gentle rinsing with saline and note the effect.
- Next, apply one drop of the fluorescein solution to the cornea of each eye (three eyes can be handled at the same time).
- Immediately rinse the corneas by turning up the saline drip.
- Immediately observe and record the findings.

**Other time intervals:**

- Repeat the procedure described above and record the findings at the respective intervals. However, fluorescein retention is no longer determined.

*NOTE. In case of test chemicals that have adhered to the cornea, fluorescein retention should also be determined whenever the test chemical can be removed. When determining the corneal thickness, a confirmation of fluorescein retention can be also obtained by observing the extent of penetration of fluorescein into or through the cornea (severe*

*fluorescein retention will result in considerable penetration). Also corneal opacity can be confirmed by observing the cross-section of the cornea during the thickness measurement. Mixed effects on the cornea such as a general slight opacity and focal spots with severe opacity can be noted on the scoring form as 1(3). This applies also to the scoring of fluorescein retention. The most severe score is used for calculation of the maximum mean score (3 in this case).*

### *2.8 Sampling and fixation of the corneas*

After the final examination the test and control eyes are preserved in a neutral aqueous phosphate buffered 4 per cent solution of formaldehyde for histopathological examination. For that purpose, the eyes are first almost cut in half with a scalpel just behind the level of the lens and through the vitreous body (not through the cornea!). The eye cut in half is placed in a container with approximately 20 mL of formalin. After fixation for at least 24 hours, the half with the cornea and lens tissue is trimmed with scissors in such a way that a thin piece containing the entire cornea without the lens is available for further processing.

Precise directions are given in Annex II of the OECD GD 160.

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# ANNEX V: DETAILED PROTOCOL FOR STUDIES USING THE BOVINE CORNEAL OPACITY AND PERMEABILITY TEST METHOD

## Preface

This protocol is based on a comprehensive test method evaluation process conducted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; ICCVAM 2010), in conjunction with the European Centre for the Validation of Alternative Methods and the Japanese Centre for the Validation of Alternative Methods, which included an international independent scientific peer review of the validation status and scientific validity of the BCOP test method. The protocol is based on information obtained from (1) the Institute for In Vitro Sciences, Inc. (IIVS), a non-profit foundation that has performed the BCOP test method since 1997 in a Good Laboratory Practice (GLP)-compliant testing facility and (2) DB-ALM Protocol 124 (1999), which represents the protocol used for the European Community sponsored prevalidation study of the BCOP test method conducted in 1997–1998. Both of these protocols are based on the BCOP test method first reported by Gautheron et al. (1992). Future studies using the BCOP test method could include further characterization of the usefulness or limitations of the BCOP test method in a weight-of-evidence approach for regulatory decision-making. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the NICEATM–ICCVAM website (http://iccvam.niehs.nih.gov/) to ensure use of the most current test method protocol.

## 1. PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the potential ocular hazard of a test chemical as measured by its ability to induce opacity and increase permeability in an isolated bovine cornea. Effects are measured by (1) decreased light transmission through the cornea (opacity); (2) increased passage of sodium fluorescein dye through the cornea (permeability); and (3) evaluation of fixed and sectioned tissue at the light microscopic level, if applicable. The opacity and permeability assessments of the cornea following exposure to a test chemical are considered individually and also combined to derive an in vitro irritancy score (IVIS), which is used to classify the irritancy level of the test chemical. Histological evaluation of the corneas can be useful for identifying damage in tissue layers that might not produce significant opacity or permeability changes. Furthermore, histopathology may be useful to identify the depth and degree of damage within the corneal layers to aid in predicting the likelihood for corneal recovery.

The focus of this protocol is on the use of the BCOP test method for the detection of serious eye damage as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS Category 1) (UN 2015), and to identify chemicals that do not require classification for eye irritation or serious eye damage, as defined by the GHS (GHS No Category). The BCOP test method is currently not considered to be adequately validated to identify test chemicals other than UN GHS Category 1 and UN GHS No Category (e.g. UN GHS Category 2 or UN GHS Categories

2A and 2B) (UN 2015), although these have been tested using this protocol. If such results are obtained with the BCOP test method, the obtained result may still be useful within an IATA approach using e.g. a weight-of-evidence analyses in conjunction with other testing and/or non-testing data, to further evaluate potential eye hazard including moderate and mild irritants (i.e., UN GHS Category 2/2A and 2B).

## 2. SAFETY AND OPERATING PRECAUTIONS

All procedures with bovine eyes and bovine corneas should follow the institution's applicable regulations and procedures for handling animal substances, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

## 3. MATERIALS, EQUIPMENT, AND SUPPLIES

### *3.1 Source of Bovine Eyes*

Eyes from cattle are obtained from an abattoir located within close proximity of the testing facility. The cattle type (breed not specified) can be cows, heifers, steers, or bulls. Because cattle have a wide range of weights depending on breed, age, and sex, there is no recommended weight for the animal at the time of sacrifice.

Eyes from very old cattle are not recommended because the corneas tend to have a greater horizontal corneal diameter and vertical corneal thickness that could affect assay performance (Doughty et al. 1995; Harbell J, personal communication). Additionally, eyes from calves are not recommended since their corneal thickness and corneal diameter are considerably less than that of eyes from adult cattle. Accordingly, eyes from cattle between 12 and 60 months old are typically used, although, the use of corneas from young animals (i.e., 6 to 12 months old) is permissible provided that it can be demonstrated that the test results obtained from the negative and positive controls routinely fall within the established acceptance ranges.

### *3.2 Equipment and Supplies*

- Corneal holders
- Dissection equipment (scissors, scalpels, forceps)
- Electric screwdriver
- Conical centrifuge tubes (50 mL)
- Incubator or water bath
- Low-residue detergent-based cleaning solution designed for cleaning healthcare and laboratory instruments (for example Alconox Liquinox®)
- Microplate reader or UV/VIS spectrophotometer
- Micropipettors and pipette tips
- Opacitometer

- Petri dishes
- Plastic containers for collection and transport of eyes
- Sample tubes (5 mL, glass) for permeability determination
- Spatula
- Specialized window-locking ring screwdriver
- Standard tissue culture and laboratory equipment
- Sterile deionised water
- Syringes (10 mL) and blunt tip needles (19 Gauge)
- Vacuum pump
- 96 well plates (polystyrene) or cuvettes of an appropriate size for UV/VIS spectrophotometer

### *3.3 Chemicals*

- Ethanol (200 proof, absolute, anhydrous, ACS/USP grade)
- Dimethylformamide
- Imidazole
- Penicillin
- Sodium chloride
- Sodium fluorescein
- Streptomycin

### *3.4 Solutions*

Follow the manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions.

- 0.9% (w/v) NaCl in sterile deionised water (saline).
- 1X Hanks' Balanced Salt Solution with Ca++ and Mg++ (HBSS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin.
- Dulbecco's Phosphate Buffered Saline (DPBS).
- Eagle's Minimum Essential Medium without phenol red, warmed to 32˚C.
- Eagle's Minimum Essential Medium with phenol red (used only for rinsing test chemicals), warmed to 32˚C.
- Sodium fluorescein (Na-fluorescein) diluted in DPBS to 4 mg/mL for liquid test articles or 5 mg/mL for solid test articles.

## 4. TEST CHEMICAL PREPARATION

Ideally, all test chemical solutions should be prepared fresh on the day of use.

### *4.1 Non-surfactant Liquid Test Chemicals*

Liquid test chemicals are tested undiluted. However, if prescribed, dilutions of aqueous soluble test chemicals should be prepared in 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system.

### *4.2 Non-surfactant Solid Test Chemicals*

Non-surfactant solid test chemicals should typically be prepared as 20% (w/v) solutions or suspensions in 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system.

### *4.3 Surfactants*

Solid and concentrated liquid surfactants should be prepared and tested as a 10% (w/v, v/v) dilution or suspension in 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system. Alternate concentrations of liquid surfactant concentrates may be tested with justification.

### *4.4 Surfactant Preparations*

Surfactant-based preparations (e.g. product formulations) are usually tested neat, or can be diluted in 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system, with justification of the selected dilution. For examples, surfactant-based cleaning solutions at end-user concentrations are usually tested neat, whilst highly concentrated liquid soaps, shampoos, and cleaning gels may be diluted to model end user concentrations, or to model an optimum surfactant activity.

## 5. CONTROLS

### *5.1 Negative Control*

During routine testing, a concurrent negative control (e.g. deionized or distilled water, or 0.9% sodium chloride) is included to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints. It ensures that the assay conditions do not inappropriately result in an abnormal response.

### *5.2 Solvent/Vehicle Control*

When testing a diluted test chemical, a concurrent solvent/vehicle control is included to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints. Only a solvent/vehicle that has been demonstrated to have no adverse effects on the test system can be used. Therefore, it is recommended to test the negative control concurrently with the solvent control to evaluate for potential solvent-induced changes.

### *5.3 Positive Control*

A substance known to induce a positive response is included as a concurrent positive control in each experiment to verify the integrity of the test system and its correct conduct. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be at either extreme.

Examples of positive controls for liquid test chemicals are 100% ethanol or 100% dimethylformamide. An example of a positive control for solid test chemicals substances is a 20% (w/v) dilution of imidazole prepared in 0.9% sodium chloride.

### *5.4 Benchmark Substances (if appropriate)*

Benchmark substances 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. Furthermore benchmark chemicals having similar physical chemical properties as the tested chemical (e.g. similar colour, state of aggregation, viscosity etc) might help to evaluate actual adverse effects of a test chemical more accurately. Appropriate benchmark substances should have the following properties:

- •A consistent and reliable source(s)
- •Structural and functional similarity to the class of the substance being tested
- •Known physical/chemical characteristics
- •Supporting data on known effects in vivo
- •Known potency in the range of the desired response

## 6. EXPERIMENTAL DESIGN

### *6.1 Collection and Transport Conditions of Bovine Eyes*

Bovine eyes are typically obtained from a local abattoir, where the eyes should be excised as soon as possible after sacrifice. Care should be taken to avoid damaging the cornea during the enucleation procedure. Eyes should be immersed completely in HBSS in a suitably sized container, and transported to the laboratory in such a manner as to minimize deterioration and/or bacterial contamination. Because the eyes are collected during the slaughter process, they might be exposed to blood and other biological substances, including bacteria and other microorganisms. Therefore, it is important to ensure that the risk of contamination is minimized (e.g. by keeping the container containing the eyes on wet ice, by adding antibiotics to the HBSS used to store the eyes during transport [e.g. penicillin at 100 IU/mL and streptomycin at 100 µg/mL]). If yeast contamination is likely, the HBSS may be supplemented with fungizone. The time interval between collection of the eyes and use of corneas should be minimized (typically collected and used on the same day) and should be demonstrated to not compromise the assay results.

### *6.2 Preparation of Corneas*

Since the whole globe enucleated eyeballs received for this assay are provided en masse as a raw material by an abattoir, typically a large number of eyes are disqualified for use in the assay by the Testing Facility due to a number of quality concerns. Multiple acceptance evaluations are performed throughout the cornea preparation procedures, from initial receipt of the whole globe enucleated eyeballs, to the final objective measurement of corneal opacity. Only those corneas which have been mounted in corneal holders and meet all of the quality criteria throughout the cornea qualification process are qualified to be defined as a BCOP Test System.

a. Carefully examine all whole globe enucleated eyes macroscopically. Those exhibiting unacceptable defects, such as opacity, scratches, pigmentation, and neovascularization are rejected.

b. For those eyes where no defects are initially detected, carefully remove the cornea from each selected eye by making an incision with a scalpel 2 to 3 mm outside the cornea, then by cutting around the cornea with dissection scissors, leaving a rim of sclera to facilitate handling. Carefully peel off the iris and lens, ensuring no fragments of these tissues are remaining on the cornea. Take care to avoid damaging the corneal epithelium and endothelium during dissection.

c. Place the isolated corneas in a Petri dish containing HBSS until they are mounted in the corneal holders. The isolated corneas are examined again for defects immediately prior to mounting the corneas in the corneal holders. If any previously unrecognized defects are observed at this point, those defective corneas are also discarded. Only those corneas which pass the initial qualification evaluations are mounted in corneal holders. Since each corneal holder is identified by a unique number the mounting of corneas into the corneal holders provides an initial opportunity to identify and track the disposition of individual corneas from this point forward.

d. Mount the corneas in holders (one cornea per holder) by placing the endothelial side of the cornea against the O-ring of the posterior chamber. Place the anterior chamber over the cornea and join the chambers together by tightening the chamber screws. Care should be taken not to shift the two chambers to avoid damaging the cornea.

e. Fill both chambers with fresh complete MEM (about 5 mL), always filling the posterior chamber first to return the cornea to its natural curvature. Care should be taken when adding or removing liquid from the posterior chamber to avoid the formation of bubbles and to minimize shear forces on the corneal endothelium.

f. Seal each chamber with plugs provided with the holders.

g. Incubate the corneal holders in a vertical position at 32 ± 1°C for at least 60 minutes to allow the corneas to equilibrate with the medium and to achieve normal metabolic activity.

h. At the end of the initial 1-hour incubation period, examine each cornea for defects, such as tears or wrinkling. Discard corneas with any observed defects.

### *6.3 Control Cornea Selection and Opacity Reading*

The final step in the qualification of the corneas for use in a study is an objective evaluation of the corneal opacity; namely, only corneas with initial opacity values ≤7 opacity units (as measured with a calibrated corneal opacitometer) are acceptable for use in a study.

a After the 1-hour incubation period, remove the medium from both chambers of each holder (anterior chamber first) and replace with fresh complete MEM.

b Take and record an initial opacity reading for each cornea, using an opacitometer or equivalent instrument that has been appropriately calibrated according to the manufacturer's specifications. This initial opacity reading will be used to calculate the final opacity value for each cornea. The testing facility should ensure the opacitometer is functioning properly each day it is used.

c Any corneas that show macroscopic tissue damage or an opacity >7 opacity units are discarded.

d Calculate the mean opacity value for all corneas.

e Select a minimum of three corneas with opacity values close to the mean value for all corneas as negative (or solvent/vehicle) control corneas. The remaining corneas are then distributed into treatment and positive control groups. The disposition of any unused corneas should be accounted for.

### *6.4 Treatment Groups*

A minimum of three corneas is treated with each test chemical solution or suspension. In addition, a minimum of three corneas per assay are treated with the positive control and a minimum of three corneas per assay are treated with the negative control. If a benchmark substance is used the day of testing, a minimum of three corneas should be treated with the benchmark.

Different treatment methods are used depending on the physical nature and chemical characteristics (liquid or surfactant versus non-surfactant solid) of the test chemical. A closed chamber method is typically used for non-viscous to slightly viscous liquid test chemicals, while an open chamber method is typically used for semi-viscous and viscous liquid test chemicals and for neat solids. The open chamber method allows direct access to the anterior side of the corneas to dose and rinse materials that cannot readily be pipetted.

### *6.5 Treatment of Corneas and Opacity Measurements*

#### *6.5.1 Closed chamber method for non-viscous to slightly viscous liquid test chemical and surfactant preparations*

a Record the initial opacity readings and label each chamber with the appropriate control or test chemical identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes using an appropriate aspiration technique (e.g. blunt needle attached to a large syringe, or alternatively to a vacuum pump).

b Add 0.75 mL of the control or test chemical (or enough test chemical to completely cover the cornea) to the anterior chamber through the dosing holes using a micropipet. The dosing holes are then resealed with the chamber plugs.

c Rotate the holders such that the corneas are in a horizontal position. The holders should be gently tilted back and forth to ensure a uniform application of the control or test chemical over the entire cornea.

d Incubate the holders in a horizontal position at 32 ± 1°C for 10 ± 1 minutes. If other exposure times are used, justification must be provided.

e Remove the control or test chemical from the anterior chamber through the dosing holes and rinse the epithelium at least three times (or until no visual evidence of test chemical is observed) with approximately 2 to 3 mL of fresh complete MEM (containing phenol red as an indicator of the effectiveness of rinsing acidic or alkaline materials). Perform one final rinse of the epithelium using fresh complete MEM (without phenol red to ensure its removal prior to the opacity measurement). If it is not possible to remove all visible signs of the test chemical, this should be documented. Refill the anterior chamber with fresh complete MEM (without phenol red).

f Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g. dissimilar opacity patterns, tissue peeling or residual test article).

g Incubate the holders in a vertical (anterior chamber facing forward) position at 32 ± 1°C for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.

h Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and record pertinent observations. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test chemical, etc.

### *6.5.2 Open chamber method for semi-viscous and viscous liquid test chemicals and surfactant preparations*

a Record the initial opacity readings and label each chamber with the appropriate control or test article identification. Just prior to treatment, remove the medium from the anterior chamber through the dosing holes.

b Remove the window-locking ring and glass window from all appropriate anterior chambers and place the holders into a horizontal position (anterior chamber facing up).

c Add test chemical to each chamber successively at a constant rate of 15 to 30 seconds between each chamber. Apply approximately 0.75 mL of the control or test chemical (or enough test chemical to completely cover the cornea) directly to the epithelial surface of the cornea using a micropipet or other appropriate device, such as a spatula. Maintain the holders in a horizontal position (anterior chamber up).

d If necessary, to aid in filling the pipette with substances that are viscous, the test article may first be transferred to a syringe. Insert the pipette tip of the positive

displacement pipette into the dispensing tip of the syringe, so that the substance can be loaded into the displacement tip under pressure. Simultaneously, depress the syringe plunger as the pipette piston is drawn upwards. If air bubbles appear in the pipette tip, the test article should be expelled and the process repeated until the tip is filled without air bubbles. This method should be used for any substances that cannot be easily drawn into the pipette (e.g. gels, toothpastes, and face creams).

e If necessary, immediately upon dosing, slightly tilt the holders to achieve a uniform application of the test article over the entire cornea.

f After all of the chambers are dosed, replace the glass windows and window-locking rings.

g Incubate the holders in a horizontal position at 32 ± 1°C for 10 ± 1 minutes. If other exposure incubation times are used, justification should be provided.

h Prior to the end of the exposure period, remove the window-locking ring and glass window from each appropriate chamber.

i At the completion of the exposure period, successively rinse each cornea at least three times (or until no visual evidence of test chemical is observed) according to the intervals that they were dosed. Using a syringe, add fresh complete MEM with phenol red to the inside wall of the anterior chamber creating a "whirlpool or vortex effect", which causes the test article to be rinsed off the cornea. Take special care not to spray the medium directly onto the cornea. Residual test article that cannot be removed from the cornea by the "whirlpool method" is removed by placing a layer of medium over the cornea (added to the inside wall of the chamber). Spray a gentle stream of medium through the medium layer, directing it towards the residual test article. Perform one final rinse of the epithelium using fresh complete MEM (without phenol red). If after several tries the test article cannot be removed, document this, and proceed to the next step.

j Once each cornea is completely rinsed of test article, replace the glass window and window-locking ring. Continue rinsing as stated previously for the "closed chamber method" (see Section 6.5.1, step e).

k Perform a post-treatment opacity reading for each cornea and record the results. Observe each cornea visually and, if applicable, record pertinent observations (e.g. dissimilar opacity patterns, tissue peeling or residual test article).

l Incubate the holders in a vertical (anterior chamber facing forward) position at 32 ± 1°C for 120 ± 10 minutes. If other post-exposure incubation times are used, justification should be provided.

m Record a post-incubation opacity reading for each cornea, which will be used to calculate the final corneal opacity value. Observe each cornea visually and record pertinent observations. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test chemical, etc.

### *6.5.3 Solid non-surfactant test chemicals*

Solid non-surfactant test chemicals are administered following one of the previously described procedures, with a few exceptions, which are noted below:

- •Solid test chemicals are tested on the cornea as a 20% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g. sterile deionised water).
- •Solid test chemicals are incubated at 32 ± 1°C for 240 ± 10 minutes.
- •There is no post-treatment incubation period. Thus, immediately following the rinsing process, both chambers are refilled (posterior chamber first) with fresh complete MEM, and the post-treatment opacity readings are taken. During the post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are these are recorded. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test article, etc. Immediately following these opacity readings and visual observations, the permeability experiment is performed.

### *6.6 Application of Sodium Fluorescein*

Following the final opacity measurement, permeability of the cornea to Na-fluorescein is evaluated. The Na-fluorescein solution is applied to the cornea by one of two methods, depending on the nature of the test chemical:

Liquid and surfactant test chemicals and surfactant preparations:

a Remove the medium from both chambers (anterior chamber first).

b Fill the posterior chamber with fresh complete MEM, and add 1 mL of a 4 mg/mL Na¬-fluorescein solution to the anterior chamber using a micropipettor.

c Reseal the dosing holes in the top of both chambers with the chamber plugs.

d Solid non-surfactant test chemicals:

i. Remove the medium from the anterior chamber only and replace with 1 mL of a 5 mg/mL Na-fluorescein solution.

ii. Reseal the dosing holes in the top of both chambers with the chamber plugs.

### *6.7 Permeability Determinations*

a. After adding the Na-fluorescein to the anterior chamber and sealing the chambers, rotate the holders into a horizontal position with the anterior chamber facing up. Tilt the holders slightly, if necessary, to achieve a uniform application of the Na-fluorescein over the entire cornea. Incubate the holders in a horizontal position for 90 ± 5 minutes at 32 ± 1°C.

b. After the 90-minute incubation period, remove the medium in the posterior chamber of each holder and place into sample tubes pre-labelled according to holder number. It is important to remove most of the medium from the posterior chamber and mix it in the tube so that a representative sample can be obtained for the OD490 determination.

c. After completing the Na-fluorescein penetration steps, the corneas may be fixed in an appropriate fixative (e.g. 10% neutral buffered formalin) at room temperature for at least 24 hours, so that the tissues are available if histology is necessary or

requested at a later time. It is important that the corneas not be allowed to dry between transfer from the holders and fixation (submersion in the fixative) (see paragraph 13, GD 160).

d. If using a microplate reader to measure optical density, transfer 360 µL of the medium from each sample tube into its designated well on a 96-well plate. The standard plate map provides two wells for each cornea. The first well receives an undiluted sample from each cornea tested. When all of the media samples have been transferred onto the plate, measure and record their OD490. Any OD490 value (of a control or test chemical sample) that is above the linear range of absorbance must be diluted to bring the OD490 into the acceptable range. [Note: The linear range of absorbance of different microplate readers can vary. Thus, each laboratory must determine the upper limit of absorbance (in the linear range) for the microplate reader used in its facility. IIVS has determined that OD490 values greater than 1.500 may not be within the linear range of absorbance for their model plate reader, and thus must be diluted to bring the OD490 into the acceptable range.] A dilution of 1:5 is generally sufficient but higher dilutions may be required. Prepare the dilution from the original sample of medium and transfer 360 µL into the second well designated for that cornea. Reread the plate and record the data from both the undiluted and diluted OD490 values. Use the values from this second reading in all calculations. The OD490 values of less than 1.500 will be used in the permeability calculation.

e. If using a UV/VIS spectrophotometer to measure optical density, adjust the spectrophotometer to read at OD490, and zero the spectrophotometer on a sample of complete MEM. Prior to reading samples from the BCOP test method, prepare and read two quality control samples of Na-fluorescein solution to ensure the Na-fluorescein calibration curve (see note below) conducted for the spectrophotometer is still acceptable. If the average of the quality control samples does not fall within the accepted range of the Na-fluorescein calibration curve, then prepare a Na-fluorescein calibration curve prior to running samples from the BCOP test method. If the average of the quality control samples falls within the accepted range of the calibration curve, then proceed to read samples from the BCOP test method. Transfer an aliquot of the mixed medium from the posterior chamber of the BCOP holder into a cuvette, then take and record an absorbance reading using the spectrophotometer. Any solutions giving an OD490 beyond the linear range of the spectrophotometer must be diluted in complete MEM, and another reading taken, repeating these steps until the OD490 is within the linear range of the spectrophotometer. Repeat these procedures for each sample from the BCOP test method, rinsing the cuvette(s) thoroughly between each sample, until all samples have been read and results recorded.

*Note: If conducting this assay for the first time, a calibration curve for the spectrophotometer must be performed, using a series of dilutions of Na¬-fluorescein solution in complete MEM. A calibration curve should be prepared and used to determine the linear range of the spectrophotometer and thus determine the upper limit of the linear range of absorbance.*

### *6.8 Histopathology*

Histological evaluation of the corneas can be useful for identifying damage in tissue layers that might not produce significant opacity or permeability changes, or when the standard BCOP endpoints produce borderline results. Furthermore, histopathology may be useful to identify the depth and degree of damage within the corneal layers to aid in predicting the likelihood for corneal recovery. Jester (1998), Maurer (1996, 2002) and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deeper injury into the stroma has more serious consequences, while a full thickness injury including damage or loss of the endothelium would be predictive of a severe injury. Similar observations were presented relating the depth of histopathologic changes in corneas treated with a series of anti-microbial products with cleaning claims (which had been previously classified in vivo) in the BCOP assay (Redden, 2009). Kolle et al. (2015) reported that histopathology of the corneas used in the BCOP assay, using a depth of injury concept, improved the sensitivity for identifying severe ocular irritant agrochemical formulations over the IVIS alone, although they concluded that for agrochemical formulations the sensitivity for identifying severe ocular irritants was not sufficient. A standardized scoring scheme using the formal language of pathology to describe any effects should be used.

a. Corneal sections will be examined for the presence of changes in the corneal epithelium, stroma, and endothelium. Particular emphasis will be placed on assessment of the depth and degree of injury into the stromal elements. Treated tissues will be compared to the negative control tissues.

b. Negative and Positive control corneas from the BCOP assay will have been processed with the relevant test article-treated corneas as a common histology batch process. The histology of the negative control corneas may thus be used to evaluate the quality/acceptability of the slides within the processing batch. Prior to conducting the evaluation of test article-induced histopathology, the quality of the stained corneal sections will be evaluated, so that the nature and degree of the artefacts of both the BCOP assay and the histology processing can be assessed. To this end, the negative control slides are used to detect artefacts at the batch level. They are also used to assess “normal” staining (degree of hematoxylin or eosin in each layer/cell type), tissue architecture and general thickness.

c. Slides will be thoroughly examined by microscopic evaluation. Each cross section of each cornea within a treatment group should be observed first under low magnification for an overall assessment of the quality of the tissue sections for conducting the histopathology. Corneal sections are evaluated across the entire section from one crush zone to the other (the crush zone is the outer corneal perimeter where the cornea was mounted against an O-ring within the corneal chamber in the BCOP assay, and is readily apparent in the histology sections). Occasional processing artefacts may render sections of tissues unacceptable for use in the histopathology.

d. The test chemical and positive control-treated corneas will be evaluated for changes in cellular morphology and tissue architecture. Since test chemicals are applied topically on the outer corneal epithelium in the BCOP assay, any changes or toxic effects would be expected to be observed in the corneal epithelium, and dependent upon the penetration of the test chemical into the cornea, may be observed deeper

into the cornea. Therefore, the evaluation of changes in the treated corneas will be performed starting superficially with the squamous epithelium at the site of test chemical exposure, and progressing into the cornea to the endothelium. Gross changes or erosion of the corneal architecture would be readily apparent, while other changes such as cellular or nuclear staining may be a bit more subtle. In general, the depth of observed changes in the bovine cornea will be reported. However, the degree of many of the observed changes may not be fully assessed, and thus the relative frequency of such observed changes relative to the negative control corneas might be presented. The interpretation of the histopathology should allow for an integration of all of the observed changes, prior to the definitive assessment.

e. Representative fields may be photographed and presented in the report solely to be illustrative of the cited changes.

### *6.9 Maintenance of the Corneal Holders*

Following completion of the assay, clean the disassembled parts of each holder as follows:

a. Soak the posterior and anterior chambers with a low-residue detergent-based laboratory cleaning solution (e.g., Liquinox®).

b. Soak the chamber plugs, O-rings, and handle screws in 70% ethanol. Rinse the chamber plugs, O-rings, and handle screws thoroughly in hot tap water, and air dry prior to reassembling the chambers.

c. Clean the interior and exterior surfaces of each pre-soaked posterior and anterior chamber by using a scrubbing sponge. Rinse each posterior and anterior chamber thoroughly in warm tap water and air dry prior to reassembling the chambers.

d. Match up each numbered posterior chamber with its corresponding anterior chamber; insert an O-ring into the appropriate place; attach a chamber handle screw to the anterior chamber; and finally insert the chamber screws into the anterior chamber.

## 7. EVALUATION OF TEST RESULTS

Results from the two test method endpoints, opacity and permeability, should be combined in an empirically derived formula that generates an in vitro irritancy score for each test chemical.

### *7.1 Opacity*

a. Calculate the change in opacity for each individual cornea (including the negative control) by subtracting the initial opacity reading from the final post-treatment opacity reading. Then calculate the average change in opacity for the negative control corneas.

b. Calculate a corrected opacity value for each treated cornea, positive control, and solvent/vehicle control (if applicable) by subtracting the average change in opacity

of the negative control corneas from the change in opacity of each treated, positive control, or solvent/vehicle control cornea.

c. Calculate the mean opacity value of each treatment group by averaging the corrected opacity values of the treated corneas for each treatment group.

### *7.2 Permeability*

*Microplate Reader Method*

a. Calculate the mean OD490for the blank wells (plate blanks). Subtract the mean blank OD490 from the raw OD490 of each well (blank corrected OD490).

b. If a dilution has been performed, correct the OD490 for the plate blank before the dilution factor is applied to the reading. Multiply each blank corrected OD490 by the dilution factor (e.g. a factor of 5 for a 1:5 dilution).

c. Calculate the final corrected OD490 value for each cornea by subtracting the mean OD490 value for the negative control corneas from the OD490 value of each treated cornea.

d. Final Corrected OD490 = (raw OD490 – mean blank OD490) -mean blank corrected negative control OD490

e. Calculate the mean OD490 value for each treatment group by averaging the final corrected OD490 values of the treated corneas for a particular treatment group.

*UV/VIS Spectrophotometer Method*

a Calculate the corrected OD490 value of each treated, positive control, or solvent/vehicle control cornea by subtracting the average value of the negative control corneas from the original OD490 value for each cornea.

b Final Corrected OD490 = raw OD490 -mean blank corrected negative control OD490

c Calculate the mean OD490 value for each treatment group by averaging the final corrected OD490 values of the treated corneas for a particular treatment group.

### *7.3 In Vitro Irritancy Score*

a. Use the mean opacity and mean permeability values (OD490) for each treatment group to calculate an in vitro irritancy score for each treatment group:

b. In Vitro Irritancy Score = mean opacity value + (15 x mean OD490 value)

## 8. CRITERIA FOR AN ACCEPTABLE TEST

A test is acceptable if the positive control gives an in vitro irritancy score that falls within two SDs of the current historical mean, which is to be updated at least every three months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e. less than once a month). In the BCOP, 20% (w/v) imidazole induces a severe response (in vitro score = 69.7-136.2 at IIVS [n=125]; mean = 103, SD = 16.6). The negative or solvent/vehicle control responses should result in opacity and permeability

values that are less than the established upper limits for background opacity and permeability values for bovine corneas treated with the respective negative or solvent/vehicle control.

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