The isolated chicken eye (ICE) test method is an in vitro assay that provides short-term (1 hour) measurements of the whole chicken eye (i.e., cornea, conjunctiva, and iris). Corneal swelling, opacity, and fluorescein retention are assessed as indicators of potential ocular irritation and corrosion.

Following examination of the validation status of the ICE, the Interagency COmmittee for the Validation of Alternative Methods (ICVAM) recommended ICE for use in a tiered-testing strategy for regulatory classification and labeling. These recommendations were made after consideration of public comments on the initial report submitted by the Institute for Laboratory Animal Research (ILAR).

To have the greatest impact as reducing animal use (CVAM), with input from representatives in the U.S., Europe (EU), and Japan, a Working Group on Organism Conservation and Development (GECID) Test Guidelines (TG) that included an ICVAM ICE test method protocol was established.

This protocol was developed following an international peer review process in which the ICE test was submitted to the Interagency COmmittee for the Validation of Alternative Methods (ICVAM) and the Japanese Center for the Validation of Alternative Methods (JCGM) for consideration for inclusion in the International Acceptance of ICE (JCGM 2006). ICE Class I and II results were nearly accepted by the ICVAM Working Group of National Coordinators. Once formally adopted by the ICVAM Council, all ICVAM member countries will accept ICE, in accordance with OC ED Mound Acceptance of Test Methods. Prinsen (2005) have further characterized the test results from ICE can be used in the U.S. instead of the isolated rabbit eye test for defining acute regulatory hazard classification tolerances.

The use of ICE will reduce the need for rabbit eye safety testing and enhance the in vivo testing of a wide range of substances likely to cause severe pain and discomfort.

Test Method Overview (see Figure 1)

- Heads are collected from chickens obtained from a slaughterhouse and preserved in an injectable fixative solution (10% formalin) in a sterile steel jar with the cranial portion ventral. The eyes are then removed for a standard superfrosting apparatus (see Table 1).
- Damage to the test substance is assessed by determination of cornal swelling, opacity, and fluorescein retention.
- ICE can be used to classify certain types of substances as ocular corrosives and severe irritants. ICE does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

ICE Test Method Performance

A complete description of the laboratory and the results accuracy and reliability analysis conducted for the ICE test method can be obtained at http://www.niehs.nih.gov/nc3i/ice/.

The accuracy of the ICE test method when compared to in vivo rabbit eye tests classifications using the United Nations (UN) classification system (UN 2007) and the EU guidelines (European Union 1998) classification system are provided in Table 2. This includes the accuracy of ICE when all substances were evaluated and when specific chemical and physical classes are excluded to increase the accuracy and decrease false positive and false negative results (see Table 2).

Table 1: Comparison of Ocular Irritancy Classification of the ICE Test Method with the Acceptance of 10 ICE

<table>
<thead>
<tr>
<th>Chemical</th>
<th>ICE Test Method</th>
<th>General Acceptance (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 1</td>
<td>83%</td>
<td>83%</td>
</tr>
<tr>
<td>Category 2</td>
<td>75%</td>
<td>67%</td>
</tr>
<tr>
<td>Category 3</td>
<td>50%</td>
<td>45%</td>
</tr>
<tr>
<td>Category 4</td>
<td>0%</td>
<td>14%</td>
</tr>
</tbody>
</table>

Test Method Solutions (see Tables 1 and 2)

- Solutions are collected from chickens obtained from a slaughterhouse and preserved in an injectable fixative solution (10% formalin) in a sterile steel jar with the cranial portion ventral. The eyes are then removed for a standard superfrosting apparatus (see Table 1).
- Damage to the test substance is assessed by determination of cornal swelling, opacity, and fluorescein retention.
- ICE can be used to classify certain types of substances as ocular corrosives and severe irritants. ICE does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

Within the ICE method, the test solution is placed on the corneal surface. The cornea is then examined for corneal swelling, opacity, and fluorescein retention.

Selection Criteria and Preparation of Eyes Used in the ICE

- Eyes with high baseline fluorescein staining (i.e., > 0.5) after they are enucleated are rejected.
- The eyes can be carefully removed and the cornea is then examined for damage, a standard microscope is placed on the eye, and the cornea is stained.
- If the fluorescein score is determined as positive for a test classification, the cornea is then exposed to the standard superfrosting apparatus.
- After a final in vivo examination, acceptable eyes are incubated for approximately 12 to 14 hours to equalize them to the test system prior to in vitro testing. Following the equalization period, a zero reference measurement is recorded for the corneal surface and opacity to serve as a baseline (i.e., k = 0).

Application of the Test Substance

- Each treatment group and positive control consists of a minimum of three eyes with the negative control consisting of one eye.
- Eyes are prepared for applying a zero reference measurement as a recorded baseline.
- The test to be assessed is placed into the superfrosting apparatus, placed in a horizontal position, and the test substance is applied to the cornea.
- Liquid test substances are typically tested using a standard volume of 25 µL.
- Solid substances should be ground as finely as possible and applied to 25 µL of the test solution on the surface of the cornea and allowed to contact the corneal surface.
- Exposure time (fix or fit) is 10 seconds.
- The eyes are then carefully washed with saline (approximately 25 mL) and fluorescein retention is measured in the superfrosting apparatus in the original weight position.

Selection of Specific Test Substances for the ICE Test Method


Decision and Study Acceptance Criteria

- Overall in vitro classification is determined by combinations of the 3 endpoints.
- A test is considered acceptable if the concurrent negative and positive controls and the concurrent positive controls give an irritation classification that falls within nonirritant and severe irritation classes, respectively.

Endpoints Measured for Rodent Transplant

- Corneal opacity (observed histologically), swelling determined from corneal thickness measurements performed by an optical pachymeter or an electron microscope, and morphology effects (e.g., effects of healing of the corneal wound and inflammatory cell response). Swelling was measured using a pachymeter at various times (e.g., 24 hours) after the post-treatment time. Fluorescein retention is determined only in permanent and 60 seconds after post-treatment.

- Photographs are available to document corneal opacity, fluorescein retention, morphological effects and, if conducted, histopathology.

- Results from corneal opacity, swelling, and fluorescein retention should be evaluated separately to generate an ocular class for each endpoint.

- The ICE classes for each endpoint are then combined to generate an irritation classification for each test substance.

Collection and Transport of Eyes to the Lab

- Because eyes are collected in the laboratory, the intact heads are obtained from the slaughterhouse and preserved in an injectable fixative solution (10% formalin) in a sterile steel jar with the cranial portion ventral. The eyes are then removed and transported to the laboratory.

Table 2: False Negativity and False Positivity Rates of ICE, by Chemical Class and Properties of Test Substance

<table>
<thead>
<tr>
<th>Chemical Property</th>
<th>False Negativity (%)</th>
<th>False Positivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 1</td>
<td>83%</td>
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</table>

Central Substances

- Central regulations on automobile headlight controls and positive controls are included in each experiment.
- Examples of positive controls for liquid test substances are 1% acetic acid or 1% benzalkonium chloride.
- Examples of positive controls for solid test substances are sodium hypochlorite or iodine.

References


Table 3: Recommended Substances for Demonstrating Technical Proficiency with ICE

<table>
<thead>
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</tbody>
</table>

- Overall in vitro classification is determined by combinations of the 3 endpoints.
- A test is considered acceptable if the concurrent negative and positive controls and the concurrent positive controls give an irritation classification that falls within nonirritant and severe irritation classes, respectively.

- Substances that are positive in this assay can be used as ocular corrosives or severe irritants without further testing in rabbits.

- A substance that tests negative would need to be tested in rabbits using a sequential testing strategy, as outlined in OECD Test Guideline 405.