APPENDIX E
ICCVAM RECOMMENDED ICE TEST METHOD PROTOCOL

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

PREFACE

The information included in this protocol was extracted from published protocols, as well as 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 ICCVAM/NICEATM 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, 1996), the European Union (EU; EU, 2001), and in the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2003). Substances other than ocular corrosives and severe irritants (e.g., nonirritants and mild/moderate ocular irritants) have been tested using this protocol; however, the accuracy and reliability of the ICE test method have not yet been formally evaluated for the other classes of ocular irritancy defined by EPA (1996), EU (2001), and the GHS (UN 2003).

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 slaughterhouse), 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 should be included in each experiment to verify that an appropriate response is induced. If the ICE test method 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 ICE. 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 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 a ocular irritant. Appropriate benchmark controls should have the following properties:

- 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 slaughterhouse). Heads should be cut off 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 $\leq$ 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 a 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, examine the eyes again with the slit-lamp microscope to ensure that they have not been damaged (i.e., no corneal opacity) 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: 1) a corneal thickness deviating more than 10% from the mean value for the eyes, 2) a fluorescein retention score of > 0.5) any additional signs of damage should be rejected as test eyes and replaced.
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

Use a minimum of three eyes to be treated with each test substance (including both positive and negative controls).

6.4 Treatment of Eyes and Observations

6.4.1 Dosing procedure

- 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.
- Immediately following the zero reference measurement, apply the test substance to the eye (see **Sections 6.4.1.1** and **6.4.1.2**).
- 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.
- Apply the test material for a total of 10 seconds and then rinse the eye with 20 ml isotonic saline at room temperature.
- After the rinse step, return the eye to the superfusion apparatus.

6.4.1.1 *Liquid test substances*

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

6.4.1.2 *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

- 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.**
- 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.
- After the final (240 minutes) examination, immerse all eyes in 4% neutral buffered formaldehyde for preservation for possible histopathological examination (if necessary).
- 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.

6.4.2.1 *Criteria and Scoring System*

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

a. 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.

b. 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.

c. 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

d. 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.

e. 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 non-irritating 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 severe 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, 1 x II*
	2 x IV, 1 x I*
	Corneal opacity ≥ 3 at 30 min (in at least 2 eyes)
	Corneal opacity = 4 at any time point (in at least 2 eyes)
	Severe loosening of the epithelium (in at least 1 eye)

*Combinations less likely to occur.

11.0 STUDY REPORT

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 positive, negative, and benchmark controls, including data from replicate repeat experiments as appropriate, and means and ± 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 2003a, 2003b; FDA 2003) should be followed.

12.0 REFERENCES

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