International Acceptance of *In Vitro* Alternative Ocular Safety Testing Methods: The Bovine Corneal Opacity and Permeability (BCOP) Test Method (draft OECD TG 437)

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Introduction

- The bovine corneal opacity and permeability (BCOP) test method is an *in vitro* method that provides short-term maintenance of physiological and biochemical function of the bovine cornea.
- Changes in opacity and permeability to fluorescein are assessed as indicators of potential ocular irritation and corrosiveness.
- Following examination of the validation status of the BCOP, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that BCOP can be used to classify positive substances as ocular corrosives and severe irritants (ICCVAM 2007) for certain chemical classes. While not a complete replacement for the *in vivo* rabbit eye test, BCOP can be used 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 stakeholders in the U.S., European Union (EU), and Japan, drafted an Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) that is based on the ICCVAM BCOP test method protocol.
- This protocol was developed following an international peer review evaluation with contributions from the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). Draft TG 437 was recently accepted by the OECD Working Group of National Coordinators. Once formally adopted by the OECD Council, all 30 OECD member countries will accept TG 437 in accordance with OECD Mutual Acceptance of Data Treaty.
- The protocol was subsequently accepted by U.S. Federal agencies, and positive results from BCOP can be used in the U.S. instead of the rabbit eye test for making certain regulatory hazard classification decisions.
- Use of BCOP will reduce rabbit use for eye safety testing and eliminate such testing in animals of most substances likely to cause severe pain and discomfort.

Test Method Overview (see Figure 1)

- Corneas are isolated from the eyes of freshly slaughtered cattle and are maintained in a corneal holder (**Figure 2)**.
    - Corneal opacity is measured quantitatively as the amount of light transmission through the cornea.
    - Permeability is measured quantitatively as the amount of sodium fluorescein dye that passes across the full thickness of the cornea, as detected in the medium in the posterior chamber.
- Damage by the test substance is assessed by quantitative measurements of changes in corneal opacity and permeability, which are used to calculate an *in vitro* irritation score (IVIS). The IVIS is used to assign a hazard classification category.
- BCOP 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**).

BCOP Test Method Performance and Limitations

- A complete description of the databases and the resulting accuracy and reliability analysis conducted for the BCOP test method can be obtained at http://www.iccvam.niehs.gov/methods/ocudocs/.
- The accuracy of the BCOP 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 BCOP when both the complete dataset was evaluated (N=147) and when discordant chemical and physical classes were excluded (N=85), increasing the accuracy and decreasing the false positive and false negative rates (see also **Table 2**).
- Positive results obtained with alcohols or ketones should be interpreted cautiously due to risk of overprediction (see **Table 2**)
- BCOP results do not correlate with conjunctival or iridal injuries.
- BCOP does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

Proficiency Chemicals for the BCOP Test Method

- Prior to routine use of BCOP, 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 BCOP reference data are available in the ICCVAM Background Review Document for BCOP (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, and there are high quality data from BCOP

Collection, Transport, Selection Criteria, and Preparation of Eyes Used in the BCOP

- Collect the eyes while preventing contact with any potentially irritant substances such as detergents.
- Immerse completely in Hanks' Balanced Salt Solution (HBSS) and transport to the laboratory on wet ice.
- Antibiotics added to the HBSS used to store the eyes during transport are recommended [e.g., penicillin at 100 IU/mL and streptomycin at 100 μg/mL].
- The time interval between collection of the eyes and use of corneas in the BCOP should be minimized (typically collected and used on the same day).
- Only corneas free of defects such as increased opacity, scratches, and neovascularization are to be used
- Acceptable corneas are dissected with a 2 to 3 mm rim of sclera remaining and placed in the corneal holders (**Figure 2**).
- Corneas are allowed to equilibrate in pre-warmed medium (32 ± 1°C) for at least one hour to achieve normal metabolic activity, to the extent possible.
- After equilibration, any corneas that show macroscopic tissue damage (e.g., scratches, pigmentation, neovascularization) or an opacity >7 opacity units are discarded.
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Figure 1. Basic Procedures for the BCOP Test Method

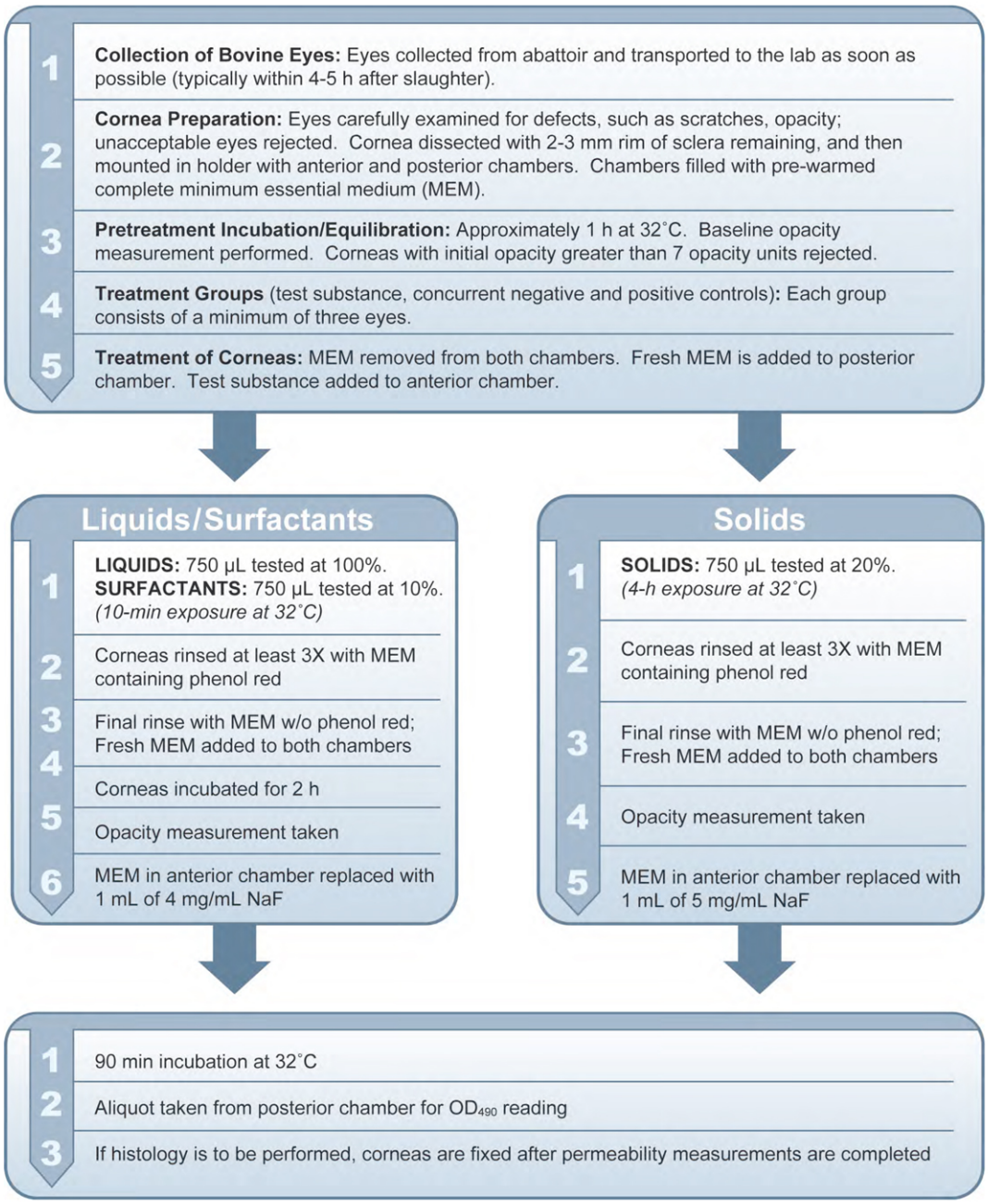

Table 1. Comparison of Performance Characteristics of the BCOP *In Vitro* Test Method for Identification of GHS Severe Ocular Irritants or Corrosives[1]

***In Vitro* Method**	**Accuracy**	**Sensitivity**	**Specificity**	**False Positive Rate**	**False Negative Rate**
BCOP – All Substances (N=147)[2]	81% (119/147)	84% (36/43)	80% (83/104)	20% (21/104)	16% (7/43)
BCOP – Excluding alcohols, ketones, and solids (N=85)	92% (78/85)	100% (27/27)	88% (51/58)	12% (7/58)	0% (0/27)

Abbreviation: BCOP = Bovine corneal opacity and permeability test method; GHS = UN Globally Harmonized System.

[1] ICCVAM (2006).

[2] N = number of substances tested; the number in parentheses in each column indicates the data on which the percentage calculation is based.

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

Category	N[1]	False Positive Rate[2]	False Negative Rate[2]
Overall	147	20% (21/104)	16% (7/43)
Chemical Class[3]			
Alcohols	18	53% (8/15)	67% (2/3)
Amine/Amidine	8	0% (0/4)	0% (0/4)
Carboxylic acids	15	38% (3/8)	14% (1/7)
Esters	12	12% (1/8)	0% (0/4)
Ether/Polyether	6	0% (0/5)	0% (0/1)
Heterocyclic compounds	12	33% (2/6)	17% (1/6)
Hydrocarbons	12	8% (1/12)	- (0/0)
Inorganic Salt	5	0% (0/3)	0% (0/2)
Ketones	10	40% (4/10)	- (0/0)
Onium compounds	11	0% (0/3)	0% (0/8)
Properties of Interest			
Liquids	92	26% (18/68)	4% (1/24)
Solids	32	10% (2/20)	42% (5/12)
Pesticide	8	33% (1/3)	40% (2/5)
Surfactant[4]	35	5% (1/21)	7% (1/14)

[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*. The data used to calculate the percentage are provided in parentheses.

[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.nlm.nih.gov/mesh/). Shaded cells indicate positive results obtained with alcohols or ketones, which should be interpreted cautiously due to risk of overprediction.

[4] Combines single chemicals labeled as surfactants along with surfactant-containing formulations.

Figure 2 BCOP corneal holder

- Made of an inert material (*e.g.*, polypropylene).
- Comprised of two halves (an anterior and posterior chamber), each chamber holds 5 mL.
- Corneas are placed endothelial side down on the posterior chambers and the anterior chambers are placed on the epithelial side of the corneas
- The end of each chamber houses a glass window which can be removed for easy access to the cornea.
- Two holes on the top of each chamber permit introduction and removal of medium and test compounds.

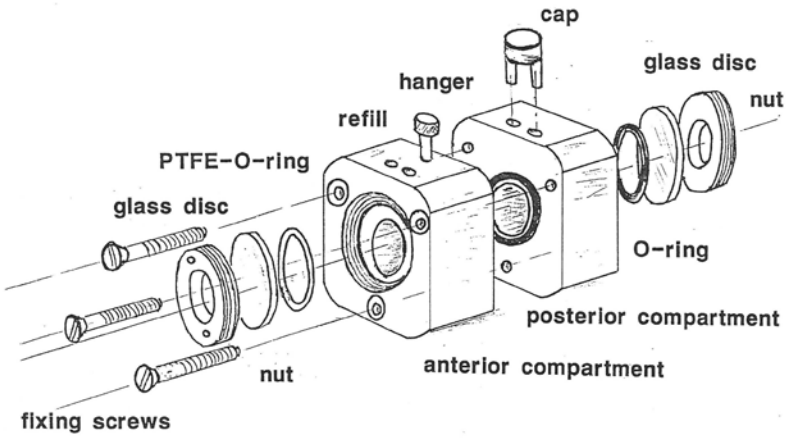

Table 3. Recommended Substances for Demonstrating Technical Proficiency with BCOP

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-Dichlorobenz-oyl 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 substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system.

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

[3] Based on results in BCOP.

Figure 3 BCOP Opacitometer

- Spectrophotometric measurement of opacity.
- Compares light transmission through control compartment versus light transmission through the experimental chamber containing the cornea.

Photo courtesy of Dr. Rodger Curren, Institute for In Vitro Sciences

Application of the Test Substance

- Each treatment group (test substance, concurrent negative and positive controls) consists of a minimum of three eyes.
- A closed-chamber method is typically used for nonviscous to slightly viscous liquid test substances, while an open-chamber method is typically used for semiviscous and viscous liquid test substances and for neat solids.
    - Closed-chamber method: 750 µL is introduced through the dosing holes and the holes are subsequently sealed with the chamber plugs during the exposure.
    - Open-chamber method: 750 µL is applied directly to the epithelial surface of the cornea using a micropipet. After dosing, the glass window is replaced on the anterior chamber to recreate a closed system.
- Two different treatment protocols are used, one for liquids and surfactants (solids or liquids), and one for non-surfactant solids.
    - Liquids are tested undiluted
    - Surfactants are tested at a concentration of 10% w/v in a 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system.
- Exposure time is 10 minutes (alternative exposure times may be used with appropriate scientific rationale)
- Non-surfactant solids are typically tested as solutions or suspensions at 20% concentration in a 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system.
    - Exposure time is 4 hours.
    - Solids may also be tested neat by direct application onto the corneal surface using the open chamber method
    - Alternative exposure times may be used with appropriate scientific rationale.
- It is critical to ensure that the test substance adequately covers the epithelial surface and that it is adequately removed during the rinsing steps.

Protocol: Post Application

Post-Exposure Incubation

- Following exposure, the substance is removed and the epithelium washed at least three times with EMEM
- After rinsing:
  - For liquids or surfactants, the corneas are incubated for an additional two hours at 32 ± 1ºC
  - For solids, corneas do not require further incubation.
- Opacity and permeability of each cornea are then recorded
- Each cornea is observed visually and pertinent observations recorded (e.g., tissue peeling, residual test substance, nonuniform opacity patterns).

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 1% sodium hydroxide or 10% dimethylformamide.
  - An example of a positive control for solid test substances is 20% (weight to volume) imidazole in 0.9% sodium chloride solution.

Endpoints Measured

- Opacity is determined by the amount of light transmission through the cornea using an opacitometer.
- Permeability is determined by the amount of sodium fluorescein dye that penetrates all corneal cell layers (i.e., the epithelium on the outer cornea surface through the endothelium on the inner cornea surface).

Data Evaluation

- The mean opacity and permeability OD_{490} values for each treatment group should be combined to calculate an in vitro irritation score (IVIS)

  IVIS = mean opacity value + (15 x mean permeability OD_{490} value)

Decision Criteria

- IVIS ≥ 55.1 is defined as a corrosive or severe irritant.
- IVIS < 55.1 requires additional testing in a method considered valid for identifying less severe ocular hazard categories.

Study Acceptance Criteria

- A test is considered acceptable if the positive control gives an IVIS that falls within two standard deviations of the current historical mean, which is to be updated at least every 3 months, or each time an acceptable test is conducted in laboratories where tests are conducted infrequently (i.e., less than once a month).
- The negative or solvent/vehicle control responses should result in opacity and permeability values that are less than the established upper limits for background opacity and permeability values for bovine corneas treated with the respective negative or solvent/vehicle control.

Summary

- The BCOP 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 BCOP is recommended for use as part of a tiered-testing strategy for regulatory classification and labelling 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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