

Appendix B

ICCVAM-Recommended Test Method Protocols

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Appendix B1

**ICCVAM-Recommended Bovine Corneal Opacity and Permeability (BCOP) Test
Method Protocol**

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ICCVAM-Recommended Protocol for Future Studies Using the Bovine Corneal Opacity and Permeability (BCOP) Test Method

PREFACE

This proposed protocol for measuring corneal damage was developed following a comprehensive test method evaluation process conducted by ICCVAM, which included an international independent scientific peer review of the validation status and scientific validity of the BCOP (ICCVAM 2006a,b). It is based primarily on information obtained from 1) the Institute for In Vitro Sciences, Inc. (IIVS), a nonprofit foundation that has performed the BCOP assay 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 assay conducted in 1997-1998. Both of these protocols are based on the BCOP assay methodology 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 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 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). Substances other than ocular corrosives and severe irritants (e.g., substances not labeled 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 (2001), and GHS (UN 2007).

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 holders¹
- 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 deionized water
- Syringes (10 mL) and blunt tip needles (19 Gauge)
- Vacuum pump
- Volumetric flasks
- 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

¹ Users should be aware of a proposed corneal holder developed by Ubels et al. (2002). The ICCVAM Test Method Evaluation Report (2006b) recommends, “Studies should be conducted to evaluate the impact of using a corneal holder that maintains normal curvature (e.g., the corneal mounting system designed by Ubels et al. 2002) on accuracy and/or reliability of the BCOP test method.”

3.4 Solutions

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

- 0.9% (w/v) NaCl in sterile deionized 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 containing 1% (v/v) Fetal Bovine Serum (complete MEM), warmed to 32°C
- Eagle's Minimum Essential Medium with phenol red containing 1% Fetal Bovine Serum (complete MEM 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

All test substance solutions should be prepared fresh on the day of use.

4.1 Nonsurfactant Liquid Test Substances

Liquid test substances are usually tested undiluted. However, if prescribed, dilutions of aqueous soluble test substances should be prepared in 0.9% sodium chloride.

4.2 Nonsurfactant Solid Test Substances

Nonsurfactant solid test substances should be prepared as 20% (w/v) solutions or suspensions in 0.9% sodium chloride.

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.

4.4 Surfactant Preparations

Surfactant-based preparations (e.g., product formulations) are usually tested neat, or can be diluted in 0.9% sodium chloride, 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 known ocular irritant is included as a concurrent positive control in each experiment to verify that an appropriate response is induced. As the BCOP assay is being used to identify corrosive or severe irritants, ideally 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 irritant response should not be excessive.

Examples of positive controls for liquid test substances are 10% sodium hydroxide or dimethylformamide. An example of a positive control for solid test substances is 20% (weight to volume) 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 the *in vivo* rabbit eye test
- 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 cattle abattoir, where the eyes are excised as soon as possible after sacrifice. Care should be taken to avoid damaging the cornea during the enucleation procedure. Eyes are collected in a suitable container in which they are immersed in HBSS containing the antibiotics penicillin (100 IU/mL) and streptomycin (100 µg/mL). The container is maintained on ice at all times throughout collection of the eyes and transportation to the testing facility (NOTE: antibiotics may not be necessary if the eyes are kept below 4°C throughout transport). The eyes are used within five hours of sacrifice.

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 $32 \pm 1^\circ\text{C}$ 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. Calculate the average opacity value for all corneas.
- d. Select a minimum of three corneas with opacity values close to the average value for all corneas as negative (or solvent/vehicle) control corneas.
- e. Corneas that display an initial opacity reading significantly greater (+ 2 standard deviations [SDs]) than the average opacity for all corneas in the batch of eyes collected the day of testing should not be used in the assay.

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 nonsurfactant solid) of the test substance. The controls used depend on which method is used.

6.5 Treatment of Corneas and Opacity Measurements

6.5.1 Closed chamber method for nonviscous 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 to the anterior chamber through the dosing holes using a micropipettor. 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 $32 \pm 1^\circ\text{C}$ for 10 ± 1 minutes. If other exposures 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 with approximately 2 to 3 mL of fresh complete MEM with phenol red. Perform one last rinse of the epithelium using fresh complete MEM. 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 $32 \pm 1^\circ\text{C}$ 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 semiviscous 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 micropipettor 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 $32 \pm 1^\circ\text{C}$ 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 in the exposure group 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. 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 $32 \pm 1^\circ\text{C}$ 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, which is noted below:

- 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 deionized water).

6.5.4 Solid nonsurfactant test substances

Solid nonsurfactant 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 deionized water).
- Solid test substances are incubated at $32 \pm 1^\circ\text{C}$ 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:

- a. Liquid and surfactant test substances and surfactant preparations: Remove the medium from both chambers (anterior chamber first). Fill the posterior chamber with fresh complete MEM, and add 1 mL of a 4 mg/mL Na-fluorescein solution to the anterior chamber using a micropipettor. Reseal the dosing holes in the top of both chambers with the chamber plugs.
- b. Solid nonsurfactant test substances: Remove the medium from the anterior chamber only and replace with 1 mL of a 5 mg/mL Na-fluorescein solution. 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 $32 \pm 1^\circ\text{C}$.
- b. After the 90-minute incubation period, remove the medium in the posterior chamber of each holder and place into sample tubes pre-labeled 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.
- e. 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.
- f. 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 assay, 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 assay. 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 assay. Transfer an aliquot of the mixed medium from the posterior chamber of the BCOP holder into a cuvette, then take and record an absorbance reading using the spectrophotometer. Any solutions giving an OD₄₉₀ beyond the linear range of the spectrophotometer must be diluted in complete MEM, and another reading taken, repeating these steps until the OD₄₉₀ is within the linear range of the spectrophotometer. Repeat these procedures for each sample from the BCOP assay, rinsing the cuvette(s) thoroughly between each sample, until all samples have been read and results recorded.

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

Microplate Reader Method

- a. Calculate the mean OD₄₉₀ for the blank wells (plate blanks). Subtract the mean blank OD₄₉₀ from the raw OD₄₉₀ of each well (blank corrected OD₄₉₀).
- b. If a dilution has been performed, correct the OD₄₉₀ for the plate blank before the dilution factor is applied to the reading. Multiply each blank corrected OD₄₉₀ by the dilution factor (e.g., a factor of 5 for a 1:5 dilution).
- c. Calculate the final corrected OD₄₉₀ value for each cornea by subtracting the mean OD₄₉₀ value for the negative control corneas from the OD₄₉₀ value of each treated cornea.
- d. **Final Corrected OD₄₉₀** = (raw OD₄₉₀ – mean blank OD₄₉₀) – mean blank corrected negative control OD₄₉₀
- e. Calculate the mean OD₄₉₀ value for each treatment group by averaging the final corrected OD₄₉₀ values of the treated corneas for a particular treatment group.

UV/VIS Spectrophotometer Method

- a. Calculate the corrected OD₄₉₀ value of each treated, positive control, or solvent/vehicle control cornea by subtracting the average value of the negative control corneas from the original OD₄₉₀ value for each cornea.
Final Corrected OD₄₉₀ = raw OD₄₉₀ - mean blank corrected negative control OD₄₉₀
- b. Calculate the mean OD₄₉₀ value for each treatment group by averaging the final corrected OD₄₉₀ values of the treated corneas for a particular treatment group.

7.3 *In Vitro* Irritancy Score

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

$$\text{In Vitro Irritancy Score} = \text{mean opacity value} + (15 \times \text{mean OD}_{490} \text{ value})$$

Additionally, the opacity and permeability values should be evaluated independently to determine whether a test substance induced 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. In the BCOP, 100% ethanol induces a moderate to severe response (*in vitro* score = 39.9 -65.4 at IIVS [n = 632]; mean = 52.7, standard deviation [SD] = 6.4), while 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 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.

For these and other substances that produce significant permeability with minimal opacity, it is recommended that permeability values > 0.600 be considered severe. 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 OD₄₉₀ 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 \pm 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.

11.0 References

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Appendix B2

ICCVAM-Recommended Cytosensor Microphysiometer (CM) Test Method Protocol

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ICCVAM-Recommended Protocol for Future Studies Using the Cytosensor Microphysiometer (CM) Test Method

PREFACE

This proposed protocol for ocular toxicity is based primarily on information obtained in INVITTOX Protocol 102 derived from the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. The information contained within INVITTOX 102 was modified based upon the COLIPA protocol (Brantom et al., 1997; Harbell et al., 1999). Future studies using the CM test method could include further characterization of the usefulness and limitations of the CM 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 study is to compare the ocular toxicity of the test material as predicted using the CM method with historical rabbit Draize eye data. The CM method evaluates the potential ocular toxicity by measuring the test material induced reduction in the metabolic rate in treated cultures of L929 cells. Change in metabolic rate is measured indirectly as a function of changes in extracellular acidification rate. The dose that induces a 50% decrease in metabolic rate is the end point of the assay.

The focus of this protocol is on the use of the CM test method for the detection of ocular corrosives and severe irritants and substances not labeled as irritants as defined by the U.S. Environmental Protection Agency (EPA; EPA, 2003a), the European Union (EU; EU, 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). Mild/moderate ocular irritants have been tested using this protocol; however, the CM test method is not currently considered to be adequately validated for these classes of ocular irritancy as defined by EPA (2003a), EU (2001), and GHS (UN 2007).

2.0 Safety and Operating Precautions

All procedures with L929 cells should follow the institution's applicable regulations and procedures for handling human or 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 Equipment and Supplies

- Aspirator
- Balance
- Beakers, disposable
- Capsules, eight with L-929 cells grown to be <80% confluent at time of use (confluent monolayer could interfere with accurate CM readings) in DMEM. To prepare these, load $5-6 \times 10^5$ cells about 18 hr prior to use and incubate in complete DMEM with 1% calf serum under standard culture conditions.

- Cell culture equipment for preparation of cells
- Cytosensor System with eight sterilized chambers, set up in the injection loop mode - Molecular Devices Corporation, Menlo Park, California, USA
- Cytosoft and the following Cytosoft protocols for toxicity testing:
 - Tox Maintenance (ii) Routine Tox 003 (4x2) (both supplied by MDC)
 - A statistics program capable of MRD_{50}
- Pipettors, rack, etc., for preparation of dilutions
- Refrigerator
- Statistical program for calculation of MRD_{50}
- Tubes, 15 ml, for preparation of dilutions (4 dilutions per test sample).
- Tube racks
- Syringes, 4 x 5 ml and a 30 ml
- Water bath

3.2 Media and Reagents

- Assay Medium: DMEM complete with 1% Fetal Bovine Serum, 5.0 $\mu\text{g/ml}$ gentamicin, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate
- Growth Medium: Dulbecco's modified Eagle's medium (DMEM) (1mg/ml glucose) complete with 10% Fetal Bovine Serum, 2.0 mM L-glutamine, and 1.0 mM sodium pyruvate
- Positive Control: sodium lauryl sulfate (SLS) 10% in water (stock)
- Treatment medium: Serum-free, Sodium Bicarbonate-free, DMEM with 5.0 $\mu\text{g/ml}$ gentamicin, 2.0 mM L-glutamine, and additional NaCl for consistent osmolarity (MDMEM). 11.1 ml of 4 M NaCl is required per liter
- Trypsin, 0.05% in Ca^{+2} and Mg^{+2} -free Hank's Balanced Salt Solution

4.0 Test Substance Preparation

- The test article will be dissolved in MDMEM. It is essential that the test material be in a single-phase solution in the highest dose used (300 mg/mL) to prepare the subsequent dilutions. If the substance cannot form a single phase solution/suspension at a concentration of 33.3 mg/mL, the test sample cannot be tested by the CM using standard techniques
- The stability of the test article under the actual experimental conditions will not be determined by the testing laboratory

5.0 Controls

5.1 Negative Control

The baseline acidification rate will serve as the internal negative control for each cell culture. Baseline rates will fall between 50 and 150 microvolts/sec after a stabilization period of at least 15 minutes. Replace the cell-containing insert in a chamber that fails to achieve these ranges.

5.2 Solvent/Vehicle Control

Untreated controls are recommended when solvents/vehicles other than 0.9% sodium chloride or distilled water are used to dissolve test substances, in order to demonstrate that the solvent/vehicle is not interfering with the test system.

5.3 Positive Control

When the 8-channel Cytosensor is used, a positive control assay will be performed with each definitive trial of the assay. When the 4-channel machine is used, a concurrent positive control trial will be performed with at least one of the definitive trials for each test material. The positive control substance is SLS prepared from a 10% stock in water.

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 the *in vivo* rabbit eye test
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Filling the Workstations with Medium

Put 8 x 50 ml tubes, each having at least 20 ml of MDMEM on the Cytosensor and fill the injection loops with MDMEM, using a 30 ml syringe. Using the "Front Panel" controls, set the flow rate to 90-100% to fill the lines, and then set the flow rate back to idle (5%).

6.2 Checking Out the Equipment

Empty Sterilant from the sensor chambers, wash them by repeated filling with, and aspiration of, distilled water, and then add about 2 ml of low-buffer DMEM to each chamber. Put them on the Cytosensor. Set flow rate to High (90-100% of max) and clear obvious bubbles. Run Cytosoft default protocol ("New") to see that system sets up and the background rate in the absence of cells settles within 10 minutes to between +5 and -5 microvolts/sec. This gives the opportunity to attend to any equipment problems before starting to use cells.

6.3 Checking Out the Cells

Exit "New" protocol and set flow rate to Normal (approx. 50%) using "Front Panel" controls. To at least 8 cell-containing cell capsules in a culture tray containing Low-Buffered DMEM, add spacers and inserts as described in the Manual. Move the tray to the Cytosensor and use forceps to transfer the completed capsules to the sensor chambers, lifting the gantries and raising the plungers one set at a time. When all the capsules are in place, set the flow rate to High and clear obvious bubbles again.

6.4 Cell Culture Maintenance and Preparation of the Capsule Cups

Stock cultures of L929 cells will be maintained and passaged in Growth Medium and incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in air. L929 cells will be seeded onto capsule cups at approximately 6.0×10^5 cells per capsule cup in Seeding Medium as described below.

Flasks of L929 cells to be passaged or seeded are selected at or near confluency. The size of flasks used will depend on the number of cells needed. The Growth Medium is decanted and the cell sheet washed twice with approximately 10 mL of PBS for each 75cm^2 of growth surface. The cells are trypsinized with approximately 3 mL of 0.05% trypsin (for each 75cm^2 of growth surface) for 15 to 30 seconds. The trypsin solution is aspirated and the cells are incubated at room temperature for

approximately 2 to 5 minutes, until the cells begin to round. The cells are dislodged by tapping the flask, which contains approximately 5mL of Seeding Medium for each 75cm² of growth surface. The cells are triturated using a pipet in order to break up clumps and are transferred by pipet to a conical centrifuge tube. If more than one flask is used, the contents of each are pooled. Cell counts are performed as required. The L929 cells will be seeded with approximately 6.0 x 10⁵ cells per each capsule cup (0.5 mL of a 1.2 x 10⁶ cell suspension) with 1.5 mL of Seeding Medium added to each outside well. The plate will be incubated at 37 ± 1°C and 5 ± 1% CO₂ in air for 16 to 32 hours. Prior to the start of the assay, the medium in capsule cups will be switched to Low-Buffered DMEM and a spacer will be added to each capsule cup and gently tapped down to the bottom. The cell capsules will be placed into the sensor chambers and exposed to Low-Buffered DMEM at 37 ± 1°C.

For routine passaging, the stock cultures are trypsinized as described above, but are dislodged and resuspended using warm (approximately 37°C) Growth Medium, seeded into a culture flask(s), and returned to the humidified incubator maintained at 37 ± 1°C and 5 ± 1% CO₂ in air.

6.5 Dose Range Finding Assay

A dose range finding assay will be performed to establish an appropriate test article dose range for the definitive CM assay. Dosing solutions will be prepared by serial three-fold dilutions (producing the same concentrations suggested in the following table) in sterile, Low-Buffered DMEM that has been allowed to equilibrate to room temperature.

IMPORTANT: Do not attempt to use preparations that separate into more than one phase in the Cytosensor. Similarly, do not attempt to use such preparations to make dilutions. At the discretion of the Study Director, a suspension that maintains a single phase may be assayed and used to prepare further dilutions.

If the sample does not go into a single phase with the medium at 10.0 mg/mL (maintaining a ratio of 100 mg/10 mL), prepare dilutions 2 or 3 as required. If a single-phase test article/medium mixture is not achieved, the Study Director and Sponsor are to be consulted.

DILUTION #	CONCENTRATION
1	10 mg/mL
2	3.33 mg/mL
3	1.11 mg/mL
4	0.370 mg/mL
5	0.123 mg/mL
6	0.0412 mg/mL
7	0.0137 mg/mL

The test article will be evaluated by exposure to L929 cells contained in sensor chambers. After the baseline data points have been taken, the exposure cycle will begin with the lowest test article concentration. From these baseline data points, the spreadsheet will compute the mean baseline value used in the MRD₅₀ calculation. Each exposure cycle will take 20 minutes.

The maximum solvent concentration (other than Low-Buffered DMEM) will be 10% unless otherwise specified.

There will be three phases in the exposure cycle, with the following parameters selected within the CM software (Cytosoft): First, a test article concentration will be introduced into the sensor chamber for 13 minutes and 30 seconds. The nominal rate of flow will be 100 μ L per minute for the first minute, and 20 μ L per minute for the next 12 minutes and 30 seconds. The second phase will be the washout phase, which will be six minutes at a nominal rate of 100 μ L per minute. The test article will be washed out of the sensor chamber during this phase. Finally, the third phase will be the measurement of the acidification rate. For 25 seconds, there will be no flow and the rate of pH change will be measured.

The exposure cycle will repeat with increasing test article concentrations until either the highest test article concentration is reached or until the MRD₅₀ value has been surpassed. Each test article concentration will be tested on a single set of cells. Positive control materials and solvent controls (for solvents other than Low-Buffered DMEM) will be tested in the same fashion. If possible, an MRD₅₀ value will be calculated from the dose range finding assay.

The test article doses for the definitive assay will be chosen so that generally seven doses (spaced as three-fold dilutions) will be available for the determination of the MRD₅₀. Generally, three concentrations will be chosen to result in expected survivals lower than 50%, one concentration will be chosen to result in an expected survival of approximately 50%, and three or more concentrations will be chosen to result in expected survivals greater than 50%. If a test article fails to cause 50% toxicity in the dose range finding CM assay, the maximum dose will generally be 270 mg/mL, or less based on its solubility/workability.

6.6 Definitive Assay

The definitive assay will be performed in the same manner as the dose range finding assay, with the exception that if the MRD₅₀ value from the dose range finding assay is > 10 mg/mL, higher doses of test article will be prepared and tested in the definitive assay. At least seven doses, spaced at three-fold dilution intervals, up to a maximum of 270 mg/mL will be prepared. The determination of the final MRD₅₀ will be based upon the results of at least two definitive assays and will generally also include the results of the dose range finding assay, if an MRD₅₀ could be determined. The results from additional definitive assays may also be incorporated into the calculation of the final MRD₅₀.

7.0 Evaluation of Test Results

The acidification rates that occur after exposure to each test article concentration are calculated by the CM software (Cytosoft) and compared to the mean acidification rate (base acidification rate) of the same cells prior to exposure to a test material. The percent of control acidification rate will be determined by comparing the dose response acidification rate to the base acidification rate. The dose response curve will be plotted with the percent of control acidification rates on the ordinate and the test article concentration on the abscissa. The concentration of the test material that results in a fifty percent reduction in acidification is interpolated from the curve and referred to as the MRD₅₀. These calculations can be performed using the Excel spreadsheet program provided for this study.

8.0 Criteria for an Acceptable Test

Assay acceptance criteria are normally based on the performance of the positive control. The CM assay would be accepted if the positive control MRD₅₀ fell within 2 standard deviations of the historical range. The acceptable range for SLS will be provided by the lead laboratory. The positive control assay will not be performed with each trial on the 4-channel machine. Therefore, acceptance of those trials, lacking a positive control, will be based on judgment of the study director.

9.0 Data Interpretation

Interpretation of MRD₅₀ values is done according to the decision criteria provided in Background Review Document: Existing Methods for Eye Irritation Testing: Silicon Microphysiometer and Cytosensor Microphysiometer (ECVAM 2008), as follows:

For the EU system (EU 2001) the proposed PM is:

	MRD ₅₀
R41	<2 mg/mL
R36	<10 mg/mL; >2 mg/mL
Not classified	>10 mg/mL

For the GHS system (UN 2007) the proposed PM is:

	MRD ₅₀
1	<2 mg/mL
2A or 2B	<10 mg/mL; >2 mg/mL
No Label	>10 mg/mL

For the EPA system (EPA 2003a) the proposed PM is:

	MRD ₅₀
I	<2 mg/mL
III	<80 mg/mL; >2 mg/mL
Not classified	>80 mg/mL

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

- Name and address of the test facility
- Name and address of the Study Director

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

Test Conditions

- Description of test system used
- Calibration information for measuring device used
- 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

- Tabulation of data from individual test samples

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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Appendix B3

ICCVAM-Recommended EpiOcular™ (EO) Test Method Protocol

ICCVAM recommends this EpiOcular™ test method protocol for nonregulatory, validation, or optimization studies to facilitate collection of consistent data and expand the available database. Exceptions and/or changes to the test method protocol should be accompanied by a scientific rationale.

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ICCVAM-Recommended Protocol for Future Studies Using the EpiOcular™ (EO) Test Method

PREFACE

This proposed protocol for ocular toxicity is based primarily on information obtained from the Institute for In Vitro Sciences, Inc. (IIVS). Future studies using the EO test method could include further characterization of the usefulness and limitations of the EO 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 evaluate the potential ocular irritation of the test substance by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye conversion by the EO human tissue construct, a proprietary three-dimensional epithelial construct available from MatTek Corporation, following topical exposure to the test substance.

The focus of this protocol is on the use of the EO test method for the detection of substances not labeled as irritants, as defined by the U.S. Environmental Protection Agency (EPA; EPA, 2003a), European Union (EU; EU, 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). However, the EO test method is not currently considered to be adequately validated for classification of ocular irritancy as defined by EPA (2003a), EU (2001), and GHS (UN 2007).

ICCVAM recommends this EO test method protocol for nonregulatory, validation, or optimization studies to facilitate collection of consistent data and expand the available database. Exceptions and/or changes to the test method protocol should be accompanied by a scientific rationale.

2.0 Safety and Operating Precautions

All procedures with the EO test method should follow the institution's applicable regulations and procedures for handling human or 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 Assay Medium and Reagents

- Assay medium is provided with the Standard EpiOcular™ kit (OCL-200). The base medium is Dulbecco's Modified Eagle's Medium (DMEM) containing epidermal growth factor, insulin, hydrocortisone, and other proprietary stimulators of non-keratinizing epithelial differentiation, gentamycin (5 µg/mL), phenol red, and amphotericin B (0.25 µg/mL). Alternative kits with various medium components removed (e.g., antibiotic-free, phenol red-free, antifungal-free) are also available.
- Dulbecco's Phosphate Buffered Saline, Ca²⁺- and Mg²⁺-Free (Ca²⁺Mg²⁺-Free-DPBS)
- Isopropanol, for extraction

- MTT Addition Medium is DMEM containing 2mM L-glutamine by Quality Biological (or equivalent)
- Sterile deionized water by Quality Biological (or equivalent)

3.2 EpiOcular™ Three-Dimensional Epithelial Tissue Construct

- Standard EpiOcular™ kit (OCL-200) is available for purchase from MatTek Corporation, Ashland, MA (<http://www.mattek.com>). Each kit consists of 24 tissues, each tissue 9 mm in diameter. Half kit (i.e., 12 tissues) and six tissue kits are also available. The tissues are screened using PCR and negative for HIV, hepatitis B, and hepatitis C. The tissues are shipped at 4°C on medium-supplemented agarose gels.
- The use of EO tissues offers features appropriate for a model for ocular irritation. First, the model is composed of stratified human keratinocytes in a three-dimensional structure. Secondly, test materials can be applied topically to the model so that water-insoluble materials may be tested.

3.3 Chemicals

- Isopropanol, reagent-grade

3.4 Solutions

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

- Triton® X-100, 0.3%

4.0 Test Substance Preparation

Test articles will generally be tested neat. End use concentrations or other forms may be used. To aid in filling the pipet for pipettable materials that are viscous, the test article may first be transferred to a syringe. The pipet tip of the positive displacement pipet will be inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipet piston is drawn upwards. If air bubbles appear in the pipet tip, the test article should be removed (expelled) and the process repeated until the tip is filled without air bubbles. This method should be used for any materials that cannot be easily drawn into the pipet (e.g., gels, toothpastes, mascaras, and face creams) and solid test articles that are creamed like lipsticks and antiperspirants/deodorant sticks. Dry powders will be ground with a mortar and pestle and passed through a #40 copper sieve, if needed. Materials that are too viscous to spread over the tissue will first be spread onto the flat end of a dosing device. When the test article must first be applied to a dosing device, approximately 30 µL or 30 mg of material will be applied to the dosing device so as to cover the dosing surface. The sample should be spread to form a relatively smooth even layer on the surface of the dosing device to maximize uniform tissue contact. Solids such as lipsticks or antiperspirant/deodorant sticks can be pre-softened by creaming a portion in a weigh boat. The softened portion can be transferred to a syringe affixed with a three way stopcock attached to a second syringe. The sample is pushed from syringe to syringe until it is of a consistency, which can be pipetted. The exact exposure conditions used for other test article forms will be determined.

Controls

5.1 Negative Control

A negative control (e.g., sterile deionized water or other solvent as appropriate) is included in each experiment in order to detect nonspecific changes in the test system, as well as to provide a baseline for the assay endpoints.

5.2 Positive Control

A known ocular irritant is included in each experiment to verify that an appropriate response is induced. For the EO test method, the positive control is 0.3% Triton[®] X-100. The selection of positive control test substances should be based on the availability of high quality *in vivo* data.

5.3 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 the *in vivo* rabbit eye test
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Test System

The EO human cell construct described in **Section 3.2** is used. According to the manufacturer, the EO tissue construct is derived from normal, human epidermal keratinocytes derived from the neonatal foreskin of a single donor and cultured using serum-free media to form a stratified squamous epithelium similar to that found in the cornea. The tissue construct consists of highly organized basal cells that progressively flatten out, as the apical surface of the tissue is approached, analogous to the normal *in vivo* corneal epithelium. EO is mitotically and metabolically active and releases many of the pro-inflammatory agents (cytokines) known to be important in ocular irritation and inflammation. Substances are applied topically, which permits the use of solid or semi-solid materials (e.g., gels) in addition to water-soluble materials.

Prior to use, each plate (6, 12, and 24-well) will be uniquely identified with a number written in permanent marker, on the plate and its cover, the test article number, and the exposure time.

The experimental design of this study consists of the determination of the pH of the neat liquid test substance if possible (and/or dosing solution as appropriate) and a single definitive assay. The toxicity of the test substance will be evaluated by the exposure time required to reduce tissue viability to 50% of controls (ET₅₀). Viability will be determined by the NAD(P)H-dependent microsomal enzyme reduction of MTT (and to a lesser extent, by the succinate dehydrogenase reduction of MTT) in control and test article-treated cultures (Berridge et al. 1996). Data will be presented in the form of relative survival (relative MTT conversion) versus test substance exposure time.

One of two exposure time ranges may be used. The standard exposure time range extends up to four hours and is used for most substances to be tested. For extremely mild substances, such as those that might be applied around or in the eyes, a long exposure assay might be used. For the long exposure study, exposure times of up to 24 hours could be used. In general, the standard exposure range will be used, unless the available information (e.g., test substance chemical class or physicochemical properties necessitates an alternative exposure time range).

6.2 Collection and Transport Conditions of EO Tissues

Including time in transit, tissues may be stored at 4°C for up to four days prior to use. However, extended storage periods are not recommended unless absolutely necessary. In addition, the best

reproducibility will be obtained if tissues are used consistently on the same day (e.g., Tuesday afternoon or following overnight storage at 4°C on Wednesday morning).

6.3 Administration of Test Substances

Test substances will generally be tested neat. End-use concentrations or other forms may be used as needed. One hundred μ L of pipettable substances, such as liquids, gels, creams, and foams, will be applied directly on the tissue so as to cover the upper surface (i.e., topical administration). Powders will be placed directly onto the culture at approximately 30 mg/culture. For viscous substances applied with the aid of a dosing device, the dosing device is placed into the Millicell[®] to bring the test substance in contact with the tissue. All exposure conditions must be documented.

6.4 pH Determination

The pH of the neat liquid test substance (and/or dosing solution as appropriate) will be determined, if possible. The pH will be determined using pH paper (e.g., with a pH range of 0 – 14 to estimate, and/or a pH range of 5-10 to determine a more precise value). The typical increments on the pH paper used to report the pH are approximately 0.3 to 0.5 pH units. The maximum increment on the pH paper is 1.0 pH unit.

6.5 Controls

Generally, at least two negative control exposure times will be used. One negative control exposure time will be selected to fit the range of the shortest test substance or positive control exposure times (the minimum negative control exposure time will be 15 minutes). The second negative control exposure time will be selected to match the longest test substance or positive control exposure time (whichever is longer, up to 240 minutes). On occasion, the second negative control exposure time may be selected to fit the longest test substance exposure time of a test substance run concurrently, but from an independent study. For the long exposure assay (exposures of greater than 240 minutes), multiple negative control exposure times may be selected to fit the range of test substance exposure times. A single negative control exposure-time may be used if all exposure times are one hour and less. Additional negative control exposure times may be selected if needed. Positive control cultures are treated with 0.3% (3 mg/mL) Triton[®]-X-100 prepared in sterile deionized water and are exposed for 15 and 45 minutes. At least two cultures will be used for each negative and positive control exposure time.

6.6 Assessment of Direct Reduction of MTT by the Test Substance

It is necessary to assess the ability of each test substance to directly reduce MTT. A 1.0 mg/mL MTT solution will be prepared in warm MTT Addition Medium. Approximately 100 μ L (liquid test substances) or 30 mg (solid test substances) will be added to 1 mL of the MTT solution and the mixture incubated in the dark at 37 \pm 1°C in a humidified atmosphere of 5 \pm 1% CO₂ in air (standard culture conditions) for approximately one hour. The negative control (100 μ L) will be run concurrently. If the MTT solution color turns blue/purple, the test substance is presumed to have reduced the MTT. Water-insoluble test substances may show direct reduction (darkening) only at the interface between the test substance and the medium.

6.7 Receipt of the EpiOcular[™] Tissue

Upon receipt of the EO assay materials, the solutions will be stored as indicated by the manufacturer. The tissue will be stored at 2-8°C until used. On the day of dosing, EO assay medium will be warmed to approximately 37°C. Nine tenths (0.9) mL of Assay Medium will be aliquoted into the appropriate wells of pre-labeled 6-well plates. The 6-well plates will be labeled with the test substance(s) and exposure time(s). Each tissue will be inspected for air bubbles between the agarose gel and Millicell[®] insert prior to opening the sealed package. Cultures with air bubbles under greater than 50% of the

Millicell[®] area will not be used. Each 24-well shipping container will be removed from its plastic bag and its surface disinfected by wiping with 70% ethanol-soaked tissue paper. An appropriate number of tissues will be transferred aseptically from the 24-well shipping containers into the 6-well plates. The EO tissues will be incubated at standard culture conditions for at least one hour. The medium will be aspirated and 0.9 mL of fresh Assay Medium will be aliquoted into each assay well below the tissue. Upon opening the bag, any unused tissues remaining on the shipping agar at the time of tissue transfer will be briefly gassed with an atmosphere of 5% CO₂/95% air, and the bag will be sealed and stored at 2-8°C for subsequent use.

6.8 Definitive MTT Assay

Four to five exposure times will be tested for each test substance. The exposure times should be chosen to maximize the response based on knowledge of the test substance (e.g., chemical class or physicochemical characteristics). In the short-term exposure assay, if the expected range of toxic response is unknown, a 20-minute exposure time may be performed first to determine the remaining exposure durations. The maximum exposure time will be 240 minutes unless there is a need to extend it, based on knowledge of the test substance.

Two tissues will be treated for each test substance at each control exposure time. The dosing procedure will be determined as indicated in **Section 6.5**. Generally, exposure times of ten minutes or greater will be incubated at standard culture conditions.

The positive control will be exposed for 15 and 45 minutes. A second negative control will be exposed for the longest exposure time used for the test or control substances up to 240 minutes.

At the end of the treatment time, the test substance will be removed by extensively rinsing both sides of the culture with room temperature Ca²⁺ and Mg²⁺-Free Dulbecco's Phosphate Buffered Saline (Ca²⁺Mg²⁺-Free-DPBS). The process will be performed until the culture appears free from test substance. If it is not possible to remove all of the visible test material, this will be noted.

After rinsing, the tissue will be transferred to 5 mL of Assay Medium for a 10 to 20 minute incubation period at room temperature. This rinse is intended to remove any test substance absorbed into the tissue.

A 10X stock of MTT prepared in PBS (filtered at time of batch preparation) will be thawed and diluted in warm MTT Addition Medium to produce the 1.0 mg/mL solution no more than two hours before use. Alternatively, a 1.0 mg/mL MTT solution will be prepared in warm MTT Addition Medium and filtered through a 0.45 μ m filter to remove undissolved crystals. Three hundred μ L of the MTT solution will be added to each designated well of a prelabeled 24-well plate. The tissue will be transferred to the appropriate wells after rinsing, and the plates incubated for 3 \pm 0.1 hours at standard culture conditions.

After 3 \pm 0.1 hours, the bottom of the EO tissue constructs will be blotted on absorbent paper, cleared of excess liquid, and transferred to a prelabeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates will be sealed with parafilm and stored in the refrigerator (2-8°C) until the last exposure time is harvested. The plates are then shaken for at least 2 hours at room temperature. At the end of the extraction period, the liquid within each Millicell[®] insert will be decanted into the well from which it was taken. The extract solution will be mixed and 200 μ L transferred to the appropriate wells of a prelabeled 96-well plate(s). Two hundred μ L of isopropanol will be added to the wells designated as blanks. The absorbance at 550 nm (OD₅₅₀) of each well will be measured with a Molecular Devices Vmax plate reader.

6.9 Killed Controls for Assessment of Residual Test Substance Reduction of MTT

In cases where the test substance is shown to reduce MTT, only test substances that remain bound to the tissue after rinsing, resulting in a false MTT reduction signal, present a problem. To demonstrate that residual test substance is not acting to directly reduce the MTT, a functional check is performed in the definitive assay to show that the test substance is not binding to the tissue and leading to a false MTT reduction signal.

To determine whether residual test substance is acting to directly reduce the MTT, a freeze-killed control tissue is used. Freeze-killed tissue is prepared by placing untreated EO constructs in the -20°C freezer at least overnight, thawing to room temperature, and then refreezing. Once refrozen, the tissue may be stored indefinitely in the freezer. To test for residual test substance reduction, killed tissues are treated with the test substances in the normal fashion. Generally, each test substance will be evaluated for at least the shortest and longest exposure times (or longest exposure time if all exposures are 1 hour or less) in single replicate killed tissues. All assay procedures will be performed as for the viable tissue. A killed control treated with sterile deionized water (negative killed control) will be tested in parallel since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue.

If little or no MTT reduction is observed in the test substance-treated killed control, the MTT reduction observed in the test substance-treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional steps must be taken to account for the chemical reduction or the test substance may be considered untestable in this system. The OD₅₅₀ values from the killed controls will be analyzed as described in **Section 7.1**.

7.0 Evaluation of Test Results

7.1 Data Evaluation and Interpretation

The raw absorbance values will be captured, and the following calculations made: The mean OD₅₅₀ of the blank control wells will be calculated. The corrected mean OD₅₅₀ of the exposure time control(s) will be determined by subtracting the mean OD₅₅₀ of the blank control from their mean OD₅₅₀'s. The corrected OD₅₅₀ of the individual test substance exposure times and the positive control exposure times will be determined by subtracting the mean OD₅₅₀ of the blank control from their respective OD₅₅₀'s. When applicable, corrected OD₅₅₀ values will be calculated for the control and test substance-treated killed controls, as well. Generally, all calculations will be performed using Microsoft Excel as follows:

$$\text{Corrected test substance exposure time } OD_{550} = \text{Test substance exposure time } OD_{550} - \text{Blank mean } OD_{550}$$

If killed controls (KC) are used, the following additional calculations will be performed to correct for the amount of MTT reduced directly by test substance residues. The OD₅₅₀ value for the negative control killed control will be subtracted from the OD₅₅₀ values for each of the test substance-treated killed controls (at each exposure time), to determine the net OD₅₅₀ values of the test substance-treated, killed controls as follows:

$$\text{Net } OD_{550} \text{ for each test substance KC} = \text{Raw } OD_{550} \text{ test substance KC} - \text{Raw } OD_{550} \text{ negative control KC}$$

The net OD₅₅₀ values represent the amount of reduced MTT due to direct reduction by test substance residues at specific exposure times. In general, if the net OD₅₅₀ value is greater than 0.150, the net amount of MTT reduction will be subtracted from the corrected OD₅₅₀ values of the viable treated tissues, at each corresponding exposure time, to obtain a final corrected OD₅₅₀ value. These final

corrected OD₅₅₀ values will be used to determine the percent of control viabilities at each exposure time as follows:

$$\text{Final corrected OD}_{550} = \text{Corrected test substance OD}_{550} (\text{viable}) - \text{Net OD}_{550} \text{ test substance (KC)}$$

Finally, the following percent of control calculations will be made as follows:

$$\text{Percent of Control} = (\text{Corrected OD}_{550} \text{ of each test substance or positive control exposure time} / \text{Corrected mean OD}_{550} \text{ of negative control}) \times 100$$

The individual % of control values are averaged to calculate the mean percent of control per exposure time. Viability calculations for test substances treated in the long exposure time assay may be performed by comparing the corrected OD₅₅₀'s of each test substance exposure time to the appropriate exposure time control(s).

Exposure time response curves may be plotted with the % of control on the ordinate and the test substance exposure time on the abscissa. Other plot forms may be used as needed. The ET₅₀ will be interpolated from each plot. To determine the ET₅₀, two adjacent points will be selected, one that shows greater than 50% survival and one that shows less than 50% survival. The two selected points will be used to determine the slope and the y-intercept for the equation $y = m(x) + b$. Finally, to determine the ET₅₀, the equation will be solved for $y = 50$. If all of the exposure time points show greater than 50% survival, the ET₅₀ will be listed as greater than the longest exposure time. If all of the exposure times show less than 50% survival, the ET₅₀ will be presented as less than the shortest exposure time. Additional assays may be performed as needed to produce the final ET₅₀ value.

8.0 Criteria for an Acceptable Test

The assay will be accepted if the positive control, 0.3% Triton-X-100, causes an ET₅₀ within two standard deviations of the historical mean. The historical mean is updated every three months. The corrected mean OD₅₅₀ value for the minimum negative control exposure time must be within 20% of the corrected mean OD₅₅₀ value for the maximum negative control exposure time (up to 240 minutes).

9.0 Data Interpretation

Test substances with an ET₅₀ ≥ 70 minutes are identified as substances not labeled as irritants.

10.0 Study Report

A report of the results of this study will be prepared by the testing laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be prepared reporting the ET₅₀ values for each test substance as well as the positive control data. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

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
- Name and address of the test facility
- Name and address of the Study Director

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

Test Conditions

- Description of test system used
- Calibration information for instrument used for optical density measurements (e.g., spectrophotometer)
- 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

- Tabulation of data from individual test samples (e.g., ET₅₀)

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 2003a, 2003b; FDA 2003) should be followed.

11.0 References

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