International Acceptance of *In Vitro* Alternative Ocular Safety Testing Methods: The Isolated Chicken Eye (ICE) Test Method (draft OECD TG 438)

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Introduction

- The ICE test method is an *in vitro* model that provides short-term (approx. 7 hours) maintenance of the whole chicken eye (cornea).
- 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 Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that ICE can be used to classify positive substances as ocular corrosives and severe irritants (ICCVAM 2007) for certain chemical classes.
- While it is not considered valid as a complete replacement for the *in vivo* rabbit eye test, ICCVAM recommended ICE for use in a tiered-testing strategy for regulatory classification and labeling.
- These recommendations were made after consideration of public comments and a report from an independent international peer review panel.
- To have the greatest impact on reducing animal use, ICCVAM, with input from stakeholders in the U.S., EU, and Japan, drafted an OECD Test Guideline (TG) that is based on the ICCVAM ICE test method protocol.
- This protocol was developed following an international peer review evaluation with contributions from ECVAM and JaCVAM. Draft TG 438 was recently accepted by the OECD WNT and once formally adopted by the OECD Council, all 30 OECD

member countries will accept TG 438, in accordance with OECD Mutual Acceptance of Data (MAD) Treaty.

- The protocol was accepted by U.S. Federal agencies, and positive results from ICE can be used in the U.S. instead of the rabbit eye test for making certain regulatory hazard classification decisions.
- The use of ICE will reduce the use of rabbits for eye safety testing and eliminate the *in vivo* testing in animals of many substances likely to cause severe pain and discomfort.

Test Method Overview (see Figure 1)

- Heads are collected from chickens obtained from a slaughterhouse and eyes carefully dissected in the laboratory are mounted in a stainless steel clamp with the cornea positioned vertically. The clamp is then transferred to a chamber of the superfusion apparatus (**Figure 2**).
- Damage by the test substance is assessed by determination of corneal swelling, opacity, and fluorescein retention.
- ICE can be used to classify certain types of substances as ocular corrosives and severe irritants as defined by national and international hazard classification systems (**Table 1**).

ICE Test Method Performance

- A complete description of the databases and the resulting accuracy and reliability analysis conducted for the ICE test method can be obtained at http://www.iccvam.niehs.gov/methods/ocudocs/.
- The accuracy of the ICE test method when compared to *in vivo* rabbit eye test classifications using the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS; UN 2007) classification system are provided in **Table 1**. This includes the accuracy of ICE when all substances were evaluated and when specific chemical and physical classes are excluded to increases the accuracy and decrease false positive and false negative rates (see also **Table 2**).

Limitations of ICE

- Positive results obtained with alcohols should be interpreted cautiously due to risk of overprediction.
- ICE does not consider conjunctival and iridal injuries.
- ICE does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

Proficiency Chemicals for the ICE Test Method

- Prior to routine use of ICE, laboratories may wish to demonstrate technical proficiency by correctly identifying the ocular corrosivity classification of the 10 substances recommended in **Table 3**.
- Irritant substances were selected from the ICCVAM recommended list of 122 reference substances for the validation of *in vitro* ocular toxicity test methods (ICCVAM 2007)
- *In vivo* and ICE reference data are available in the ICCVAM Background Review Document for ICE (ICCVAM 2006).
- These substances were selected to represent the range of responses for local eye irritation/corrosion, which is based on results in the *in vivo* rabbit eye test (OECD TG 405, GHS Categories 1, 2A, 2B, or Not Labeled).
- Other selection criteria were that:
    - Substances are commercially available
    - There are high quality *in vivo* reference data available
    - There are high quality data from ICE

Collection and Transport of Eyes to the Laboratory

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

Table 1. Comparison of Performance Characteristics of the ICE Test Method for Identification of GHS Severe Ocular Irritants or Corrosives[1]

In Vitro Method	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
ICE – All Substances (N=144)[2,5]	83% (120/144)	50% (15/30)	92% (105/114)	8% (9/114)	50% (15/30)
Prinsen and Koëter (1993)	80 (8/10)	100 (2/2)	75 (6/8)	25 (2/8)	0 (0/2)
Balls et al. (1995)[3,4]	69 (37/54)	50 (11/22)	81 (26/32)	19 (6/32)	50 (11/22)
Balls et al. (1995)[3]	70 (150/215)	46 (40/87)	86 (110/128)	14 (18/128)	54 (47/87)
Prinsen (1996)	97 (35/36)	50 (1/2)	100 (34/34)	0 (0/34)	50 (1/2)
Prinsen (2005)	89 (41/46)	0 (0/4)	98 (41/42)	2 (1/42)	100 (4/4)
ICE – Excluding alcohols, surfactants, and solids (N=85)	92% (69/75)	71% (5/7)	94% (64/68)	6% (4/68)	29% (2/7)

Abbreviations: ICE = isolated chicken eye test method; GHS = United Nations Globally Harmonized System

[1] ICCVAM (2006).

[2] N = number of substances in the database; the numbers in parenthesis indicate the data on which the % value is based.

[3] One chemical (benzalkonium chloride, 1%) was tested *in vivo* twice within the same laboratory. The results were discordant with respect to GHS classification; the analysis was performed assuming Category 1 classification.

[4] Performance calculated using the overall *in vitro* classification based on the majority and/or most severe classification among the four laboratories.

[5] Includes the data from Balls et.al. (1995) using the overall *in vitro* classification based on the majority and/or most severe classification among the four laboratories.

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Figure 1. Basic Procedures for the ICE Assay

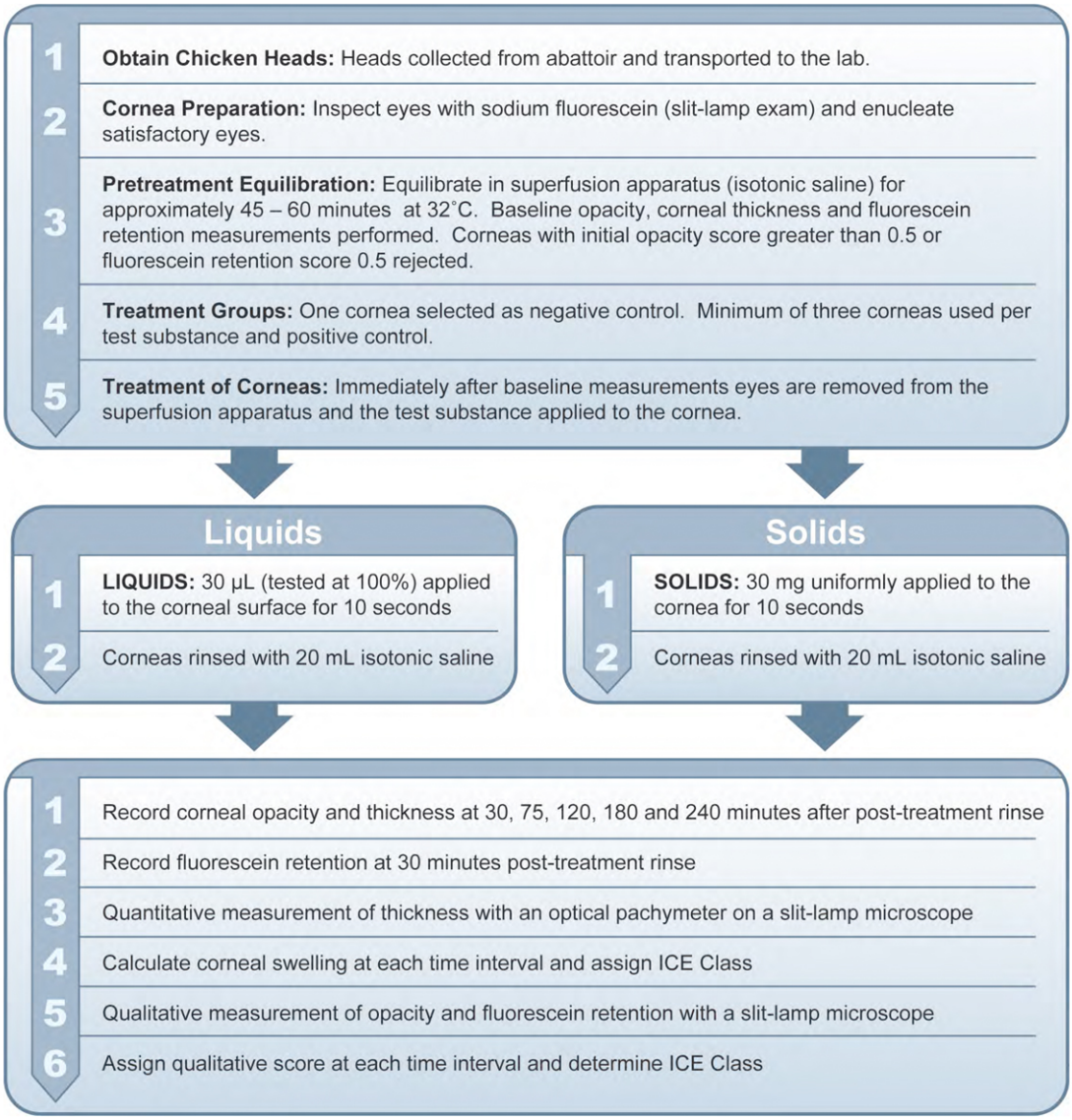


Table 2. False Negative and False Positive Rates of ICE, by Chemical Class and Properties of Interest, for the GHS Classification System

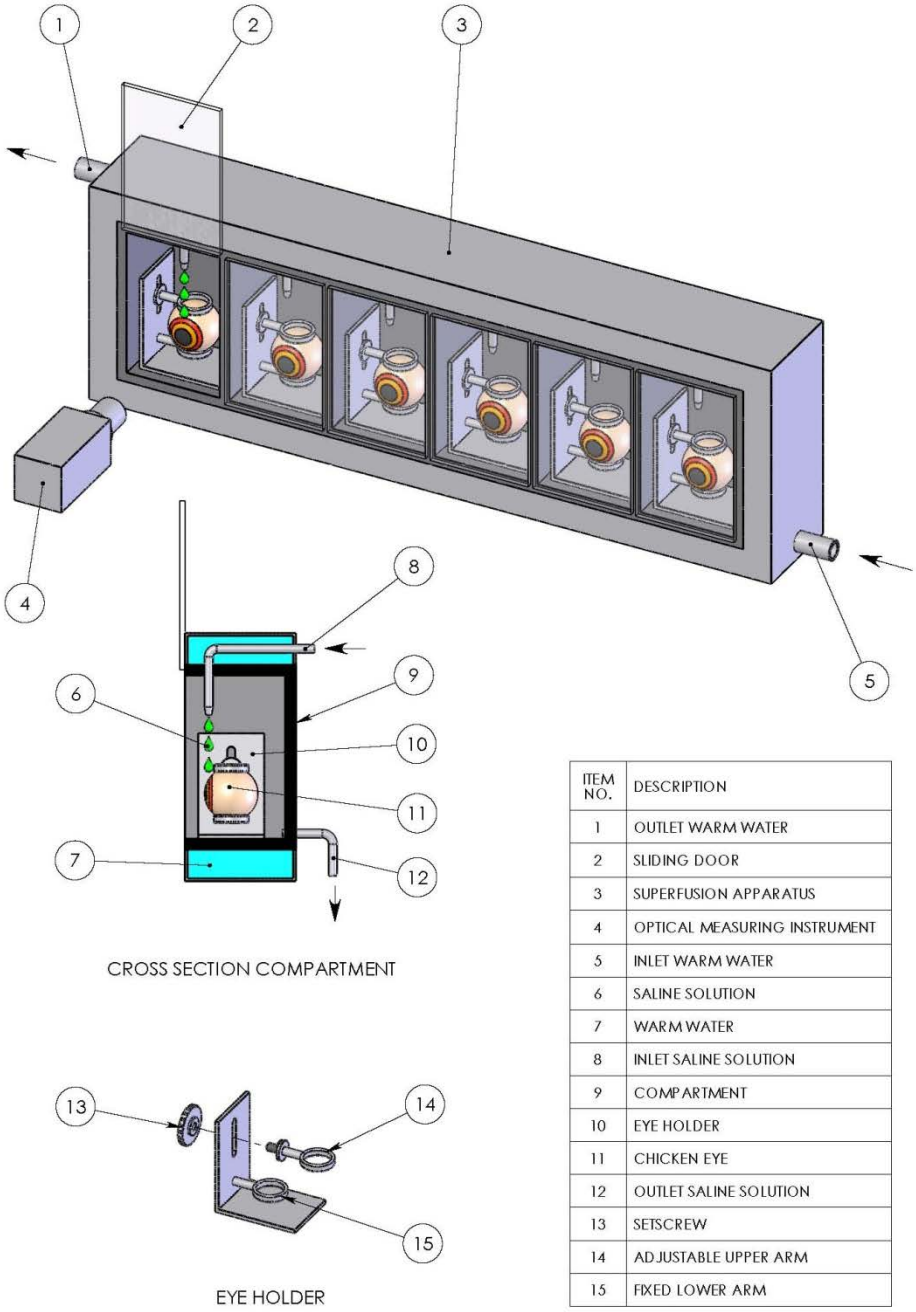
Category	N[1]	False Positive Rate[2]	False Negative Rate[2]
Overall	**144**	**8% (9/114)**	**50% (15/30)**
Chemical Class[3]			
Alcohols	12	50% (5/10)	50% (1/2)
Carboxylic acids	10	0% (0/3)	43% (3/7)
Esters	9	13% (1/8)	0% (0/1)
Heterocycles	9	0% (0/3)	33% (2/6)
Onium compounds	8	0% (0/2)	33% (2/6)
Properties of Interest			
Liquids	108	10% (9/90)	44% (8/18)
Solids	36	0% (0/24)	58% (7/12)
Pesticides	11	0% (0/6)	60% (3/5)
Surfactants	21	0% (0/12)	56% (5/9)

[1] N = number of substances tested

[2] False Positive Rate = the proportion of all negative substances that are falsely identified as positive *in vitro*; False Negative Rate = the proportion of all positive substances that are falsely identified as negative *in vitro*. Shaded cells indicate positive results obtained with alcohols, which should be interpreted cautiously due to the risk of overprediction.

[3] Chemical classes included in this table are represented by at least five substances tested by the method and assignments are made based on the Medical Subject Heading categories (http://www.nim.nih.gov/mesh/)

Figure 2 ICE Superfusion Apparatus and Eye Clamps



ITEM NO.	DESCRIPTION
1	OUTLET WARM WATER
2	SLIDING DOOR
3	SUPERFUSION APPARATUS
4	OPTICAL MEASURING INSTRUMENT
5	INLET WARM WATER
6	SALINE SOLUTION
7	WARM WATER
8	INLET SALINE SOLUTION
9	COMPARTMENT
10	EYE HOLDER
11	CHICKEN EYE
12	OUTLET SALINE SOLUTION
13	SETSCREW
14	ADJUSTABLE UPPER ARM
15	FIXED LOWER ARM

Diagram courtesy of Mr. Menk Prinsen, TNO

Selection Criteria and Preparation of Eyes Used in the ICE

- Eyes that have high baseline fluorescein staining (*i.e.,* > 0.5) or corneal opacity score (*i.e.,* > 0.5) after they are enucleated are rejected.
- The eyelids are carefully excised and the cornea is quickly assessed for damage with a slit-lamp microscope after application of sodium fluorescein.
- If undamaged, the eye is removed from the orbit, further trimmed and mounted in a stainless steel clamp with the cornea positioned vertically. The clamp is then transferred to a chamber of the superfusion apparatus (**Figure 2**) maintained at 32 ± 1.5°C.
- After a final slit-lamp examination, acceptable eyes are incubated for approximately 45 to 60 minutes to equilibrate them to the test system prior to dosing. Following the equilibration period, a zero reference measurement is recorded for corneal thickness and opacity to serve as a baseline (*i.e.,* time = 0). The fluorescein score determined at dissection is used as the baseline measurement for that endpoint.

Application of the Test Substance

- Each treatment group and positive control consists of a minimum of three eyes while the negative control consists of one eye.
- Immediately prior to dosing a zero reference measurement is recorded for corneal thickness and opacity.
- 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.
- Liquid test substances are typically tested undiluted at a standard volume of 30 µL.
- Solid substances should be ground as finely as possible and applied (30 mg) to the cornea such that the surface is uniformly covered with the test substance.
- Exposure time (liquid or solid) is 10 seconds.
    - The eye is then rinsed with isotonic saline (approximately 20 mL) at ambient temperature and returned to the superfusion apparatus in the original upright position.

Table 3. Recommended Substances for Demonstrating Technical Proficiency with ICE

Chemical	CASRN	Chemical Class[1]	Physical Form	*In Vivo* Classification[2]	*In Vitro* Classification[3]
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid	Category 1	Corrosive/Severe Irritant
Chlorhexidine	55-56-1	Amine, Amidine	Solid	Category 1	Corrosive/Severe Irritant
Dibenzoyl-L-tartaric acid	2743-38-6	Carboxylic acid, Ester	Solid	Category 1	Corrosive/Severe Irritant
Imidazole	288-32-4	Heterocyclic	Solid	Category 1	Corrosive/Severe Irritant
Trichloroacetic acid (30%)	76-03-9	Carboxylic Acid	Liquid	Category 1	Corrosive/Severe Irritant
2,6-Dichloro-benzoyl chloride	4659-45-4	Acyl halide	Liquid	Category 2A	Noncorrosive/ Nonsevere irritant
Ethyl-2-methylaceto-acetate	609-14-3	Ketone, Ester	Liquid	Category 2B	Noncorrosive/ Nonsevere irritant
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2A	Noncorrosive/ Nonsevere irritant
Glycerol	56-81-5	Alcohol	Liquid	Not Labeled	Noncorrosive/ Nonsevere irritant
n-Hexane	110-54-3	Hydrocarbon (acyclic)	Liquid	Not Labeled	Noncorrosive/ Nonsevere irritant

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

[1] Chemical classes were assigned to each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http//www.nlm.nih.gov/mesh)

[2] Based on results from the *in vivo* rabbit eye test (OECD TG 405) and using the UN GHS.

[3] Based on results in ICE.

Control Substances

- Concurrent negative or solvent/vehicle controls and positive controls are included in each experiment.
  - Examples of positive controls for liquid test substances are 10% acetic acid or 5% benzalkonium chloride.
  - An example of a positive control for solid test substances is sodium hydroxide or imidazole.

Endpoints Measured and Data Evaluation

- Corneal opacity (slit-lamp examination), swelling (determined from corneal thickness measurements made with an optical pachymeter on a slit-lamp microscope), and morphological effects (e.g., pitting or loosening of the epithelium) are evaluated pretreatment and 30, 75, 120, 180, and 240 minutes (± 5 minutes) after the post-treatment rinse. Fluorescein retention is determined only at pretreatment and 30 minutes after post-treatment rinse.
- Photographs are advisable 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 ICE class for each endpoint.
- The ICE classes for each endpoint are then combined to generate an Irritancy Classification for each test substance.

Decision Criteria and Study Acceptance Criteria

- Once each endpoint has been evaluated, ICE classes are be assigned based on a predetermined range.

Corneal Thickness

Mean Corneal Swelling (%)*	ICE Class
0 to 5	I
>5 to 12	II
>12 to 18 (>75 min after treatment)	II
>12 to 18 (≤75 min after treatment)	III
>18 to 26	III
>26 to 32 (>75 min after treatment)	III
>26 to 32 (≤75 min after treatment)	IV
>32	IV

Corneal Opacity

Mean Maximum Opacity Score*	ICE Class
0.0-0.5	I
0.6-1.5	II
1.6-2.5	III
2.6-4.0	IV

Fluorescein Retention

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

- Overall *in vitro* irritancy classification is determined by combinations of the three endpoints.

Classification	Combinations of the 3 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.

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

Summary

- The ICE test method can be used, under certain circumstances and with specific limitations, to classify substances as ocular corrosives and severe irritants, as defined by the EPA, EU, and GHS hazard classification systems.
- While it is not considered valid as a complete replacement for the *in vivo* rabbit eye test, the ICE is recommended for use as part of a tiered testing strategy for regulatory classification and labeling within a specific applicability domain.
- Substances that are positive in this assay can be classified 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.

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