Appendix B

ICCVAM-Recommended Test Method Protocols

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Appendix B1

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

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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 holders[1]
- 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 1% sodium hydroxide or 10% 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 ± 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. 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 are 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 ± 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 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 ± 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 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 ± 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 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 ± 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 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 ± 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 Na-fluorescein solution to the anterior chamber using a micropipettor.

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

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 prelabeled 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 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 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 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 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.

UV/VIS Spectrophotometer Method

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* 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 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 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.

11.0 References

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Appendix B2

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

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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 x 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 μg/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 μg/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 $37 \pm 1^{o}C$ 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 75cm^2 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 10^5 cells per each capsule cup (0.5 mL of a 1.2 x 10^6 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% CO_2 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^{o}C.

For routine passaging, the stock cultures are trypsinized as described above, but are dislodged and resuspended using warm (approximately 37^{o}C) Growth Medium, seeded into a culture flask(s), and returned to the humidified incubator maintained at 37 ± 1^{o}C and 5 ± 1% CO_2 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 MRD_{50} 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 µL per minute for the first minute, and 20 µL per minute for the next 12 minutes and 30 seconds. The second phase will be the washout phase, which will be 6 minutes at a nominal rate of 100 µL 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 MRD_{50} 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 MRD_{50} 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 MRD_{50}. 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 MRD_{50} 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 MRD_{50} 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 MRD_{50} could be determined. The results from additional definitive assays may also be incorporated into the calculation of the final MRD_{50}.

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 MRD_{50}. 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 MRD_{50} 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 MRD_{50} values is done according to the decision criteria provided in Background Review Document: Existing Methods for Eye Irritation Testing: Silicon Microphysiometer and CM (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}
1	<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 Protocol for Future Studies Using the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method

ICCVAM recommends this HET-CAM 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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PREFACE

The protocol was adapted from the protocol previously described by Spielmann and Liebsch (INVITTOX 1992). Examples of the use of this protocol can be found in Luepke (1985), Balls et al. (1995), Gilleron et al. (1996, 1997), and Spielmann et al. (1996). Future studies using the HET-CAM test method could include further characterization of the usefulness and limitations of the HET-CAM 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 components and procedures used to evaluate the potential ocular irritancy of a test substance as measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. Effects are measured by the onset of (1) hemorrhage; (2) coagulation; and (3) vessel lysis. These assessments are considered individually and then combined to derive a score, which is used to classify the irritancy level of the test substance.

The focus of this protocol is on the use of the HET-CAM test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA 2003a), European Union (EU; EU 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). However, the HET-CAM 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).

This HET-CAM test method protocol has been modified from a generic description of the Irritation Score (IS) analysis method to include a more detailed IS(A) analysis method to be used for prospective studies. However, a description of the IS(B) analysis method, which was described in 2006 (ICCVAM 2006) is included for retrospective analyses, where IS(B) analysis method data could be converted to fixed time points similar to those used for the IS(A) analysis method described in **Section 7.0**.

ICCVAM recommends this HET-CAM 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 chicken eggs 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 Eggs

Fertile White Leghorn chicken eggs should be obtained from commercial sources. Fresh (not older than seven days), fertile, clean eggs weighing between 50 and 60 grams should be used. Eggs should

be candled prior to use and nonviable or defective eggs should be discarded. Excessively misshapen eggs or eggs with cracked or thin shells should not be used. Transport of eggs should occur under conditions that will not affect embryo viability or development.

3.2 Equipment and Supplies

- Candling light
- Deionized/Distilled Water
- Dentist's rotating saw blade
- Incubator with an automatic rotating device
- Micropipette(s) and disposable tips appropriate for recommended volumes
- Mortar and pestle (or comparable grinding tools for test substances)
- Stop clock or electronic chronometer
- Standard general biological laboratory equipment and supplies (e.g., microcentrifuge tubes for measurement of substance volume), as needed
- Tapered forceps
- Volumetric flasks

3.3 Solutions

The manufacturer’s recommendations should be followed with regard to storage temperature and shelf life of stock solutions. Solutions should be prepared volumetrically.

- 0.9% (w/v) sodium chloride (NaCl) in deionized/distilled water
- 1% (w/v) sodium dodecyl sulfate (SDS) in deionized/distilled water
- 0.1 N sodium hydroxide (NaOH) in deionized/distilled water

4.0 Test Substance Preparation

All test substances should be evaluated undiluted unless dilution is justified. If dilution is justified, then 0.9% NaCl or olive oil should be used as the diluent, depending on substance solubility. Use of a different solvent should be justified. Dilutions should be prepared on the same day as the test.

Paste, particulate, or granular test substances or formulations should be evaluated without dilution. Solid test substances should be ground to a fine dust to obtain a volume of 0.3 mL after gentle compaction of the particulates in a measuring container (e.g., microcentrifuge tube).

5.0 Controls

5.1 Negative Control

A 0.9% NaCl negative control should be included in each experiment in order to provide a baseline for the assay endpoints and to ensure that the assay conditions do not inappropriately result in an irritant response.

5.2 Solvent Control (if appropriate)

If the test substance is diluted in olive oil, then this solvent should be included as a control substance in order to provide a baseline for the assay endpoints and to ensure that the assay conditions do not inappropriately result in an irritant response. If a solvent other than 0.9% NaCl or olive oil is used, than both the solvent and 0.9% NaCl should be included as controls to ensure that the alternative solvent does not result in an irritant response.

5.3 Positive Control

A known ocular irritant should be included in each experiment to verify that an appropriate response is induced. If the HET-CAM assay is being used only to identify corrosive or severe irritants, then the positive control should be a substance (e.g., 1% SDS, NaOH) that induces a severe response *in vivo* as well as in HET-CAM. 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. The selection of positive control test substances should be based on the availability of high quality *in vivo* data.

5.4 Benchmark Control (if appropriate)

Benchmark controls may be useful in demonstrating that the test method is functioning properly for detecting the ocular irritancy potential of chemicals of a specific chemical class or a specific range of responses, or for evaluating the relative irritancy potential of an ocular irritant. Appropriate benchmark controls 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 Treatment Groups

Use at least three eggs per group (negative and positive controls, test substance, and, if included, benchmark and solvent controls). To the extent possible, eggs from the same hen should be randomized among treatment groups.

6.2 CAM Preparation

a. Select fresh (not older than 7 days), clean, fertile 50-60 g White Leghorn chicken eggs. Candle the eggs and discard any eggs that are nonviable or defective. Excessively misshapen eggs or eggs with cracked or thin shells should not be used. Shaking, unnecessary tilting, knocking, and all other mechanical irritation of the eggs should be avoided when preparing.

b. Place eggs in an incubator with a rotating tray. Incubate eggs at 38.3 ± 0.2°C and 58 ± 2% relative humidity when incubating in a still-air incubator or at 37.8 ± 0.3ºC and 58 ± 2% relative humidity when incubating in a forced-air incubator. Hand rotate eggs five times per day until day 8.

c. Candle the eggs on incubation day 8 and remove any nonviable or defective eggs. Eggs are returned to the incubator (without hand rotation) with the large end of the eggs upwards for an additional day.

d. Remove eggs from the incubator on day 9 for use in the assay. Candle eggs and discard any nonviable or defective eggs.

e. Mark the air cell of the egg. Cut the section marked as the air cell with a rotating dentist saw blade and then pare it off. Care should be taken when removing the eggshell to ensure that the inner membrane is not injured.

f. Moisten the inner membrane with 0.9% NaCl. A disposable glass pipette can be used to apply the solution. Place the egg into the incubator for a maximum of 30 minutes.

g. Remove the egg from the incubator, prior to its use in the assay, and decant the 0.9% NaCl solution. Carefully remove the inner membrane with forceps, ensuring that the inner membrane is not injured.

6.3 Treatment of Eggs with Test Substances

Depending on the physical form of the test substance, the following form-specific application protocols should be followed.

6.3.1 Liquid or diluted test substances or formulations

Apply 0.3 mL of liquid substances or diluted substances directly onto the CAM surface.

6.3.2 Solid, particulate, or granular test substances or formulations

Apply 0.3 mL of solid, particulate, or granular substances (which have been ground to a fine dust) directly onto the CAM, ensuring that at least 50 % of the CAM surface area is covered. In cases where the total weight of the test substance at this volume is greater that 0.3 g, 0.3 g of the solid, particulate, or granular test substance should be used. In either case, the weight of the test substance should be recorded.

6.3.3 Paste test substances or formulations

Apply 0.3 mL of paste substances or formulations directly onto the CAM, ensuring that at least 50% of the CAM surface area is covered. In cases where the total weight of the test substance at this volume is greater that 0.3 g, 0.3 g of the paste test substance should be used. In either case, the weight of the test substance should be recorded.

6.4 Observations

Observe the reactions on the CAM over a period of 300 seconds. The time for the appearance of each of the noted endpoints should be monitored and recorded, in seconds. Endpoints that should be observed are:

- Hemorrhage (bleeding from the vessels)
- Vascular lysis (blood vessel disintegration)
- Coagulation (intra- and extra-vascular protein denaturation)

Hemorrhage time = observed start (in seconds) of hemorrhage reactions on CAM

Lysis time = observed start (in seconds) of vessel lysis on CAM

Coagulation time = observed start (in seconds) of coagulation formation on CAM

Collection of additional information and data may be useful in further analyses and conducting retrospective studies. To maximize the likelihood of obtaining reproducible results, reference photographs for all endpoints should be available.

7.0 Evaluation of Test Results

The ICCVAM-recommended HET-CAM protocol for prospective studies is the IS(A) analysis method, which is based on development of each of the three HET-CAM endpoints at fixed time intervals of 0.5, 2, and 5 minutes (Luepke 1985).

The numerical time-dependent scores for lysis, hemorrhage, and coagulation (**Table 7-1**) are summed to give a single numerical value indicating the irritation potential of the test substance on a scale with a maximum value of 21.

Table 7-1 Scoring Scheme for Irritation Testing with the HET-CAM Test Method

Effect	Score		
	0.5 min	**2 min**	**5 min**
Lysis	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

For retrospective analyses, data from the HET-CAM test method protocol using the IS(B) analysis method (ICCVAM 2006) could be converted to fixed time points similar to those used for the IS(A) analysis method.

8.0 Criteria for an Acceptable Test

A test is considered acceptable if the negative and positive controls each induce a response that falls within the classification of nonirritating and severely irritating, respectively. Historical control studies indicate that using 0.9% NaCl, as a negative control, the IS value was 0.0. Historical control studies indicate that using 1% SDS and 0.1 N NaOH, as positive controls, the IS values ranged between 10 and 19, respectively.

9.0 Data Interpretation

When using the IS analysis method, the severe irritancy classification for a test substance is assigned when the value is greater than nine.

10.0 Study Report

Information and data that should be included in study reports for the HET-CAM test method include, but are not limited to:

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)
- 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

- Experimental starting and completion dates
- 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., irritancy scores for the test substance and the various controls, including data from replicate repeat experiments as appropriate, and means and ± the standard deviation for each test)

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 B4

ICCVAM-Recommended Protocol for Future Studies Using the Isolated Chicken Eye (ICE) Test Method

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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 ICE (ICCVAM 2006a,b). It is based primarily on the current protocol used by Menk Prinsen, the original developer of the test method (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 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 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 ICE 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 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

Spring chickens obtained from a local source (e.g., poultry abattoir), approximately 7 weeks old, male or female, with a weight range of 2.5–3.0 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)
- Volumetric flasks
- 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. Assay solutions should be prepared volumetrically.

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

4.0 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 solvents for diluted substances are either deionized/distilled water or physiological saline. However, alternative solvents may also be used under controlled conditions, but the appropriateness of solvents other than deionized/distilled water or 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.

5.0 Controls

5.1 Negative Controls

A negative control (e.g. deionized/distilled water, isotonic saline, other assay medium) should be included in each experiment in order 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

Solvent/vehicle controls are recommended when solvents/vehicles other than deionized/distilled water, saline, or other assay medium are used to dissolve test substances, in order to demonstrate that the solvent/vehicle is not interfering with the test system.

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 assay 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 controls may be useful to demonstrate that the test method is functioning properly for detecting the ocular irritancy potential of chemicals of a specific chemical class or a specific range of responses, or for evaluating the relative irritancy potential of an ocular irritant. 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 animal models
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Collection and Transport Conditions of Chicken Eyes

Heads of spring chickens should be obtained from a local source (e.g., poultry abattoir). Heads should be removed immediately after sedation of the animals by electric shock and incision of the neck for bleeding. Chicken heads may then be transported to the laboratory at ambient temperature in humidified plastic boxes (i.e., sealed with tissues moistened with isotonic saline) within two hours after they are humanely killed. Once at the laboratory, the eyes may be dissected from each chicken head.

6.2 Preparation of Eyes

a. Carefully remove the eyelids without damaging the cornea. Place a drop of fluorescein sodium BP 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 underpad 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 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, apply the test substance to the eye (see Sections 6.4.1.1 and 6.4.1.2).

c. During the dosing procedure, remove the clamp holding the eye from the superfusion apparatus and place it on tissue paper with the cornea facing upwards.

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

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

Liquid test substances

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

Solid test materials
If necessary, grind solid test substances into a fine powder with a mortar and pestle, or comparable grinding tools. Apply 0.03 g of a solid test substance evenly over the entire surface of the cornea.

6.4.2 Endpoint observations

a. Examine the control and test eyes at 30, 75, 120, 180, and 240 minutes (± 5 minutes) after treatment using the criteria and scoring system as indicated in Section 6.4.2.1.

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 4% neutral buffered formaldehyde for preservation 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.

Criteria and scoring system
The following criteria and scoring system are applied for the assessment of possible effects:

- Corneal swelling is expressed as a percentage and is calculated according to the following formula:

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

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.

- 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.

- Fluorescein retention
  The mean fluorescein retention value for all test eyes is calculated for the 30-minute 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

- 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. On the basis of severity of the observed findings, these effects are divided into four categories: 1 = none; 2 = slight; 3 = moderate; 4 = severe.
- 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.

7.0 Evaluation of Test Results

Results from the three test method endpoints, corneal opacity, corneal swelling, and fluorescein retention should be evaluated separately (as in Section 9.0), and also combined to generate an Irritancy Classification for a test material (as in Section 10.0).

8.0 Criteria for an Acceptable Test

A test is considered acceptable if the negative and positive controls give an Irritancy Classification that falls within nonirritating and severely irritating, respectively

9.0 Data Interpretation

Interpretation of corneal thickness, corneal opacity, and fluorescein retention using four irritancy categories is done according to the following scales:

9.1 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

9.2 Corneal 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

9.3 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

10.0 Assessment of the Eye Irritancy

The 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 3 Endpoints
Severely Irritating	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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Appendix B5

ICCVAM-Recommended Protocol for Future Studies Using the Isolated Rabbit Eye (IRE) Test Method

ICCVAM recommends this IRE 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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PREFACE

The information included in this protocol was derived from protocols used at Unilever Safety and Environmental Assurance Centre, Colworth, United Kingdom (Jones P, personal communication) and at SafePharm Laboratories, Derby, United Kingdom (Whittingham A, personal communication) and from evaluation of IRE protocols reported in the literature (Burton et al. 1981; Price and Andrews 1985; Whittle et al. 1992; INVITTOX 1994; Balls et al. 1995; Chamberlain et al. 1997; Cooper et al. 2001; Jones et al. 2001; Guerriero et al. 2004). Future studies using the IRE test method could include further characterization of the usefulness or limitations of the IRE 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 the protocol is to provide details of the essential procedures required to (1) insure induction of corneal irritancy in the enucleated eye of the rabbit by a potentially irritating test substance, (2) evaluate the degree of irritancy, and (3) enable assignment of an appropriate regulatory classification on the potential ocular irritancy of a test substance. Toxic effects in the isolated rabbit eye are measured by (1) subjective assessment of changes in corneal opacity, (2) uptake of fluorescein dye within the cornea (permeability), (3) increased corneal thickness (swelling), and (4) corneal epithelial changes (pitting, sloughing, mottling, etc.) evaluated macroscopically or by slit-lamp. The opacity, swelling, and permeability assessments following exposure to a test substance are assessed individually and are used to determine if the test substance has the potential to induce ocular corrosion or severe irritation.

The focus of this protocol is on the use of the IRE test method for the detection of ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection Agency (EPA 2003a), European Union (EU 2001), and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2007). However, the IRE 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 IRE 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 rabbit eyes should follow the institution's applicable regulations and procedures for handling of 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 Material, Equipment, and Supplies

3.1 Source of Rabbit Eyes

Rabbits should not be bred and sacrificed specifically for use in the IRE test method. Eyes should be obtained from healthy New Zealand White rabbits of either sex weighing 2.5-4.0 kg. To reduce

animal usage, rabbits may be obtained from intra- or extramural laboratories where rabbits may have been used for other purposes (e.g., isolated organ bath, controls) that would not affect ocular tissue, or from a local abattoir where rabbits are typically sacrificed as a food source. Isolated rabbit eyes of exceptional quality without corneal surface defects may be purchased and shipped overnight from a reputable source such as Pel-Freeze Biologicals (Edelhauser H, personal communication). For rapid transfers from laboratory to laboratory within close proximity to each other (1 hour or less), the eyes may be wetted with isotonic saline, or an appropriate buffer (e.g., HBSS without phenol red), secured in position in a hydrated container at room temperature and sealed for shipment. For longer shipments (up to 4 hours), storage at 4°–8°C is recommended. For overnight shipment, storage at 4°–8°C in isotonic saline, or an appropriate buffer (e.g., HBSS without phenol red) with optional antibiotics and an antimycotic is recommended (Vafeas et al. 1998; Chandrasekher et al. 2002).

3.2 Equipment and Supplies

- Chamber, superfusion, Perspex® or similar inert material, water-jacketed temperature-controlled at 32 ± 1.5°C (Burton et al. 1981)
- Drip tubes made from stainless steel tubing (for saline rinsing of cornea)
- Forceps, tissue
- Holders, eye, Perspex or stainless steel with moveable upper jaw
- Magnifying glass
- Plastic tubing, medical or food-grade to supply lines for saline drip tubes
- Pump, peristaltic, 0.1-0.4 mL/minute flow rate adjusted to pump saline in flask in water bath through the saline drip tube
- Pump, peristaltic, approximately 4 L/minute flow rate to pump water through superfusion apparatus and maintain temperature control
- Scissors, fine surgical
- Scissors, surgical enucleation
- Slit-lamp biomicroscope or equivalent
- Optical or ultrasonic pachymeter to quantitatively measure corneal thickness. The optical pachymeter is used in conjunction with the slit-lamp whereas the ultrasonic pachymeter is a stand-alone device.
- Syringe, plastic, 20 ml for eye wash
- Syringe for sodium pentobarbitone administration
- Thermistor (e.g., YSI thermistor, Yellow Spring Co., Inc, OH, USA) to check saline drip temperature
- Tubing, food or medical grade for pumping saline and for connecting to water supply in circulator, sizes may vary with hose fittings
- Water bath, recirculating (capable of maintaining a temperature of 32 ± 1.5°C)
- Weigh Boat, plastic disposable, or a 1 mL disposable plastic syringe with the narrow tip removed

3.3 Solutions

Solutions may be obtained ready prepared from a commercial supplier. Follow the manufacturer's recommendations with regard to storage temperature and shelf life of stock solutions. If necessary, prepare assay solutions volumetrically and store at room temperature unless otherwise noted. Buffers or solutions containing glucose or temperature-sensitive components should be stored at 4°–8°C and equilibrated to room temperature just before use.

- Buffers, physiological salt solution (Hank's, Krebs, etc.)
- Fluorescein, sodium BP (1%–2%), prepared fresh on the day of the experiment

- Physiological (isotonic) saline (0.9%)
- Sodium pentobarbitone
- Sterile deionized/distilled water

4.0 Test Substance Preparation

4.1 Liquid Test Substances

Apply liquid test substances undiluted, although liquid test substances may be diluted if deemed necessary (e.g., as part of the study design). Isotonic saline or standard buffered physiological salt solutions (e.g., Hank's, Krebs, etc.) are the recommended solvents. The appropriateness of solvents other than isotonic saline or standard buffered physiological salt solutions must be demonstrated.

4.2 Solid, Particulate or Granular Test Substances

Grind solid, particulate or granular test substances as fine as possible in a mortar and pestle. The material may be sprinkled on the cornea using a weigh boat or gently compacted in a syringe with the narrow tip removed and then applied. The substance may need to be prewetted and the pH measured (Guest R, personal communication).[2]

5.0 Controls

5.1 Negative Control

A negative control (e.g., distilled water, isotonic saline, other assay medium) is included in each experiment in order to detect non-specific changes in the test system, as well as to provide a baseline for the assay endpoints, and ensure that the assay conditions do not inappropriately result in an irritant response.

5.2 Solvent/Vehicle Controls

Solvent/vehicle controls are recommended when solvents/vehicles other than deionized/distilled water, saline, or other assay medium are used to dissolve test substances, in order to demonstrate that the solvent/vehicle is not interfering with the test system.

5.3 Positive Controls

A known ocular irritant is included in each experiment to verify that an appropriate response is induced. If the IRE assay is being used only to identify corrosive or severe irritants, then the positive control should be a reference substance that induces a severe response *in vivo* as well as in the IRE. 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. The selection of positive control test substances should be based on the availability of high quality *in vivo* data. For test substances being tested in liquid or solid form, a corresponding liquid or solid positive control should be included in the test.

5.4 Benchmark Controls

Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the ocular irritancy potential of substances of a specific chemical class or a specific range of

[2] Since the isolated eye has less moisture content than the eye *in situ* and compounds that dissociate or hydrolyze could produce false negatives due to reduced dissociation or hydrolysis in the isolated eye.

responses, or for evaluating the relative irritancy potential of an ocular irritant. Appropriate benchmark controls should be chosen based on high quality *in vivo* test results and have the following properties:

- A consistent and reliable source(s)
- Structural and functional similarity to the class of 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 Treatment Groups

Use at least three eyes for each test substance and three eyes for each of the controls in the study. The controls must be tested concurrently with the test substance.

6.2 Eye Selection and Preparation

a. For each assay, use a number of animals adequate to provide at least three eyes for each test substance and three eyes for each of the various controls considering rejection levels of suitable eyes to be as high as 25% in some cases. All isolated eyes should be randomly distributed within experimental groups, particularly when both eyes from the same rabbit are used.

b. Examine the rabbit corneas *in vivo* macroscopically and microscopically and, if the eyes are accepted to be free of imperfections, measure the initial corneal thickness (Reading T-2; *in vivo* reading, if possible). In some cases, rabbits may be euthanized commercially and this *in vivo* reading may not be possible. In those cases, a pre-equilibration reading (T-1) is sufficient (**Section 6.3**).

c. Euthanize the rabbits humanely by injection of a lethal dose of sodium pentobarbitone into the marginal ear vein. Follow the institution's applicable regulations and procedures regarding euthanasia. A typical lethal dose for rabbits is 200 mg/kg, administered intravenously. Remove each eye by dissection of the conjunctiva and the optic nerve (leave approximately a 5–10 mm section of the nerve to prevent loss of intraocular pressure) after deflection of the nictitating membrane.

d. Rinse the orbit occasionally with saline during the dissection to prevent drying and afterwards to remove any adherent tissue.

e. Ship eyes obtained from external sources in saline or an appropriate buffer (e.g., HBSS without phenol red) at an appropriate temperature (4 -8°C for shipment over periods greater than 1 hour or 25 ± 5°C for shipment over a period of 1 hour or less) in a humidified, sealed container to prevent drying of the corneas. For longer shipments periods (e.g., overnight), antibiotics with an antimycotic may be needed (Vafeas et al. 1998; Chandrasekher et al. 2002).

f. The method of euthanasia and any prior pharmacological or physiological treatment of the animals for eyes shipped from external sources are noted and the eyes are inspected microscopically and macroscopically for imperfections.

g. If there is any doubt that the cornea is free of imperfections, apply a 1%–2% solution of sodium fluorescein BP followed immediately by a gentle, but thorough rinse with

physiological saline (a time insufficient for actual penetration of fluorescein) to identify corneal imperfections.

h. Once they have been inspected and are deemed to be free of corneal defects, the eyes are clamped into the holders (one eye per holder) with the cornea in a vertical position, without altering the *in vivo* orientation of the eyeball, and placed in the maintenance chamber (see **Figure 6-1** and **Figure 6-2**).

i. The eyes are equilibrated for 30 to 45 minutes at 32 ± 1.5°C.

Figure 6-1 Isolated Rabbit Eye Equilibration Apparatus

Photo provided courtesy of R. Guest

Figure 6-2 Isolated Rabbit Eye Holder

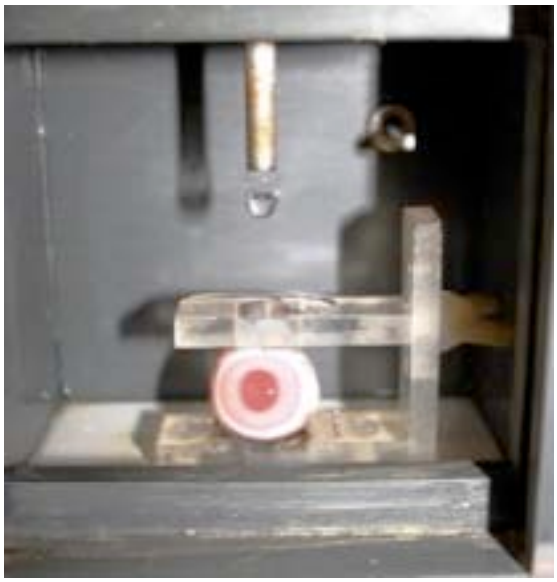

Photo provided courtesy of R. Guest

6.3 Pretreatment Measurements

a. Measure the corneal thickness (Reading T-1) before equilibration. Any eyes in which corneal swelling has exceeded 7% relative to *in vivo* values are discarded and replaced (Reading T-2; **Section 6.2**).

b. The corneal thickness is measured again after equilibration and just prior to application of the test substance. This will become Reading T0 (**Section 6.3**). If a significant amount of time (3 ± 1 hours) has elapsed between post-equilibration and application, any eyes that have swelling >7% relative to the post-equilibration value (T0) should be replaced. If an ultrasonic pachymeter is used which requires direct contact with the cornea, an initial measurement and a post-equilibration reading may be necessary to minimize the possibility of damage to the cornea (Guest R, personal communication).

6.4 Application of Test Substances

a. Remove the holder from the cell where the eye is held in a vertical position, then reposition the eye with the cornea in the horizontal plane (i.e., facing upward) and apply the test substance (premoistened, if necessary) directly on the corneal surface immediately.

b. For liquid substances, apply 0.1 mL of undiluted test substance using a syringe over as much of the entire corneal surface as possible.

c. For solid substances, sprinkle a volume of 0.1 mL (not exceeding 100 mg) of neat test substance pulverized to a fine powder or dust over the entire cornea using a plastic weigh boat or other means of delivery (e.g., from a 1 mL disposable syringe with the tip removed). Record the mean weight of material that is applied to each eye.

d. Adjust the concentration, volume or weight if necessary for compounds with known physical characteristics that may interfere with the test (e.g., viscous substances or solids that irreversibly adhere to the cornea and cannot be washed off).

e. Apply 0.1 mL of physiological saline (prewarmed to 32°C) to the control eye.

f. For liquid positive control substances, apply 0.1 mL using a syringe over as much of the entire corneal surface as possible.

g. For solid positive control substances, sprinkle 0.1 g pulverized to a fine powder or dust over the entire cornea using a plastic weigh boat or other means of delivery.

h. Allow the test substance, the positive control, and the negative control to remain in contact with the cornea for 10 ± 2 seconds.

i. Rinse each eye with 20 ml of physiological saline (prewarmed to 32°C) using a syringe and place the eye holder back in the cell of the maintenance chamber.

j. Return the saline drip tube to its original position to bathe the cornea between measurement periods.

k. Repeat these procedures for subsequent treated and control eyes.

6.5 Endpoint Observations

6.5.1 Corneal opacity and area

a. With the aid of the light source from the slit-lamp (diffuse illumination), examine each eye macroscopically at each time point (0.5, 1, 2, 3, 4 hours), assess the extent of corneal injury, noting signs of sloughing, mottling, pitting or other signs of epithelial damage. Identify focal areas for slit-lamp evaluation.

b. Examine each eye microscopically at each time point (0.5, 1, 2, 3 and 4 hours) using a slit-lamp set with a narrow slit width and score corneal opacity and area involvement according to the scoring system found in **Table 6-1**.

6.5.2 Corneal swelling

a. Measure corneal thickness using the depth measuring attachment or ultrasonic pachymeter before treatment (as described previously) and at each time point post-treatment.

b. Calculate corneal swelling based on the percent change in corneal thickness over time according to the following formula:
[(Corneal Thickness at Time T/Corneal Thickness at Time T0)-1] x 100%

6.5.3 Corneal epithelial observations

a. Examine the cornea macroscopically or by slit-lamp microscopically at each time point for sloughing, mottling, pitting or other signs of epithelial damage.

b. 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.

Table 6-1 Evaluation of Corneal Irritation[1]

Description	
Cornea	**Individual Score**
Normal cornea. Appears with the slit-lamp adjusted to a narrow slit image as having a bright gray line on the epithelial surface and a bright gray line on the endothelial surface with a marble-like gray appearance of the stroma.	0
Some loss of transparency. Only the anterior half of the stroma is involved as observed with an optical section of the slit-lamp. The underlying structures are clearly visible with diffuse illumination, although some cloudiness can be readily apparent with diffuse illumination.	1
Moderate loss of transparency. In addition to involving the anterior stroma, the cloudiness extends all the way to the endothelium. The stroma has lost its marble-like appearance and is homogenously white. With diffuse illumination, underlying structures are clearly visible.	2
Involvement of the entire thickness of the stroma with endothelium intact. With optical section, the endothelial surface is still visible. However, with diffuse illumination the underlying structures are just barely visible (to the extent that the observer is still able to grade flare and iritis, observe for pupillary response, and note lenticular changes).	3
Involvement of the entire thickness of the stroma with endothelium damaged. With the optical section, cannot clearly visualize the endothelium. With diffuse illumination, the underlying structures cannot be seen. Cloudiness removes the capability for judging and grading flare, iritis, lenticular changes, and pupillary response.	4
Corneal area	**Individual Score**
Normal cornea with no area of cloudiness	0
1% to 25% area of stromal cloudiness	1
26% to 50% area of stromal cloudiness	2
51% to 75% area of stromal cloudiness	3
76% to 100% area of stromal cloudiness	4
Overall Corneal Opacity/Area	**Product Score**
Corneal Opacity x Area[2]	Maximum of 16

[1] From: Hackett and McDonald (1991).

[2] The overall corneal opacity score is the product of the corneal opacity score and the corneal area score. The product of individual scores of 1 and 4 (Product Score of 4) or 2 and 2 (Product Score of 4), for example, would each qualify for a severe irritant rating based on the overall corneal opacity/area score.

c. Additional endpoints such as histopathology to look at each of the various corneal tissue layers (i.e., epithelium, Bowman's layer, stroma, Descemet's layer, and endothelium) or confocal microscopy with live/dead cell staining may be used to corroborate or to re-evaluate the actual depth of injury, particularly where equivocal results may have been obtained by use of existing endpoints or where the irritancy of a substance falls into the interface between a severe and nonsevere irritant. A standardized scoring scheme using the formal language of pathology to describe any effects should be included.

6.5.4 Fluorescein penetration

- At the end of the 4-hour testing period or earlier score each cornea for fluorescein penetration using a 10 ± 2.0 seconds application followed by a thorough rinse with physiological saline or negative control buffer (**Table 6-2**).

Table 6-2 Fluorescein Penetration Scoring System[1]

Description	**Individual Scores (Area/Intensity)**
Negligible — No staining.	0
Slight staining confined to small focal area. Some loss of detail in underlying structures with diffuse illumination.	1
Moderate staining confined to a small focal area. Some loss of detail in underlying structures on diffuse illumination.	2
Marked staining involving a larger portion of the cornea. Underlying structures are barely visible but not completely obliterated with diffuse illumination.	3
Extreme staining with no visibility of underlying structures.	4
Fluorescein Penetration	**Product Score**
Fluorescein Area x Intensity	**Maximum of 16**

[1] From: Hackett and McDonald (1991).

7.0 Evaluation of Test Results

Using the scores obtained from the endpoints evaluated (as described above), determine if the test substance meets the criteria for a corrosive or severe ocular irritant using the decision criteria provided in **Table 8-1**.

8.0 Criteria for an Acceptable Test

- If, in the course of evaluation of three eyes, there is significant disagreement in the results between eyes, repeat the experiment and calculate the mean for all six determinations to assess overall damage.
- Changes in control eyes greater than 7% during the 4-hour observation period warrant rejection of the experiment.
- A test is considered acceptable if the negative control produces either no effect or only slight or marginal effects on the various parameters and the positive control produces a severe irritant effect as defined in **Table 8-1**.

Table 8-1 Decision Criteria for Determination of Severe Irritants: Overall Scoring System for Corneal Damage and Irritation[1]

Ocular Parameter	Cut-off Value to Detect Severe Eye Irritants
Maximum Corneal Opacity[2] Cloudiness x Area	Greater than or equal to a score of 3
Maximum Fluorescein Uptake[3] Intensity x Area	Greater than or equal to a score of 4
Mean Corneal Swelling[4] 0.5 hours 1 hour 2 hours 3 hours 4 hours	Greater than or equal to 25%
Corneal Epithelial Observations[5]	Any pitting, mottling, or sloughing

[1] From: Guerriero et al., 2002

[2] Represents maximum score obtained in 3 eyes

[3] Represents maximum score obtained in 3 eyes

[4] Represents mean swelling calculated for 3 eyes

[5] Represents information obtained for any single animal

- Control charts should be used to monitor historical responses and calculate acceptable ranges for negative and positive controls, and benchmark controls when used, over time and across laboratories. These ranges should be updated frequently to adjust test acceptance criteria for individual control substances. An acceptable test would then have positive or benchmark controls that fell within these acceptable ranges.

9.0 Data Interpretation

Test substances meeting or exceeding the criteria for severe irritation defined in **Table 8-1** in an acceptable test (as defined in **Section 8.0**) are identified as severe irritants. Test substances not meeting these cut-off criteria in an acceptable test are identified as nonsevere irritants. Benchmark substances are recommended for comparing the responses of test substances of different product or chemical classes. It may be useful to carefully evaluate the pattern of responses in the four endpoints.

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)
- 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 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
- Complete supporting information for the enucleated rabbit 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

- Tabulation of data from individual test samples (e.g., irritancy scores for the test substance and the various controls, including data from replicate repeat experiments as appropriate, and means and ± the standard deviation for each trial)

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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