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GUIDANCE DOCUMENT ON "THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) AND ISOLATED CHICKEN EYE (ICE) TEST METHODS: COLLECTION OF TISSUES FOR HISTOLOGICAL EVALUATION AND COLLECTION OF DATA ON NON-SEVERE IRRITANTS

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GUIDANCE DOCUMENT ON THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) AND ISOLATED CHICKEN EYE (ICE) TEST METHODS: COLLECTION OF TISSUES FOR HISTOLOGICAL EVALUATION AND COLLECTION OF DATA ON NON-SEVERE IRRITANTS

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No. 143, *Peer Review Report for the Validation of the Fish Sexual Development Test and Agreement of the Working Group of National Co-ordinators of the Test Guideline Programme on the Follow-up of the Peer Review (2011)*

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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80 E-mail: ehscont@oecd.org

E-mail: ehscont@oecd.org

FOREWORD

This Guidance Document (GD) was developed 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 project was led by the United States. The draft GD was submitted to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) for comments in March and November 2010. It was approved by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 October 2011.

This document is published under the responsibility of the Joint Meeting.

TABLE OF CONTENTS

I. INTRODUCTION 19
II. HISTOPATHOLOGICAL EVALUATION IN OCULAR SAFETY TEST METHODS 20
Background 20
Source of Tissue for Histopathological Evaluation 20
Sample Identification 21
Tissue Preparation 21
Tissue Preservation 21
Post-fixation Tissue Trimming 22
Tissue Processing and Embedding 22
Tissue Sectioning and Slide Preparation 23
Staining of the Tissues 23
Evaluation of Quality and Acceptability of the Corneal Sections 23
III. DATA AND REPORTING 24
Preparation of Digital Images 24
Test Report 24
Decision Criteria for All Ocular Hazard Categories 25
The BCOP Test Method 25
Study Acceptance Criteria 26
The ICE Test Method 26
Study Acceptance Criteria 30
Test Report 30
IV. LITERATURE 31
ANNEX I 35
ANNEX II 39
ANNEX III 41
ANNEX IV 42
ANNEX V 55

I. INTRODUCTION

1. This Guidance Document (GD) accompanies the OECD Test Guideline (TG) 437 on the bovine corneal opacity and permeability (BCOP) test method (OECD 2009a) and TG 438 on the isolated chicken eye (ICE) test method (OECD 2009b). This document 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 in order to support future evaluation of its usefulness and limitations as an additional endpoint to improve the accuracy of *in vitro* ocular safety test methods; ii) to provide specific guidance on using the BCOP and ICE test methods to generate data that can be evaluated to determine and optimize their usefulness for identifying additional hazard categories (ICCVAM 2010a); iii) for those substances that test negative in the BCOP or ICE test method for severe/irreversible effects and are tested *in vivo*, to provide standard procedures for enucleating, fixing, and processing eyes from *in vivo* rabbit eye studies for histopathological evaluation.

2. Histopathological evaluation may be useful for (i) assessment of the histological damage of chemical classes or formulations that are not well characterized in these test methods; (ii) assisting with determination of a mode of action where it cannot be easily predicted; (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). Histopathological evaluation may be used as an additional endpoint in the *in vivo* rabbit eye test 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 will 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, sampling of the eyes for histopathology is advised.

4. This GD describes the general procedures for the collection, preservation, and preparation of ocular tissues for use in performing histopathological evaluations. However, if differences exist, laboratories that routinely perform histopathological evaluations of ocular tissue can employ their existing procedures. This document does not provide guidance on the evaluation or interpretation of histopathological data or the associated decision criteria to be used for ocular hazard classification. When relevant information becomes available, this GD will be updated.

5. As described in TG 437 and 438, BCOP and ICE data are accepted for the hazard classification and labelling of ocular corrosives/severe irritants (OECD 2009a) (OECD 2009b). However, even if an ocular corrosive or severe irritant classification is not obtained, BCOP or ICE data can be useful, in conjunction with *in vivo* data or other valid *in vitro* test data, to further evaluate the usefulness and limitations of the BCOP or ICE test methods for identifying non-severe irritants (i.e. moderate and mild irritants) and substances not classified as irritants. Therefore, this GD provides provisional decision criteria to classify non-severe irritants and substances not classified as irritants. These data can then be reported in parallel with any other data obtained (i.e. from the *in vivo* rabbit eye test or an adequately validated *in vitro* test method).

6. Definitions are provided in **Annex 1**.

II. HISTOPATHOLOGICAL EVALUATION IN OCULAR SAFETY TEST METHODS

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 does not provide guidance on the evaluation or 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, *in vitro* 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 Centre for the Validation of Alternative Methods [EC-ECVAM], or the Japanese Center for the Validation of Alternative Methods [JaCVAM]).

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 the institution's applicable regulations and 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).

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 e*t al.* 2006)(ICCVAM 2010b).

Tissue Preparation

12. Corneas to be used for histopathological evaluation following *in vivo* studies 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.

Tissue Preservation

13. 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, Fredenburgh, and Myers 2008).

14. To prevent the tissues from drying out, which would induce substantial artefacts, they should remain immersed in fixative before processing and embedding.

15. 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. 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).

16. 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, Fredenburgh, and Myers 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).

17. The fixatives most commonly used for ocular tissues are 10% NBF and Davidson's (see **Annex II**). Davidson’s fixative is suggested for whole eyes in the ICE test method due to the rapid penetration into the deeper tissues by the alcoholic component of the fixative (Latendresse et al. 2002). However, 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), 4% formalin (Prinsen M, personal communication), 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).

Post-fixation Tissue Trimming

18. 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.

Tissue Processing and Embedding

19. 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)

20. A routine schedule for processing 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.

21. 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.

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 Material 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 always be dehydrated and cleared at room temperature.

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. Processed tissues should be embedded so as to maintain the appropriate orientation in the hardened tissue block once the paraffin cools. For example, true corneal cross-sections (*i.e.* anterior to posterior) are usually desired to permit an accurate measurement of the corneal thickness due to swelling caused by the test substance relative to the negative control. Therefore, the tissue should be embedded in the block on its edge in the correct orientation to permit such sections.

Tissue Sectioning and Slide Preparation

26. 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). 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).

27. 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). 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.

28. 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).

29. 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).

Staining of the Tissues

30. For routine histopathological evaluations, tissues are most commonly stained with hematoxylin and eosin (H&E) (Gamble 2008). See **Annex II** for H&E stain recipes and **Annex III** for sample H&E staining protocols. For the ICE test method, the use of Periodic Acid-Schiff (PAS) staining is preferred for histopathological evaluation because it clearly defines the basement membrane, in contrast to other routinely used stains including H&E (Prinsen *et al.* 2009). 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).

Evaluation of Quality and Acceptability of the Corneal Sections

31. Tissues from animals/samples treated with test substance should be processed together with positive and negative control tissues. Negative control tissues 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). Positive control data from the testing laboratory may be used to develop a historical database for ocular damage produced by severe irritants. 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).

III. DATA AND REPORTING

Preparation of Digital Images

32. Digital images or, preferably, digital slide scans of all tissue sections should if feasible be prepared for archival purposes.

33. All histopathological evaluations should be performed by personnel trained to identify the relevant morphological changes in treated corneas.

Test Report

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

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known;
- The CAS Registry Number (RN), if known;
- Purity and composition of the substance or preparation (in percentage[s] by weight), to the extent this information is available;
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study;
- Treatment of the test/control substances prior to testing, if applicable (*e.g.* warming, grinding);
- Stability, if known.

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 (*i.e.* 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 substance and control (*e.g.* n=3);
- Fixative, dehydration and clarifying agents, and protocols used (to be available as raw data/standard operating procedure (SOP);
- Embedding material, infiltration solvents, and concentrations used (to be available as raw data/SOP);

- Thickness of tissue sections;
- Stain (in report) and the associated staining protocol used (to be available as raw data/SOP);
- Information on instruments used (to be available as raw data/SOP).

Results

- Digital images or digital slide scans for archival purposes, if feasible;
- Detailed descriptions of all lesions and artefacts using standard histopathological terminology;
- Description of the scoring scheme used in the evaluation;
- Summary and individual specimen data tables.

Decision Criteria for All Ocular Hazard Categories

35. As described in TG 437 (OECD 2009a) and 438 (OECD 2009b), BCOP and ICE can be used, under certain circumstances and with specific limitations, to classify substances as ocular corrosives and severe irritants. However, even if an ocular corrosive or severe irritant classification is not obtained, BCOP or ICE data can be useful, in conjunction with *in vivo* data or valid *in vitro* test data, to further evaluate the usefulness and limitations of the BCOP or ICE test methods for identifying non-severe irritants (*i.e.* moderate and mild irritants) and substances not classified as irritants. Therefore, the following provisional decision criteria are provided to correspond to hazard categories for non-severe irritants and substances not classified as irritants. These data can then be reported in parallel with any other data obtained (*i.e.* from the *in vivo* rabbit eye test or an adequately validated *in vitro* test method).

The BCOP Test Method

36. A detailed protocol for BCOP is provided in **Annex IV**. As described in OECD TG 437 (OECD 2009a), the mean opacity and permeability OD_{490} 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 OD_{490} value).

37. A test substance that induces an IVIS ≥ 55.1 is defined as an ocular corrosive or severe irritant. The recommended decision criteria for using BCOP to identify non-severe irritants and substances not classified as irritants are provided in **Table 1**.

Table 1: Overall BCOP classification criteria[1]

***In Vitro* Classification**	**IVIS Score Range**
Not Classified[2]	0 to 3
Mild[3]	3.1 to 25
Moderate[4]	25.1 to 55
Severe[5]	≥ 55.1

[1]As described in TG 437 (OECD 2009a), BCOP data are only accepted for regulatory hazard classification and labelling of ocular corrosives/severe irritants.

[2]EPA Category IV; GHS Not Classified; EU Not Classified
[3]EPA Category III; GHS Category 2B; EU Not Classified
[4]EPA Category II; GHS Category 2A; EU Category 2
[5]EPA Category I; GHS Category 1; EU Category 1

38. 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 2009), was evaluated by ICCVAM (2010a). Based on the current BCOP validation database (n=211 substances), which was updated since the ICCVAM evaluation of the BCOP test method for identifying ocular corrosives and severe irritants (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 for identifying all categories of ocular irritation (i.e. EPA Category II, III, IV; GHS Category 2A, 2B, Not Classified; EU Category 2, Not Classified).

39. However, to further evaluate the usefulness and limitations of the BCOP test method for identifying all categories of ocular irritation, it is recommended that the complete classification scheme of the BCOP test method (see **Table 1**) be applied and that these data are reported in parallel with any other data obtained (i.e. from the *in vivo* rabbit eye test or an adequately validated *in vitro* test method).

40. When such data are generated, the criteria described above may need to be modified in order to optimize the BCOP test method for identifying non-severe irritants (*i.e.* moderate and mild irritants) and substances not classified as irritants.

Study Acceptance Criteria

41. A test is acceptable if the positive control gives an IVIS that falls within two standard deviations 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). 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.

The ICE Test Method

42. A detailed protocol for ICE is provided in **Annex V**. As described in OECD TG 438 (OECD 2009b), the overall *in vitro* irritancy classification for a test substance is assessed by reading the irritancy classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention (see **Table 7)**.

43. 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:

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

44. 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 overall category score is then given for each test substance (**Table 2**).

Table 2: ICE classification criteria for corneal thickness

Mean Corneal Swelling (%)	Category
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

45. The above mean corneal swelling scores are only applicable if thickness is measured with a Haag-Streit BP900 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.

46. Corneal opacity is calculated by using the area of the cornea that is most densely opacified for scoring (**Table 3)**. 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 overall category score is then given for each test substance (**Table 4).**

Table 3: 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 4: ICE classification criteria for opacity

Mean Maximum Opacity Score	Category
0.0–0.5	I
0.6–1.5	II
1.6–2.5	III
2.6–4.0	IV

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

Table 5 : 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 6: ICE classification criteria for mean fluorescein retention

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

48. 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 generate an Irritancy Classification for each test substance (see **Table 7**).

49. The overall *in vitro* irritancy classification for a test substance is assessed by reading the irritancy classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention and applying the scheme presented in **Table 7**.

Table 7: Overall ICE classification criteria[1]

Irritant Classification	Combinations of Three Endpoints
Not Classified[2]	3 x I
	2 x I, 1 x II
Mild[3]	3 x II
	2 x II, I x I
	2 x II, 1 x III
	2 x I, 1 x IV
	1 x I, 1 x II, 1 x III
Moderate[4]	3 x III
	2 x III, 1 x II
	2 x III, 1 x IV
	2 x III, 1 x 1
	2 x II, 1 x IV*
	1 x II, 1 x III, 1 x IV*
Severe[5]	3 x IV
	2 x IV, 1 x III
	2 x IV, 1 x II*
	2 x IV, 1 x I*

[1]As described in TG 438 (OECD 2009b), ICE data are only accepted for regulatory hazard classification and labelling of ocular corrosives/severe irritants.
[2]EPA Category IV; GHS Not Classified; EU Not Classified
[3]EPA Category III; GHS Category 2B; EU Not Classified
[4]EPA Category II; GHS Category 2A; EU Category 2
[5]EPA Category I; GHS Category 1; EU Category 1
*Combinations less likely to occur.

50. The ability of the ICE 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 2009), was evaluated by ICCVAM (2010a). No new ICE data were available since the ICCVAM evaluation of the ICE test method for identifying ocular corrosives and severe irritants (ICCVAM 2006b). The overall correct classification ranged from 59% (83/141) to 77% (118/153) when evaluating the entire database, depending on the hazard classification system used. Based on these performance statistics, the ICE test method is not considered valid as a complete replacement for the *in vivo* rabbit eye test for identifying all categories of ocular irritation (i.e. EPA Category II, III, IV; GHS Category 2A, 2B, Not Classified; EU Category 2, Not Classified.

51. However, 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 7**) be applied and that these data are reported in parallel with any other data obtained (*i.e.* from the *in vivo* rabbit eye test or an adequately validated *in vitro* test method).

52. When such data are generated, the criteria described above may need to be modified in order to optimize the ICE for identifying non-severe irritants (*i.e.* moderate and mild irritants) and substances not classified as irritants.

Study Acceptance Criteria

53. A test is acceptable if the concurrent negative or vehicle/solvent controls and concurrent positive controls fall within the Not Classified and Severe irritant classification, respectively.

Test Report

54. For the BCOP and ICE test methods, the information to be included in the test report is outlined in TG 437 (OECD 2009a) and 438 (OECD 2009b), respectively.

IV. LITERATURE

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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.

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

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.

Classification system: An arrangement of quantified results or data into groups or categories according to previously established criteria.

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 substances being examined.

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 substance. 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.

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 μM 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 globes).

Descemet's membrane: The posterior lamina of the cornea that lies at the posterior end of the stroma and precedes the endothelial layer.

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.

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).

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).

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

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

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 of the extent of fluorescein sodium that is retained by epithelial cells in the cornea following exposure to a test substance. Increased fluorescein retention is indicative of damage to the corneal epithelium.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): 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 2009).

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: The potential for an adverse health or ecological effect. The adverse effect is manifested only if there is an exposure of sufficient level.

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:** 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 *In Vitro* Irritancy Score = mean opacity value + (15 x mean permeability value).

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

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.

Negative control: An untreated sample containing all components of a test system, except the test substance solvent, which is replaced with a known nonreactive material, such as water. This sample is processed with test substance-treated samples and other control samples to determine whether the solvent 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: Substances that are not classified as EPA Category I, II, or III; EU 1 or 2; or GHS Category 1, 2A, or 2B ocular irritants (EPA 2003a)(EU 2008)(UN 2009).

Ocular corrosive: (a) A substance that causes irreversible tissue damage to the eye; (b) Substances that are classified as EPA Category I, EU Category 1, or GHS Category 1 ocular irritants (EPA 2003a)(EU 2008)(UN 2009).

Ocular irritant: (a) A substance that produces a reversible change in the eye following application to the anterior surface of the eye; (b) Substances that are classified as EPA Category II or III, EU Category 2, or GHS Category 2A or 2B ocular irritants (EPA 2003a)(EU 2008)(UN 2009).

Ocular severe irritant: (a) A substance that causes tissue damage in the eye following application to the anterior surface of the eye that does not resolve within 21 days of application or causes serious physical decay of vision; (b) Substances that are classified as EPA Category I, EU Category R41, or GHS Category 1 ocular irritants (EPA 2003a)(EU 2008)(UN 2009).

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. The difference between photocell signals in the two compartments is measured electronically as a change in voltage, and is displayed digitally, generating numerical opacity values with arbitrary units.

Performance: The accuracy and reliability characteristics of a test method (see “accuracy’, “reliability”).

Positive control: A sample containing all components of a test system and treated with a substance known to induce a positive response, which is processed with the test substance-treated and other control samples to demonstrate the sensitivity of each experiment and to allow for an assessment of variability in the conduct of the assay over time.

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.

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 substances 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.

Solvent control: An untreated sample containing all components of a test system, including the solvent that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same solvent. 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 substances 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.

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.

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.

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

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."

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.

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.

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

Weight of evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

ANNEX II

Formulations (Bancroft and Cook 1994)

Fixatives

10% Neutral Buffered Formalin (NBF)

Formaldehyde, (37-40%)	10 mL
Distilled water	90 mL
Sodium dihydrogen phosphate (NaH_2PO_4)	0.35 g
Disodium hydrogen phosphate (Na_2HPO_4) (anhydrous)	0.65 g

The pH should be 7.2 to 7.4,

Paraformaldehyde

Dissolve paraformaldehyde in distilled water to make a 4% solution by heating in a fume hood, stirring constantly until only a faint cloudiness persists. Clarify this solution by adding 10N NaOH drop by drop until the solution is clear. Cool to room temperature and then to 4°C in a refrigerator. Filter after cooling to 4°C. If the solution is being used only for subsequent paraffin sections, adding sodium pyrophosphate can increase the longevity of the 4% paraformaldehyde.

6% Glutaraldehyde

Glutaraldehyde (25%)	24 mL
0.1 M Phosphate buffer, pH 7.4	76 mL

On storage, the glutaraldehyde will become acidic, pH 2.5-3.0. The final pH of the above fixative should be checked and adjusted to 7.0-7.2 if necessary with sodium hydroxide.

Davidson's (Spencer and Bancroft 2008)

Formalin (37-40%)	40 mL
Alcohol	60 mL
Glacial acetic acid	20 mL
Tap water	60 mL

Mix before using, immerse for less than 24 hr and transfer to 70% alcohol prior to embedding.

Stains

Mayer's Hematoxylin (Bancroft and Cook 1994)

Hematoxylin	1 g
Distilled water	1000 mL
Potassium or ammonium alum (hydrated aluminium [ammonium or potassium] sulphate)	50 g
Sodium iodate	0.20 g
Citric acid	1 g
Chloral hydrate (Standard Laboratory Reagent)	50 g
OR Chloral hydrate (Analytical Reagent)	30 g

Dissolve hematoxylin, potassium or ammonium alum (alum accurately weighed to ± 0.01 g), and sodium iodate in distilled water by warming and stirring or allow the solution to stand at room temperature overnight. Add chloral hydrate and citric acid. If chloral hydrate (Analytical Reagent grade) is used, the amount may be reduced, as indicated above. Gently boil the mixture for 5 minutes, then cool and filter. The stain is then ready for immediate use, but should be stored in an amber bottle and always filtered prior to use.

Harris's Hematoxylin (Bancroft and Gamble 2008)

Hematoxylin (BSC certified)	2.5 g
Absolute alcohol	25 mL
Potassium alum (hydrated aluminium potassium sulphate)	50 g
Distilled water	500 mL
Sodium iodate	0.5 g
Glacial acetic acid	20 mL

Dissolve hematoxylin in absolute alcohol. Add alum after hematoxylin has been dissolved in warm distilled water in a 2-liter flask. Then, bring the mixture to a boil; slowly and carefully add sodium iodate. The stain is rapidly cooled by immersion of the flask into cold water or a sink containing chipped ice. When the solution is cold, add acetic acid. The stain may be used immediately. The use of glacial acetic acid is optional, but provides more precise and selective staining of nuclei.

Eosin (Bancroft and Gamble 2008)

Eosin Y, referred to either as water or ethanol soluble, is typically used as the counter stain to hematoxylins for H&E staining. It is prepared as a 0.5% or 1.0% solution in distilled water with a crystal of thymol added to prevent the growth of fungi. Staining may be sharpened by the addition of acetic acid (e.g. 0.5 mL/1000 mL of eosin solution).

The basic dye hematoxylin colours basophilic structures (e.g. those containing nucleic acids) and acidic components with a blue-purple hue, while the alcohol-based acidic eosin Y, colours eosinophilic structures (those composed of intracellular or extracellular protein) or basic cell components such as cytoplasm bright pink. Thus, cell nuclei appear blue-purple, while the cytoplasm is pink (Banks 1993)(Gamble 2008).

ANNEX III

Sample Protocols

Sample Protocol #1 for Routine Hematoxylin and Eosin Staining of Paraffin-Embedded Sections (Gamble 2008)

1. De-paraffinize sections; hydrate through graded alcohols to water.
2. Remove fixation pigments (i.e. from bloody deposits or acidic formalin, but not a concern if NBF is used).
3. Stain in an alum hematoxylin of choice for a suitable time (e.g. 20-45 min for Ehrlich's)
4. Wash well in running tap water until sections 'blue' for 5 min or less.
5. Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 sec.
6. Wash well in tap water until sections are again 'blue' (10-15 min).
7. Blue by dipping in an alkaline solution (e.g. ammonia water), followed by a 5-min tap water rinse.
8. Stain in 1% eosin Y for 10 min.
9. Wash in running tap water for 1-5 min.
10. Dehydrate through alcohols, clear, and mount.

Sample Protocol #2 for Routine Hematoxylin and Eosin Staining of Paraffin-Embedded-Sections (Fischer et al. 2008)

1. Hydrate the tissue:
   - Fix a microscope slide holding rehydrated tissue sections in either alcohol or an aldehyde-based fixative.
   - Immerse the slide in water for 30 sec with gentle agitation by hand to prevent precipitation with salts or buffers.
2. Dip the slide into a Coplin jar containing Mayer's hematoxylin and agitate for 30 sec.
3. Rinse the slide in water for 1 min.
4. Stain the slide with 1% eosin Y solution for 10-30 sec with agitation.
5. Dehydrate the sections with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each.
6. Extract the alcohol with two changes of xylene to clear.
7. Add one or two drops of mounting medium and cover with a cover slip. Glycerol can be used if alcohols cannot be used.

ANNEX IV

Proposed Detailed Protocol for Future Studies Using the Bovine Corneal Opacity and Permeability (BCOP) Test Method

Please Note: *The examples of positive control substances to be used for liquid test substances and the intended purpose of the positive control in the assay have been updated and clarified (see Section 5.3 [Positive Control]). A Standard Project Submission Form (SPSF) that provides extensive scientific rationale to support these revisions has been submitted to the OECD Test Guidelines Programme. This SPSF for updating TG 437 (Bovine Corneal Opacity and Permeability [BCOP] Test Method for Identifying Ocular Corrosives and Severe Irritants) is scheduled for discussion at the OECD meeting in April 2011. The final protocol appended to this Guidance Document will be consistent with the updated TG 437 based on the outcome of the OECD review,*

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) INVITTOX 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.

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the potential ocular corrosivity or severe irritancy of a test substance 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 substance are considered individually and also combined to derive an *in vitro* irritancy score, which is used to classify the irritancy level of the test substance. Histological evaluation of the corneas can be useful for identifying damage in tissue layers that does not produce significant opacity or permeability.

The focus of this protocol is on the use of the BCOP test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA 2003a), European Union (EU; EU 2008), and United Nations Globally Harmonized System

(GHS) of Classification and Labelling of Chemicals (UN 2009). Substances other than ocular corrosives and severe irritants (e.g. substances not labelled as irritants and mild/moderate ocular irritants) have been tested using this protocol; however, the BCOP test method is not currently considered to be adequately validated for these classes of ocular irritancy as defined by EPA (2003a), EU (2008), and GHS (UN 2009).

2.0 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.

3.0 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.

3.2 Equipment and Supplies

- Corneal holders
- Dissection equipment (scissors, scalpels, forceps)
- Electric screwdriver
- Falcon tubes (50 mL)
- Incubator or water bath
- Liquinox (or equivalent)
- 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)
- 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 substances), 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.

4.0 TEST SUBSTANCE PREPARATION

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

4.1 Non-surfactant Liquid Test Substances

Liquid test substances are tested undiluted. However, if prescribed, dilutions of aqueous soluble test substances 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 Substances

Non-surfactant solid test substances 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.

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.

5.0 CONTROLS

5.1 Negative Control

When testing a liquid substance at 100%, a concurrent negative control (e.g. 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.

5.2 Solvent/Vehicle Control

When testing a diluted liquid, surfactant, or solid, 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.

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

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. 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

6.0 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]). 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.

Under conditions where contamination of the bovine eyes with yeast occurs, immersion of the eyes in HBSS containing fungizone should be evaluated.

6.2 Preparation of Corneas

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

b. 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. Store the isolated corneas in a petri dish containing HBSS until they are mounted in holders. Examine the corneas before use, and discard those with defects.

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 holders in a vertical position at 32 ± 1°C for at least 60 minutes.

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

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.

6.4 Treatment Groups

A minimum of three corneas is treated with each test substance solution or suspension. In addition, three corneas per assay are treated with the positive control and three corneas per assay are treated with the negative control. If a benchmark substance is used the day of testing, 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 substance. A closed chamber method is typically used for non-viscous to slightly viscous liquid test substances, while an open chamber method is typically used for semi-viscous and viscous liquid test substances and for neat solids.

6.5 Treatment of Corneas and Opacity Measurements

6.5.1 Closed chamber method for non-viscous to slightly viscous liquid test substances

a. Record the initial opacity readings and label each chamber with the appropriate control or test substance 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 vacuum pump).

b. Add 0.75 mL of the control or test substance (or enough test substance 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 substance 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 substance from the anterior chamber through the dosing holes and rinse the epithelium at least three times (or until no visual evidence of test substance 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 substance, document the observation in the study notebook. Refill the anterior chamber with fresh complete MEM.

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 in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.2 Open chamber method for semi-viscous and viscous liquid test substances 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 substance 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 substance (or enough test substance 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 substance 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). If after several tries the test article cannot be removed, document this in the study notebook, 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 in the study notebook. Special attention is taken to observe dissimilar opacity patterns, tissue peeling or residual test substance, etc.

6.5.3 Solid and liquid surfactant test substances

Surfactant test substances are administered following one of the previously described procedures, with one exception: Surfactant test substances are tested on the cornea as a 10% (w/v) solution or suspension prepared in an appropriate solvent/vehicle (e.g. sterile deionised water).

6.5.4 Solid non-surfactant test substances

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

- Solid test substances 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 substances 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

recorded in the workbook. 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 substance:

Liquid and surfactant test substances 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 Nafluorescein solution to the anterior chamber using a micropipettor.

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

Solid nonsurfactant test substances:

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

b. 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 OD_{490} determination.

c. After completing the Na-fluorescein penetration steps, the corneas should 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).

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 OD_{490}. Any OD_{490} value (of a control or test substance sample) that is 1.500 or greater must be diluted to bring the OD_{490} 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 OD_{490} values. Use the values from this second reading in all calculations. The OD_{490} values of less than 1.500 will be used in the permeability calculation.

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.

e. If using a UV/VIS spectrophotometer to measure optical density, adjust the spectrophotometer to read at OD_{490}, 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 OD_{490} beyond the linear range of the spectrophotometer must be diluted in complete MEM, and another reading taken, repeating these steps until the OD_{490} 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 Nafluorescein 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 absorbance.

6.8 Histopathology

A histopathological evaluation of the corneal tissue might be useful when the standard BCOP endpoints (i.e. corneal opacity and permeability) produce borderline results. A standardized scoring scheme using the formal language of pathology to describe any effects should be used.

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 in a solution of warm tap water and a dime-size or greater amount of Liquinox (or equivalent).

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.

7.0 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 substance.

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

<u>Microplate Reader Method</u>

a. Calculate the mean $OD_{490}$for the blank wells (plate blanks). Subtract the mean blank OD_{490} from the raw OD_{490} of each well (blank corrected OD_{490}).

b. If a dilution has been performed, correct the OD_{490} for the plate blank before the dilution factor is applied to the reading. Multiply each blank corrected OD_{490} by the dilution factor (e.g. a factor of 5 for a 1:5 dilution).

c. Calculate the final corrected OD_{490} value for each cornea by subtracting the mean OD_{490} value for the negative control corneas from the OD_{490} value of each treated cornea.

   Final Corrected OD_{490} = (raw OD_{490} – mean blank OD_{490}) -mean blank corrected negative control OD_{490}

d. Calculate the mean OD_{490} value for each treatment group by averaging the final corrected OD_{490} values of the treated corneas for a particular treatment group.

<u>UV/VIS Spectrophotometer Method</u>

a. Calculate the corrected OD_{490} value of each treated, positive control, or solvent/vehicle control cornea by subtracting the average value of the negative control corneas from the original OD_{490} value for each cornea.

   Final Corrected OD_{490} = raw OD_{490} -mean blank corrected negative control OD_{490}

b. Calculate the mean OD_{490} value for each treatment group by averaging the final corrected OD_{490} values of the treated corneas for a particular treatment group.

7.3 *In Vitro* Irritancy Score

Use the mean opacity and mean permeability values (OD_{490}) for each treatment group to calculate an *in vitro* irritancy score for each treatment group:

In Vitro Irritancy Score = mean opacity value + (15 x mean OD_{490} value)

Additionally, the opacity and permeability values should be evaluated independently to determine whether a test substance induced corrosivity or severe irritation through only one of the two endpoints.

8.0 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.

9.0 DATA INTERPRETATION

The following classification system was established by Sina et al. (1995) based on studies with pharmaceutical intermediates exposed for 10 minutes (liquids) or 4 hours (solids).

In Vitro Irritancy Score: 55.1 and above = severe irritant

While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of substances. For example, the Sina et al. (1995) scoring scale is not appropriate for anionic and nonionic surfactants since they produce appreciable permeability while inducing little direct opacity.

Benchmark substances are recommended for assaying the responses of test substances of different product or chemical classes. Histological evaluation of the corneas may be instrumental in identifying additional changes (e.g. peroxide-induced stromal damage).

10.0 STUDY REPORT

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

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known;
- The CAS Registry Number (RN), if known;
- Purity and composition of the substance or preparation (in percentage[s] by weight), to the extent this information is available;
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study;
- Treatment of the test/control substances prior to testing, if applicable (*e.g.* warming, grinding);
- Stability, if known.

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility, and study director;
- Identification of the source of the eyes (*i.e.* 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, transport media and temperature conditions, any antibiotics used);
- If available, specific characteristics of the animals from which the eyes were collected (*e.g.* age, sex, strain, weight of the donor animal).

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (*i.e.* accuracy and reliability) of the test method over time (*e.g.* periodic testing of proficiency substances, use of historical negative and positive control data).

Criteria for an Acceptable Test

- Acceptable concurrent positive and negative control ranges based on historical data;
- If applicable, acceptable concurrent benchmark control ranges based on historical data.

Test Conditions

- Description of test system used;
- Type of corneal holder used;
- Calibration information for devices used for measuring opacity and permeability (*e.g.* opacitometer and spectrophotometer);
- Information on the bovine corneas used, including statements regarding their quality;
- Details of test procedure used;
- Test substance concentration(s) used;
- Description of any modifications of the test procedure;
- Reference to historical data of the model (*e.g.* negative and positive controls, proficiency substances, benchmark substances);
- Description of evaluation criteria used.

Results

- Tabulation of data from individual test samples (*e.g.* opacity and OD_{490} values and calculated *in vitro* irritancy score for the test substance and the positive, negative, and benchmark controls [if included], reported in tabular form, including data from replicate repeat experiments as appropriate, and means ± the standard deviation for each experiment);
- Description of other effects observed.

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the study director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (*e.g.* OECD 1998; EPA 2003b, 2003c; FDA 2003) should be followed.

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ANNEX V

Detailed Protocol for Future Studies Using the Isolated Chicken Eye (ICE) 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 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. The protocol is based on information obtained from published protocols, as well as the current protocol used by TNO Quality of Life Department of Toxicology and Applied Pharmacology (Prinsen and Koeter 1993; INVITTOX 1994; Balls et al. 1995; Prinsen 1996; Chamberlain et al. 1997). Future studies using the ICE test method could include further characterization of the usefulness or limitations of the ICE 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.

1.0 PURPOSE AND APPLICABILITY

The purpose of this protocol is to describe the procedures used to evaluate the potential ocular irritancy of a test substance as measured by its ability to induce toxicity in an enucleated chicken eye. Toxic effects are measured by (1) qualitative assessment of corneal opacity; (2) qualitative measurement of increased retention of fluorescein dye within the eye (permeability); (3) quantitative measurement of increased corneal thickness (swelling); and (4) qualitative evaluation of macroscopic morphological damage to the corneal surface. The opacity, swelling, and permeability assessments following exposure to a test article are assessed individually and then combined to derive an Eye Irritancy Classification.

The focus of this protocol is on the use of the ICE test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA 2003a), European Union (EU; EU 2008), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2009). Substances other than ocular corrosives and severe irritants (*e.g.* substances not labelled as irritants and mild/moderate ocular irritants) have been tested using this protocol; however, the ICE test method is not currently considered to be adequately validated for these classes of ocular irritancy as defined by EPA (2003a), EU (2008), and GHS (UN 2009).

2.0 SAFETY AND OPERATING PRECAUTIONS

All procedures with chicken eyes should follow the institution's applicable regulations and procedures for handling of human or animal materials, 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.

3.0 MATERIALS, EQUIPMENT, AND SUPPLIES

3.1 Source of Chicken Eyes

Although a controlled study to evaluate the optimum chicken age has not been conducted, the age and weight of the chickens used historically in this test method are that of spring chickens traditionally obtained from a local source (*e.g.* poultry abattoir), approximately 7 weeks old, male or female, with a weight range of 1.5–2.5 kg (breed not specified).

3.2 Equipment and Supplies

- Custom superfusion apparatus (that will accommodate the eye holders) with a water pump for temperature control
- Dissection equipment (*e.g.* scissors and forceps)
- Electronic balance
- Eye holders (custom stainless steel clamps)
- Micropipettor and pipette tips
- Mortar and pestle
- Physiological saline
- Slit-lamp microscope with an optical pachymeter equipped with centering lights
- Tissue paper
- Transportation chambers (humidified plastic boxes containing tissues moistened with isotonic saline or water)
- Peristaltic pump for the saline drip onto the eye

3.3 Solutions

The manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions should be followed.

- Fluorescein sodium BP, 2% w/v (also available commercially)
- Isotonic saline (*i.e.* 0.9% NaCl)
- 4% neutral buffered formaldehyde

TEST SUBSTANCE PREPARATION

4.1 Liquid Test Substances

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

4.2 Solid Test Substances

Prior to testing, solid, particulate or granular test substances should be ground as finely as possible in a mortar and pestle, or comparable grinding tool.

CONTROLS

5.1 Negative Controls

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

5.2 Solvent/Vehicle Controls

When testing diluted liquids, a concurrent solvent/vehicle control is included in the test method to detect non-specific changes in the test system, and to ensure that the assay conditions do not inappropriately result in an irritant response.

5.3 Positive Controls

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

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

5.4 Benchmark Controls

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. Appropriate benchmark controls should have the following properties:

- A consistent and reliable source(s) for the chemical
- 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

EXPERIMENTAL DESIGN

6.1 Collection and Transport Conditions of Chicken Eyes

Heads should be removed immediately after sedation of the chickens, usually by electric shock, and incision of the neck for bleeding. Heads of spring chickens should be obtained from a local source (*e.g.* poultry abattoir). The time interval between collection of the chicken heads and use of eyes in the ICE test method should be minimized (typically within two hours) and should be demonstrated to not compromise the assay results.

Because eyes are dissected in the laboratory, the intact heads are transported from the abattoir at ambient temperature in plastic boxes humidified with towels moistened with isotonic saline.

6.2 Preparation of Eyes

a. Carefully remove the eyelids without damaging the cornea. Place a drop of sodium fluorescein 2% w/v onto the corneal surface for 10-20 seconds, and then immediately rinse the eye with 20 mL isotonic saline. Examine the fluorescein-treated cornea with a slit-lamp microscope to ensure that the cornea is undamaged (*i.e.* fluorescein retention and corneal opacity scores ≤ 0.5).

b. If undamaged, further dissect the eye from the eye socket, taking care not to damage the corneal epithelium. When removing the eye from the orbit, a visible portion of the optic nerve should be left attached to the eye.

c. Once removed from the orbit, place the eye on an absorbent pad and cut away the nictitating membrane and other connective tissue.

d. Mount the eyes in stainless steel clamps (one eye per clamp), with the cornea positioned vertically and then transfer each clamp to a chamber in the superfusion apparatus. The chambers of the superfusion apparatus should be temperature controlled at 32 ± 1.5°C with a water pump. Position the clamp in the superfusion apparatus such that the entire cornea is supplied with isotonic saline from a bent stainless steel tube at a rate of 0.10-0.15 mL/minute via a peristaltic pump.

e. After being placed in the superfusion apparatus, the eyes are again examined with a slit-lamp microscope to ensure that they have not been damaged during the dissection procedure. Corneal thickness should also be measured at this time at the corneal apex using the depth-measuring device on the slit-lamp microscope. Eyes with (i), a fluorescein retention score of > 0.5; (ii) corneal opacity > 0.5; or, (iii), any additional signs of damage should be replaced. For eyes that are not rejected based on any of these criteria, individual eyes with a corneal thickness deviating more than 10% from the mean value for all eyes are to be rejected. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different. The slit-width should be set at 9½, equaling 0.095 mm.

f. Once all eyes have been examined and approved, incubate eyes at 32 ± 1.5 °C for 45-60 minutes to equilibrate them to the test system prior to dosing.

6.3 Treatment Groups

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

6.4 Treatment of Eyes and Observations

6.4.1 Dosing procedure

a. After the equilibration period, record a zero reference measurement for corneal thickness and corneal opacity to serve as a baseline (*i.e.* time = 0). The fluorescein retention score determined at dissection is used as the baseline measurement.

b. Immediately following the zero reference measurement, the eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test substance is applied to the cornea (see **Sections 6.4.1.1** and **6.4.1.2**).

c. Apply the test material for a total of 10 seconds and then rinse the eye with 20 mL isotonic saline at room temperature.

d. After the rinse step, return the eye to the superfusion apparatus.

6.4.1.1 Liquid test substances

Apply a liquid test substance at 0.03 mL with a micropipettor such that the entire surface of the cornea is evenly covered with the test substance.

6.4.1.2 Solid test materials

If possible, grind solid test substances as finely as possible with a mortar and pestle, or comparable grinding tool. Apply 0.03 g of a solid test substance such that the entire surface of the cornea is evenly covered with the test substance.

6.4.2 Endpoint observations

a. Examine the control and test eyes at 30, 75, 120, 180, and 240 minutes (± 5 minutes) after the post-treatment rinse using the scoring system and criteria as indicated in **Section 9.0**.

b. Corneal opacity, corneal thickness, and any morphological effects should be evaluated at each time point, while fluorescein retention is determined only at the 30-minute time point.

c. After the final (240 minutes) examination, immerse all eyes in an appropriate fixative (*e.g.* neutral buffered formalin) for possible histopathological examination (if necessary).

d. To maximize the likelihood of obtaining reproducible results, reference photographs for all subjective endpoints (*i.e.* corneal opacity, fluorescein retention, morphological effects, histopathology) should be readily available.

7.0 EVALUATION OF TEST RESULTS

Results from the three test method endpoints, corneal opacity, corneal swelling, and fluorescein retention should be evaluated separately to generate an ICE class for each endpoint (see **Section 9.0**). The ICE classes for each endpoint are then combined to generate an Irritancy Classification for each test substance (see **Section 10.0**).

8.0 CRITERIA FOR AN ACCEPTABLE TEST

A test is considered acceptable if the concurrent negative or vehicle/solvent controls and positive controls give an Irritancy Classification that falls within non-irritating and severe irritant/corrosive classes, respectively.

9.0 DATA INTERPRETATION

9.1 Corneal Thickness

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:

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

The mean percentage of 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 overall category score is then given for each test substance.

Mean Corneal Swelling (%)	**Category**
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

9.2 Corneal Opacity

Corneal opacity is calculated by using the area of the cornea that is most densely opacified for scoring.

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

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 overall category score is then given for each test substance.

Mean Maximum Opacity Score	**Category**
0.0–0.5	I
0.6–1.5	II
1.6–2.5	III
2.6–4.0	IV

9.3 Fluorescein Retention

Fluorescein retention is evaluated at the 30minute observation time point only. When test substances have adhered to the cornea, fluorescein retention can be determined whenever the test substance has sufficiently loosened. The following scale is used for scoring:

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

The mean fluorescein retention score for all test eyes is calculated and an overall category score is then given for each test substance.

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

Morphological effects include “pitting” of corneal epithelial cells, “loosening” of epithelium, “roughening” of the corneal surface and “sticking” of the test substance 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.

A histopathological evaluation of the corneal tissue should be included when the standard ICE endpoints (i.e. corneal opacity, swelling, and fluorescein retention) produce borderline results. A standardized scoring scheme using the formal language of pathology to describe any effects should be included.

10.0 ASSESSMENT OF THE EYE IRRITANCY

The overall *in vitro* irritancy classification for a test substance is assessed by reading the irritancy classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention, as presented in the scheme below.

Classification	Combinations of the Three Endpoints
Corrosive/Severe Irritant	3 x IV
	2 x IV, 1 x III
	2 x IV, I x II*
	2 x IV, I 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)
* Combinations less likely to occur.	

11.0 STUDY REPORT

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

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known;
- The CAS Registry Number (RN), if known;
- Purity and composition of the substance or preparation (in percentage[s] by weight), to the extent this information is available;

- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study;
- Treatment of the test/control substances prior to testing, if applicable (*e.g.* warming, grinding);
- Stability, if known.

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility, and study director;
- Identification of the source of the eyes (*i.e.* 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).

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e. accuracy and reliability) of the test method over time (*e.g.* periodic testing of proficiency substances, use of historical negative and positive control data).

Criteria for an Acceptable Test

- If applicable, acceptable concurrent benchmark control ranges based on historical data.

Test Conditions

- Description of test system used:
- Slit-lamp microscope used (*e.g.* model);
- Instrument settings for the slit-lamp microscope used:
- Information for the chicken eyes used, including statements regarding their quality;
- Details of test procedure used;
- Test concentration(s) used;
- Description of any modifications of the test procedure;
- Reference to historical data of the model (*e.g.* negative and positive controls, proficiency substances, benchmark substances);
- Description of evaluation criteria used.

Results

- Description of other effects observed;
- If appropriate, photograph of the eye.

Discussion of the Results

Conclusion

A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

- This statement indicates all inspections made during the study, and the dates any results were reported to the study director. This statement also serves to confirm that the final report reflects the raw data.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g. OECD 1998; EPA 2003b, 2003c; FDA 2003) should be followed.

12.0 REFERENCES

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