

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

Preface

This protocol is based on comprehensive test method evaluations conducted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which included international independent scientific peer reviews of the validation status of the ICE test method, and expert comments and suggested revisions received during the review process for Organisation for Economic Co-operation and Development (OECD) documents relevant to the ICE test method. Future studies using the ICE test method could include further characterization of the usefulness or limitations of the ICE test method in a weight-of-evidence approach for regulatory decision-making. Users should be aware that the test method protocol could be revised based on 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.

The ICE test method was evaluated by ICCVAM, in conjunction with the European Centre for the Validation of Alternative Methods and the Japanese Center for the Validation of Alternative Methods, in 2006 and 2010 (ICCVAM 2006, 2010). As part of the first evaluation (ICCVAM 2006) an ICCVAM-recommended protocol was developed based on information obtained from published protocols, as well as the current protocol used by TNO Quality of Life Department of Toxicology and Applied Pharmacology (Prinsen and Koeter 1993; INVITTOX 1994; Balls et al. 1995; Prinsen 1996; Chamberlain et al. 1997). As part of the second ICCVAM evaluation (2010), the ICCVAM-recommended protocol was updated to reflect expert comments and suggested revisions received during the review process for OECD Test Guideline (TG) 438 (OECD 2009), which describes use of the ICE test method for identifying ocular corrosives and severe irritants. Since then, the protocol was revised in response to expert comments and suggested revisions received on OECD Guidance Document (GD)(OECD 2011). This GD, which accompanies OECD TG 438, provides users with guidelines for collecting histopathology data for *in vitro* and/or *in vivo* eye safety test methods.

Relevant Document	Date	Basis for Protocol Revisions/Updates
ICCVAM Test Method Evaluation Report (ICCVAM 2006)	November 2006	Developed from TNO protocol, Prinsen and Koeter 1993, INVITTOX Protocol 80 (1994), Balls et al. 1995, Prinsen 1996, and Chamberlain et al. 1997
OECD TG 438 (OECD 2009)	September 2009	Based on ICCVAM-recommended protocol (ICCVAM 2006)
ICCVAM Test Method Evaluation Report (ICCVAM 2010)	September 2010	Updated based on expert comments received during OECD TG 438 review process
OECD GD 160 (ICE protocol, Annex V) (OECD 2011)	October 2011	Based on ICCVAM-recommended protocol (ICCVAM 2010); Updated based on expert comments received during OECD GD 160 review process

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) and United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (UN 2009). Substances other than ocular corrosives and severe irritants (e.g., substances not 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) and GHS (UN 2009).

2.0 Safety and Operating Precautions

All procedures with chicken eyes should follow the institution's applicable regulations and procedures for handling of human or animal materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended, including the use of laboratory coats, eye protection, and gloves. If available, additional precautions required for specific study substances should be identified in the Material Safety Data Sheet for that substance.

3.0 Materials, Equipment, and Supplies

3.1 Source of Chicken Eyes

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

3.2 Equipment and Supplies

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

3.3 Solutions

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

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

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 solvent is physiological saline. However, alternative solvents may also be used under controlled conditions, but the appropriateness of solvents other than physiological saline must be demonstrated.

4.2 Solid Test Substances

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

5.0 Controls

5.1 Negative Controls

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

5.2 Solvent/Vehicle Controls

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

5.3 Positive Controls

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

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

5.4 Benchmark Controls

Benchmark substances are useful for evaluating the ocular irritancy potential of unknown chemicals of a specific chemical or product class, or for evaluating the relative irritancy potential of an ocular irritant within a specific range of irritant responses. Appropriate benchmark controls should have the following properties:

- A consistent and reliable source(s) for the chemical
- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects *in vivo*
- Known potency in the range of the desired response

6.0 Experimental Design

6.1 Collection and Transport Conditions of Chicken Eyes

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

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

6.2 Preparation of Eyes

- a. Carefully remove the eyelids without damaging the cornea. Place a drop of sodium fluorescein 2% w/v onto the corneal surface for 10-20 seconds, and then immediately rinse the eye with 20 mL isotonic saline. Examine the fluorescein-treated cornea with a slit-lamp microscope to ensure that the cornea is undamaged (i.e., fluorescein retention and corneal opacity scores ≤ 0.5).
- b. If undamaged, further dissect the eye from the eye socket, taking care not to damage the corneal epithelium. When removing the eye from the orbit, a visible portion of the optic nerve should be left attached to the eye.
- c. Once removed from the orbit, place the eye on an absorbent pad and cut away the nictitating membrane and other connective tissue.
- d. Mount the eyes in stainless steel clamps (one eye per clamp), with the cornea positioned vertically and then transfer each clamp to a chamber in the superfusion apparatus. The chambers of the superfusion apparatus should be temperature controlled at $32 \pm 1.5^\circ\text{C}$ 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, the eye (in its holder) is removed from the superfusion apparatus, placed in a horizontal position, and the test substance is applied to the cornea (see **Sections 6.4.1.1** and **6.4.1.2**).
- c. Apply the test material for a total of 10 seconds and then rinse the eye with 20 mL isotonic saline at room temperature.
- d. After the rinse step, return the eye to the superfusion apparatus.

6.4.1.1 Liquid test substances

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

6.4.1.2 Solid test materials

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

6.4.2 Endpoint observations

- a. Examine the control and test eyes at 30, 75, 120, 180, and 240 minutes (± 5 minutes) after the post-treatment rinse using the scoring system and criteria as indicated in **Section 9.0**.
- b. Corneal opacity, corneal thickness, and any morphological effects should be evaluated at each time point, while fluorescein retention is determined only at the 30-minute time point.
- c. After the final (240 minutes) examination, immerse all eyes in an appropriate fixative (e.g., neutral buffered formalin) for possible histopathological examination (if necessary).
- d. To maximize the likelihood of obtaining reproducible results, reference photographs for all subjective endpoints (i.e., corneal opacity, fluorescein retention, morphological effects, histopathology) should be readily available.

7.0 Evaluation of Test Results

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

8.0 Criteria for an Acceptable Test

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

9.0 Data Interpretation

9.1 Corneal Thickness

Corneal swelling is determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope. It is expressed as a percentage and is calculated from corneal thickness measurements according to the following formula:

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

The mean percentage of swelling for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal swelling, as observed at any time point, an overall category score is then given for each test substance.

Mean Corneal Swelling (%)	Category
0 to 5	I
> 5 to 12	II
> 12 to 18 (>75 minutes after treatment)	II
> 12 to 18 (≤75 minutes after treatment)	III
> 18 to 26	III
> 26 to 32 (>75 minutes after treatment)	III
> 26 to 32 (≤75 minutes after treatment)	IV
> 32	IV

9.2 Corneal Opacity

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

Score	Observation
0	No opacity
0.5	Very faint opacity
1	Scattered or diffuse areas; details of the iris are clearly visible
2	Easily discernible translucent area; details of the iris are slightly obscured

3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

The mean corneal opacity value for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal opacity, as observed at any time point, an overall category score is then given for each test substance.

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

9.3 Fluorescein Retention

Fluorescein retention is evaluated at the 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:

<i>Score</i>	<i>Observation</i>
0	No fluorescein retention
0.5	Very minor single cell staining
1	Single cell staining scattered throughout the treated area of the cornea
2	Focal or confluent dense single cell staining
3	Confluent large areas of the cornea retaining fluorescein

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

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

Morphological effects include “pitting” of corneal epithelial cells, “loosening” of epithelium, “roughening” of the corneal surface and “sticking” of the test substance to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the interpretation of the investigator.

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

10.0 Assessment of the Eye Irritancy

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

Classification	Combinations of the Three Endpoints
Corrosive/Severe Irritant	3 x IV
	2 x IV, 1 x III
	2 x IV, 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

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

Balls M, Botham PA, Bruner LH, Spielmann H. 1995. The EC/HO international validation study on alternatives to the Draize eye irritation test. *Toxicol In Vitro* 9:871-929.

Chamberlain M, Gad SC, Gautheron P, Prinsen MK. 1997. IRAG Working Group I: Organotypic models for the assessment/prediction of ocular irritation. *Food Chem Toxicol* 35:23-37.

EPA. 2003a. Label Review Manual: 3rd ed. EPA737-B-96-001. Washington, DC: U.S. Environmental Protection Agency.

EPA. 2003b. Good Laboratory Practice Standards. 40 CF R792.

EPA. 2003c. Good Laboratory Practice Standards. 40 CFR 160.

FDA. 2003. Good Laboratory Practice for Nonclinical Laboratory Studies. 21 CFR 58.

ICCVAM. 2006. ICCVAM Test Method Evaluation Report: *In Vitro* Ocular Toxicity Test Methods for Identifying Severe Irritants and Corrosives. NIH Publication No. 07-4517. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmmer.htm.

ICCVAM. 2010. ICCVAM Test Method Evaluation Report: Current Validation Status of *In Vitro* Test Methods Proposed for Identifying Eye Injury Hazard Potential of Chemicals and Products. NIH Publication No. 10-7553. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: <http://iccvam.niehs.nih.gov/methods/ocutox/MildMod-TMER.htm>.

- INVITTOX. 1994. Protocol No. 80: Chicken enucleated eye test (CEET). Available: <http://ecvam-sis.jrc.it/>.
- OECD. 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring Number 1: OECD Principles on Good Laboratory Practice [as revised in 1997]. ENV/MC/CHEM(98)17. Paris:Organisation for Economic Co-operation and Development. Available: <http://www.oecd.org>.
- OCED. 2009. Test Guideline 438. Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants, adopted September 7, 2009. In: OECD Guidelines for Testing of Chemicals. Paris:Organisation for Economic Co-operation and Development.
- OECD. 2011. Guidance Document on The Bovine Corneal Opacity and Permeability (BCOP) and Isolated Chicken Eye (ICE) Test Methods: Collection of Tissues for Histological Evaluation and Colleection of Data on Non-severe Irritants. Series on Testing and Assessment, No. 160, adopted October 25, 2011. Paris:Organisation for Economic Co-operation and Development.
- Prinsen MK. 1996. The chicken enucleated eye test (CEET): A practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. Food Chem Toxicol 34:291-296.
- Prinsen MK, Koëter BWM. 1993. Justification of the enucleated eye test with eyes of slaughterhouse animals as an alternative to the Draize eye irritation test with rabbits. Food Chem Toxicol 31:69-76.
- UN (2009), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva. Available: http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html.