

**ICCVAM-Recommended Test Method Protocol
Isolated Rabbit Eye Test Method**

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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 $32 \pm 1.5^\circ\text{C}$ (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 $32 \pm 1.5^\circ\text{C}$)
- 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).¹

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

¹ 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 shipment 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 $32 \pm 1.5^\circ\text{C}$.

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 Irritation¹

Description	
Cornea	Individual Score
<i>Normal cornea.</i> 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
<i>Some loss of transparency.</i> 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
<i>Moderate loss of transparency.</i> 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
<i>Involvement of the entire thickness of the stroma with endothelium intact.</i> 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
<i>Involvement of the entire thickness of the stroma with endothelium damaged.</i> 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 System¹

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 Irritation¹

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 \pm 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.

11.0 References

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