



Section 4
Health effects

Test Guideline No. 437
Bovine Corneal Opacity And Permeability
Test Method For Identifying i) Chemicals
Inducing Serious Eye Damage
And ii) Chemicals Not Requiring
Classification For Eye Irritation Or
Serious Eye Damage

4 July 2023

**OECD Guidelines for the
Testing of Chemicals**



OECD GUIDELINE FOR TESTING OF CHEMICALS

Bovine Corneal Opacity And Permeability Test Method For Identifying i) Chemicals Inducing Serious Eye Damage And ii) Chemicals Not Requiring Classification For Eye Irritation Or Serious Eye Damage

INTRODUCTION

1. The Bovine Corneal Opacity and Permeability (BCOP) OP-KIT test method was evaluated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM), in 2006 and 2010 (1)(2). In the first evaluation, the BCOP test method was evaluated for its usefulness to identify chemicals (substances and mixtures) inducing serious eye damage (1). In the second evaluation, the BCOP test method was evaluated for its usefulness to identify chemicals (substances and mixtures) not classified for eye irritation or serious eye damage (2). The BCOP validation database contained 113 substances and 100 mixtures in total (2)(3). From these evaluations and their peer review it was concluded that the BCOP OP-KIT test method can correctly identify chemicals (both substances and mixtures) inducing serious eye damage as well as those not requiring classification for eye irritation or serious eye damage, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (4), and it was therefore endorsed as scientifically valid for both purposes. Serious eye damage is the production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Test chemicals inducing serious eye damage are classified as UN GHS Category 1. Chemicals not classified for eye irritation or serious eye damage are defined as those that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B), i.e. they are referred to as UN GHS No Category. This Test Guideline (adopted in 2009 and updated in 2013) includes the recommended use and limitations of the BCOP test method based on its evaluations. The main differences between the original 2009 version and the 2013 updated version concern, but are not limited to: the use of the BCOP test method to identify chemicals not requiring classification according to UN GHS (paragraphs 2 and 7); clarifications on the applicability of the BCOP test method to the testing of alcohols, ketones and solids (paragraphs 6 and 7) and of substances and mixtures (paragraph 8); clarifications on how surfactant substances and surfactant-containing mixtures should be tested (paragraph 28); updates and clarifications regarding the positive controls (paragraphs 39 and 40); an update of the BCOP test method decision criteria (paragraph 47); an update of the study acceptance criteria (paragraph 48); an update to the test report elements (paragraph 49); an update of Annex 1 on definitions; the addition of Annex 2 for the predictive capacity of the BCOP test method under various classification systems; an update of Annex 3 on the list of proficiency substances; and an update of Annex 4 on the BCOP corneal holder (paragraph 1) and on the opacitometer (paragraphs 2 and 3). In 2020, the Test Guideline was updated to allow the use of a laserlight-based opacitometer (LLBO) which was shown to have similar performance as the OP-KIT opacitometer used

in the validation of the BCOP test method (see paragraphs 6, 7, 42, 45, 48, Annex 2 and 5). Annex 3 on the list of proficiency substances has also been updated in 2020.

2. The Test method(s) described in this Test Guideline cannot be used on their own to replace the *in vivo* Draize eye test to predict across the full range of serious eye damage/eye irritation responses for different chemical classes. It is therefore recommended to make use of alternative testing strategies such as those described in TG 467 and 492B to address the required ranges of irritation potential. Strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test (6). The Top-Down approach (6) is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential, while the Bottom-Up approach (6) is designed to be used when, based on existing information, a chemical is expected not to cause sufficient eye irritation to require a classification. The BCOP test method is an *in vitro* test method that can be used under certain circumstances and with specific limitations for eye hazard classification and labeling of chemicals. While it is not considered valid as a stand-alone replacement for the *in vivo* rabbit eye test, the BCOP test method is recommended as an initial step within a testing strategy such as the Top-Down approach suggested by Scott *et al.* (6) to identify chemicals inducing serious eye damage, i.e. chemicals to be classified as UN GHS Category 1, without further testing (4). The BCOP test method is also recommended to identify chemicals that do not require classification for eye irritation or serious eye damage, as defined by the UN GHS (UN GHS No Category) (4) within a testing strategy such as the Bottom-up approach (6). However, a chemical that is not predicted as causing serious eye damage or as not classified for eye irritation/serious eye damage with the BCOP test method would require additional testing (*in vitro* and/or *in vivo*) to establish a definitive classification. The choice of the most appropriate test method and the use of this Test Guideline should be seen in the context of the OECD Guidance Document on an Integrated Approaches on Testing and Assessment for Serious Eye Damage and Eye irritation (7).

3. The purpose of this Test Guideline is to describe the procedures used to evaluate the eye hazard potential of a test chemical as measured by its ability to induce opacity and increased permeability in an isolated bovine cornea. Toxic effects to the cornea are measured by: (i) decreased light transmission (opacity), and (ii) increased passage of sodium fluorescein dye (permeability). The opacity and permeability assessments of the cornea following exposure to a test chemical are combined to derive an *In Vitro* Irritancy Score (IVIS) or LLBO Irritancy Score (LIS), which is used to classify the irritancy level of the test chemical.

4. Definitions are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. This Test Guideline is based on the ICCVAM BCOP test method protocol (8)(9), which was originally developed from information obtained from the Institute for In Vitro Sciences (IIVS) protocol and INVITTOX Protocol 124 (10). The latter represents the protocol used for the European Community-sponsored prevalidation study conducted in 1997-1998. Both of these protocols were based on the BCOP test method first reported by Gautheron *et al.* (11).

6. The BCOP OP-KIT and LLBO test methods can be used to identify chemicals inducing serious eye damage as defined by UN GHS, i.e. chemicals to be classified as UN GHS Category 1 (4). When used for this purpose, the BCOP OP-KIT test method has an overall accuracy of 79% (n=191), a false positive rate of 25% (n=126), and a false negative rate of 14% (n=65), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (3) (see Annex 2, Table 1). When test chemicals within certain chemical (i.e., alcohols, ketones) or physical (i.e., solids) classes are excluded from the database, the BCOP OP-KIT test method has an overall accuracy of 85% (n=131), a false positive rate of 20% (n=81), and a false negative rate of 8% (n=50) for the UN GHS classification system (3). The BCOP

LLBO test method has an overall accuracy of 78% (n=145), a false positive rate of 21% (n=89), and a false negative rate of 24% (n=56), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (12) (see Annex 2, Table 3). When test chemicals within certain chemical (i.e., alcohols, ketones) or physical (i.e., solids) classes are excluded from the database, the BCOP LLBO test method has an overall accuracy of 85% (n=55), a false positive rate of 17% (n=32), and a false negative rate of 13% (n=23) for the UN GHS classification system (12). Moreover, test chemicals inducing persistent non severe effects *in vivo* may also risk underprediction in the BCOP test method (13). The potential shortcomings of the BCOP test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) are based on the high false positive rates for alcohols and ketones and the high false negative rate for solids observed in the validation database (1)(2)(3)(12). However, since not all alcohols and ketones are over-predicted by the BCOP test method and some are correctly predicted as UN GHS Category 1, these two organic functional groups are not considered to be out of the applicability domain of the test method. It is up to the user of this Test Guideline to decide if a possible over-prediction of an alcohol or ketone can be accepted or if further testing should be performed in a weight-of-evidence approach. Regarding the false negative rates for solids, it should be noted that solids may lead to variable and extreme exposure conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (14). It should also be noted that none of the false negatives identified in the ICCVAM validation database (2)(3) or in the LLBO evaluation study (12), in the context of identifying chemicals inducing serious eye damage (UN GHS Category 1), were predicted as UN GHS No Category. Moreover, BCOP false negatives in this context are not critical since all test chemicals for which no stand-alone prediction can be made according to the UN GHS would be subsequently tested with other adequately validated *in vitro* tests, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. Given the fact that some solid chemicals are correctly predicted by the BCOP test method as UN GHS Category 1, this physical state is also not considered to be out of the applicability domain of the test method. End users can therefore use this test method for all types of test chemicals, whereby a predicted UN GHS Category 1 can be used for classification according to the UN GHS Category 1 without further testing. However, as already mentioned, positive results obtained with alcohols or ketones should be interpreted cautiously due to potential over-prediction.

7. The BCOP test method can also be used to identify chemicals that do not require classification for eye irritation or serious eye damage under the UN GHS classification system (4). When used for this purpose, the BCOP OP-KIT test method has an overall accuracy of 69% (n=196), a false positive rate of 69% (n=89), and a false negative rate of 0% (n=107), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (3) (see Annex 2, Table 2). The BCOP LLBO test method has an overall accuracy of 83% (n=145), a false positive rate of 45% (n=41), and a false negative rate of 6% (n=104), when compared to *in vivo* rabbit eye test method data classified according to the UN GHS classification system (12) (see Annex 2, Table 4). The false positive rate obtained (*in vivo* UN GHS No Category chemicals, see paragraph 48) is considerably high, but not critical in this context since all test chemicals that result in no stand-alone prediction would be subsequently tested with other adequately validated *in vitro* tests, or as a last option in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. The BCOP test method shows no specific shortcomings for the testing of alcohols, ketones and solids when the purpose is to identify chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (3)(12). Investigators could consider using this test method for all types of chemicals, whereby a negative result (UN GHS No Category) should be accepted as indicative that no classification is required (UN GHS No Category). Since the BCOP OP-KIT and LLBO test method can only identify correctly 31% and 55 % of the chemicals, respectively, that do not require classification for eye irritation or serious eye damage, this test method should not be the first choice to initiate a Bottom-Up approach (6), if other validated and accepted *in vitro* methods with similar high sensitivity but higher specificity are available.

8. In the BCOP, using the OP-KIT device for opacity, the validation database contained 113 substances and 100 mixtures in total (2)(3). In the BCOP, with the LLBO device for opacity, the evaluation database contained 145 substances (12). So far no mixtures were tested using the LLBO, but both devices are considered applicable to the testing of substances and mixtures.

9. The BCOP test method is not recommended for the identification of test chemicals that should be classified as irritating to eyes (UN GHS Category 2 or Category 2A) or test chemicals that should be classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassified as UN GHS Category 2, 2A or 2B (2)(3)(12). For this purpose, further testing with another suitable method may be required. The BCOP test method was found to be under-predictive for the identification of agrochemicals formulations that should be classified as UN GHS Category 1 (15).

10. Sources of eyes and corneas include but are not limited to: slaughterhouses, commercial suppliers, and research institutions. The providers are to comply with all applicable laws, regulations, policies, guidelines, and permit requirements. In regards to the laboratory, all procedures with bovine eyes and bovine corneas should follow the testing facility's applicable regulations and procedures for handling animal-derived materials, which include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions are recommended (16).

11. Whilst the BCOP test method does not consider conjunctival and iridal injuries, it addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification. The reversibility of corneal lesions cannot be evaluated *per se* in the BCOP test method. It has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury may be used to identify some types of irreversible effects (17). However, further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur. Finally, the BCOP test method does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

12. This Test Guideline will be updated periodically as new information and data are considered. For example, histopathology may be potentially useful when a more complete characterization of corneal damage is needed. As outlined in OECD Guidance Document No. 160 (18), users are encouraged to preserve corneas and prepare histopathology specimens that can be used to develop a database and decision criteria that may further improve the accuracy of this test method.

13. For any laboratory initially establishing this test method, the proficiency substances provided in Annex 3 should be used. A laboratory can use these chemicals to demonstrate their technical competence in performing the BCOP test method prior to submitting BCOP test method data for regulatory hazard classification purposes.

PRINCIPLE OF THE TEST

14. The BCOP test method is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the bovine cornea *in vitro*. In this test method, damage by the test chemical is assessed by quantitative measurements of changes in corneal opacity and permeability with an opacitometer and a visible light spectrophotometer, respectively. Both measurements are used to calculate an IVIS or LIS, which is used to assign an *in vitro* irritancy hazard classification category for prediction of the *in vivo* ocular irritation potential of a test chemical (see Decision Criteria in paragraph 48).

15. The BCOP test method uses isolated corneas from the eyes of freshly slaughtered cattle (calf or bovine). 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. Test chemicals are applied to the epithelial surface of the cornea by addition to the anterior chamber of the corneal holder. Annex 4 provides a description and a diagram of a corneal holder used in the BCOP test method. Corneal holders can be obtained commercially from different sources or can be constructed.

Source and Age of Bovine Eyes and Selection of Animal Species

16. Cattle sent to slaughterhouses are typically killed either for human consumption or for other commercial uses. Only healthy animals considered suitable for entry into the human food chain are used as a source of corneas for use in the BCOP test method. Because cattle have a wide range of weights, depending on breed, age, and sex, there is no recommended weight for the animal at the time of slaughter.

17. Variations in corneal dimensions can result when using eyes from animals of different ages. Corneas with a horizontal diameter >30.5 mm and central corneal thickness (CCT) values $\geq 1100 \mu\text{m}$ are generally obtained from cattle older than eight years, while those with a horizontal diameter < 28.5 mm and CCT <900 μm are generally obtained from cattle less than five years old (19). For this reason, eyes from cattle greater than 60 months old are not typically used. Eyes from cattle less than 12 months of age have not traditionally been used since the eyes are still developing and the corneal thickness and corneal diameter are considerably smaller than that reported for eyes from adult cattle. However, the use of corneas from young animals (*i.e.*, 6 to 12 months old) is permissible since there are some advantages, such as increased availability, a narrow age range, and decreased hazards related to potential worker exposure to Bovine Spongiform Encephalopathy (BSE) (20); domestic regulations should be observed in relation to potential worker exposure to BSE. As further evaluation of the effect of corneal size or thickness on responsiveness to corrosive and irritant substances would be useful, users are encouraged to report the estimated age and/or weight of the animals providing the corneas used in a study.

Collection and Transport of Eyes to the Laboratory

18. Eyes are collected by trained employees of slaughterhouses, commercial suppliers, and research institutions. To minimize mechanical and other types of damage to the eyes, the eyes should be enucleated as soon as possible after death and cooled immediately after enucleation and during transport. To prevent exposure of the eyes to potentially irritant substances, the slaughterhouse employees should not use detergent when rinsing the head of the animal .

19. Eyes should be immersed immediately and completely in cooled Hanks' Balanced Salt Solution (HBSS) in a suitably sized container, and transported to the laboratory in such a manner as to minimize deterioration and/or bacterial contamination. Because the eyes are collected during the slaughter process, they might be exposed to blood and other biological substances, including bacteria and other microorganisms. Therefore, it is important to ensure that the risk of contamination is minimized (*e.g.*, by keeping the container containing the eyes on wet ice during collection and transportation and by adding antibiotics to the HBSS used to store the eyes during transport [*e.g.*, penicillin at 100 IU/mL and streptomycin at 100 $\mu\text{g/mL}$]).

20. The time interval between collection of the eyes and use of corneas in the BCOP test method should be minimized (typically collected and used on the same day) and should be demonstrated to not compromise the assay results. These results are based on the selection criteria for the eyes, as well as the positive and negative control responses. All eyes used in the assay should be from the same group of eyes collected on a specific day.

Selection Criteria for Eyes Used in the BCOP Test Method

21. The eyes, once they arrive at the laboratory, are carefully examined for defects including increased opacity, scratches, and neovascularization. Only corneas from eyes free of such defects are to be used.
22. The quality of each cornea is also evaluated at later steps in the assay. For the OP-KIT, untreated corneas that have opacity greater than seven opacity units or equivalent after an initial one hour equilibration period (t_0) are to be discarded. For the LLBO untreated corneas at time t_0 with a laser measurement less than 1200 lux or greater than 1850 lux should be discarded (NOTE: the opacitometer should be calibrated with opacity standards that are used to establish the opacity units, see Annex 5).
23. Each treatment group (test chemical, concurrent negative and positive controls) consists of a minimum of three eyes. Three corneas should be used for the negative control corneas in the BCOP test method. Since all corneas are excised from the whole globe, and mounted in the corneal chambers, there is potential for artefacts from handling upon individual corneal opacity and permeability values (including negative control). Furthermore, the opacity and permeability values from the negative control corneas are used to correct the test chemical-treated and positive control-treated corneal opacity and permeability values in the IVIS/LIS calculations.

PROCEDURE

Preparation of the Eyes

24. Corneas, free of defects, are dissected with a 2 to 3 mm rim of sclera remaining to assist in subsequent handling, with care taken to avoid damage to the corneal epithelium and endothelium. Isolated corneas are mounted in specially designed corneal holders that consist of anterior and posterior compartments, which interface with the epithelial and endothelial sides of the cornea, respectively (Annex 4). Both chambers are filled to excess with pre-warmed phenol red free Eagle's Minimum Essential Medium (EMEM) (posterior chamber first), ensuring that no bubbles are formed. The device is then equilibrated at $32 \pm 1^\circ\text{C}$ for at least one hour (t_0) to allow the corneas to equilibrate with the medium and to achieve normal metabolic activity, to the extent possible (the approximate temperature of the corneal surface *in vivo* is 32°C).
25. Following the equilibration period, fresh pre-warmed phenol red free EMEM is added to both chambers and baseline opacity readings are taken for each cornea. Any corneas that show macroscopic tissue damage (*e.g.*, scratches, pigmentation, neovascularization) or for the opacitometers an opacity for OP-KIT greater than seven opacity units and for LLBO a laser measurement less than 1200 lux or greater than 1850 lux or equivalent are discarded. A minimum of three corneas are selected as negative (or solvent) control corneas. The remaining corneas are then distributed into treatment and positive control groups.
26. Because the heat capacity of water is higher than that of air, water provides more stable temperature conditions for incubation. Therefore, the use a water bath for maintaining the corneal holder and its contents at $32 \pm 1^\circ\text{C}$ is recommended. However, air incubators might also be used, assuming precaution to maintain temperature stability (*e.g.*, by pre-warming of holders and media).

Application of the Test Chemical

27. Two different treatment protocols are used, one for liquids and surfactants (solids or liquids), and one for non-surfactant solids.
28. Liquids are tested undiluted. Semi-solids, creams, and waxes are typically tested as liquids. Neat surfactant substances 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. Appropriate justification should be provided for alternative dilution concentrations. Mixtures containing surfactants may be tested undiluted or diluted to an appropriate concentration depending on the relevant exposure scenario in humans. Appropriate justification should be provided for the concentration tested. Corneas are exposed to liquids and surfactants for 10 minutes. Use of other exposure times should be accompanied by adequate scientific rationale. Please see Annex 1 for a definition of surfactant and surfactant-containing mixture.

29. Non-surfactant solids are typically tested as solutions or suspensions at 20% w/v 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. In certain circumstances and with proper scientific justification, solids may also be tested neat by direct application onto the corneal surface using the open chamber method (see paragraph 32). Corneas are exposed to solids for four hours, but as with liquids and surfactants, alternative exposure times may be used with appropriate scientific rationale.

30. Different treatment methods can be used, depending on the physical nature and chemical characteristics (e.g., solids, liquids, viscous vs. non-viscous liquids) of the test chemical. The critical factor is ensuring that the test chemical adequately covers the epithelial surface and that it is adequately removed during the rinsing steps. A closed-chamber method is typically used for non-viscous to slightly viscous liquid test chemicals, while an open-chamber method is typically used for semi-viscous and viscous liquid test chemicals and for neat solids.

31. In the closed-chamber method, sufficient test chemical (750 µL) to cover the epithelial side of the cornea is introduced into the anterior chamber through the dosing holes on the top surface of the chamber, and the holes are subsequently sealed with the chamber plugs during the exposure. It is important to ensure that each cornea is exposed to a test chemical for the appropriate time interval.

32. In the open-chamber method, the window-locking ring and glass window from the anterior chamber are removed prior to treatment. The control or test chemical (750 µL, or enough test chemical to completely cover the cornea) is applied directly to the epithelial surface of the cornea using a micro-pipet. If a test chemical is difficult to pipet, the test chemical can be pressure-loaded into a positive displacement pipet to aid in dosing. The pipet tip of the positive displacement pipet is inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipet piston is drawn upwards. If air bubbles appear in the pipet tip, the test article is removed (expelled) and the process repeated until the tip is filled without air bubbles. If necessary, a normal syringe (without a needle) can be used since it permits measuring an accurate volume of test chemical and an easier application to the epithelial surface of the cornea. After dosing, the glass window is replaced on the anterior chamber to recreate a closed system.

Post-Exposure Incubation

33. After the exposure period, the test chemical, the negative control, or the positive control substance is removed from the anterior chamber and the epithelium washed at least three times (or until no visual evidence of test chemical can be observed) with EMEM (containing phenol red). Phenol red-containing medium is used for rinsing since a color change in the phenol red may be monitored to determine the effectiveness of rinsing acidic or alkaline materials. The corneas are washed more than three times if the phenol red is still discolored (yellow or purple), or the test chemical is still visible. Once the medium is free of test chemical, the corneas are given a final rinse with EMEM (without phenol red). The EMEM (without phenol red) is used as a final rinse to ensure removal of the phenol red from the anterior chamber prior to the opacity measurement. The anterior chamber is then refilled with fresh EMEM without phenol red.

34. For liquids or surfactants, after rinsing, the corneas are incubated for an additional two hours at $32 \pm 1^\circ\text{C}$. Longer post-exposure time may be useful in certain circumstances and could be considered on a case-by-case basis. Corneas treated with solids are rinsed thoroughly at the end of the four-hour exposure period, but do not require further incubation.

35. At the end of the post-exposure incubation period for liquids and surfactants and at the end of the four-hour exposure period for non-surfactant solids, the opacity and permeability of each cornea are recorded. Also, each cornea is observed visually and pertinent observations recorded (e.g., tissue peeling, residual test chemical, non-uniform opacity patterns). These observations could be important as they may be reflected by variations in the opacitometer readings.

Control Substances

36. Concurrent negative or solvent/vehicle controls and positive controls are included in each experiment.

37. When testing a liquid substance at 100%, a concurrent negative control (e.g., 0.9% sodium chloride solution or distilled water) is included in the BCOP test method so that nonspecific changes in the test system can be detected and to provide a baseline for the assay endpoints. It also ensures that the assay conditions do not inappropriately result in an irritant response.

38. When testing a diluted liquid, surfactant, or solid, a concurrent solvent/vehicle control group is included in the BCOP test method so that nonspecific changes in the test system can be detected and to provide a baseline for the assay endpoints. Only a solvent/vehicle that has been demonstrated to have no adverse effects on the test system can be used.

39. A substance known to induce a positive response is included as a concurrent positive control in each experiment to verify the integrity of the test system and its correct conduct. However, to ensure that variability in the positive control response across time can be assessed, the magnitude of irritant response should not be excessive.

40. Examples of positive controls for liquid test chemicals are 100% ethanol or 100% dimethylformamide. An example of a positive control for solid test chemicals is 20% w/v imidazole in 0.9% sodium chloride solution.

41. 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.

Endpoints Measured

42. Opacity is determined by the amount of light transmission through the cornea. Corneal opacity is measured quantitatively with the aid of an opacitometer, resulting in opacity values measured on a continuous scale. The OP-KIT opacitometer is the standard device that was used in the validation of the BCOP test method (9)(21). Other devices that were evaluated are the Duratec opacitometer (22) and the LLBO (Laser Light-Based Opacitometer) (12)(21)(23) (see Annex 5). Similar opacitometers, demonstrated to be acceptable by testing the proficiency substances, can also be used.

43. 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). One mL sodium fluorescein solution of 4 mg/ml when testing liquids and surfactants, and 5 mg/ml for non-surfactant solids, is added to the anterior chamber of the corneal holder, which interfaces with the epithelial side of the cornea, while the posterior chamber, which interfaces with the endothelial side of the cornea, is

filled with fresh EMEM. The holder is then incubated in a horizontal position for 90 ± 5 min at 32 ± 1 °C. The amount of sodium fluorescein that crosses into the posterior chamber is quantitatively measured with the aid of UV/VIS spectrophotometry. Spectrophotometric measurements evaluated at 490 nm are recorded as optical density (OD490) or absorbance values, which are measured on a continuous scale. The fluorescein permeability values are determined using OD490 values based upon a visible light spectrophotometer using a standard 1 cm path length (11).

44. Alternatively, a 96-well microtiter plate reader may be used provided that; (i) the linear range of the plate reader for determining fluorescein OD490 values can be established; and (ii), the correct volume of fluorescein samples are used in the 96-well plate to result in OD490 values equivalent to the standard 1 cm path length (this could require a completely full well [usually 360µL]).

DATA AND REPORTING

Data Evaluation

45. Once the opacity and mean permeability (OD490) values have been corrected for background opacity and the negative control permeability OD490 values, the mean opacity and permeability OD490 values for each treatment group should be combined in an empirically-derived formula to calculate an *in vitro* irritancy score (IVIS). For LLBO the unit for measurement is lux, the SI unit of illuminance, and the opacity is defined as mean lux/7 (23).

- IVIS with OP-KIT = mean opacity value (read-out OP-KIT) + (15 x mean permeability OD490 value)
- LIS with LLBO = mean opacity value (read-out of LLBO in lux/7) value + (15 x mean permeability OD490 value)

46. Sina *et al.* (24) reported that the OP-KIT formula was derived during in-house and inter-laboratory studies. The data generated for a series of 36 compounds in a multi-laboratory study were subjected to a multivariate analysis to determine the equation of best fit between *in vivo* and *in vitro* data. Scientists at two separate companies performed this analysis and derived nearly identical equations. Verstraelen *et al.* (23) reported the LLBO-formula derived during the initial in-house study with 29 test chemicals, and this was confirmed in additional in-house studies with 80 and 65 chemicals (12) and the interlaboratory study with the 13 proficiency chemicals (25).

47. The opacity and permeability values should also be evaluated independently to determine whether a test chemical induced corrosivity or severe irritation through only one of the two endpoints (see Decision Criteria).

Decision Criteria

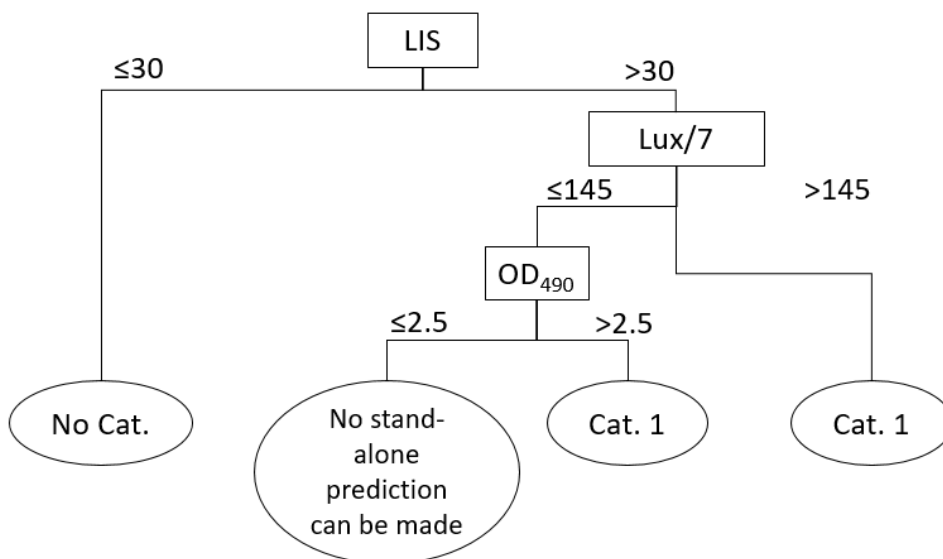
48. The prediction model for identifying test chemicals as inducing serious eye damage (UN GHS Category 1) and test chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category) are given hereafter:

Opacitometer 1	Opacitometer 2	
OP-KIT* and Duratec	LLBO **	UN GHS
IVIS \leq 3	LIS \leq 30	No Category
3 < IVIS \leq 55	LIS > 30 and lux/7 \leq 145 and OD ₄₉₀ \leq 2.5	No stand-alone prediction can be made
IVIS > 55	<ul style="list-style-type: none"> LIS > 30 and lux/7 \leq 145 and OD₄₉₀ > 2.5 or LIS > 30 and lux/7 > 145 	Category 1

*Based on ICCVAM database containing 113 substances and 100 mixtures (1) (2) (3) (22)

** Based on LLBO evaluation study with 145 substances (12)

Figure 1: Prediction model for BCOP LLBO test method



Study Acceptance Criteria

49. A test is considered acceptable if the positive control gives a value that falls within two standard deviations of the historical mean of the IVIS for the OP-KIT device. When using the LLBO device, a test is considered acceptable if the positive control gives a value that falls within two standard deviations of the historical mean of the opacity (as defined by lux/7). The positive control is to be updated at least every three 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. A single testing run composed of at least three corneas should be sufficient for a test chemical when the resulting prediction is unequivocal. However, in cases of borderline results in the first testing run, a second testing run should be considered (but not necessarily required), as well as a third one in case of discordant predictions between the first two testing runs. In this context, a result in the first testing run is considered borderline if the predictions from the 3 corneas were non-concordant, such that:

- 2 of the 3 corneas gave discordant predictions from the mean of all 3 corneas, OR,
- 1 of the 3 corneas gave a discordant prediction from the mean of all 3 corneas, AND the discordant result for
 - OP-KIT: was >10 IVIS units from the cut-off threshold of 55 ;
 - LLBO: UN GHS Cat. 1 prediction based on opacity (Lux/7, mean opacity > 145), but 1 of 3 corneas with opacity (Lux/7) < 130(12);
 - LLBO: UN GHS Cat. 1 prediction based on OD (mean OD > 2.5), but 1 of 3 corneas with OD < 2.0 (12);
 - LLBO: UN GHS No Cat. prediction based on LIS (mean LIS ≤ 30), but 1 out of 3 corneas with LIS > 40 (12).

If the repeat testing run corroborates the prediction of the initial testing run, then a final decision can be taken without further testing. If the repeat testing run results in a non-concordant prediction from the initial testing run, then a third and final testing run should be conducted to resolve equivocal predictions, and to classify the test chemical. It may be permissible to waive further testing for classification and labeling in the event any testing run results in a UN GHS Category 1 prediction.

Test Report

50. The test report should include the following information, if relevant to the conduct of the study:

Test chemical and Control Substances

- Mono-constituent substance : chemical identification, such as IUPAC or CAS name(s), CAS registry number(s), SMILES or InChI code, structural formula, and/or other identifiers;
- Multi-constituent substance, UVCB and mixture: Characterization as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

- Physical state, volatility, pH, LogP, molecular weight, chemical class, and additional relevant physicochemical properties relevant to the conduct of the study, to the extent available;
- Purity, chemical identity of impurities as appropriate and practically feasible, etc;
- Treatment prior to testing, if applicable (e.g., warming, grinding).
- - Storage conditions and stability to the extent available..

Information Concerning the Sponsor and the Test Facility

- Name and address of the sponsor, test facility and study director.

Test Method Conditions

- Opacimeter used (e.g., model and specifications) and instrument settings;
- Calibration information for devices used for measuring opacity and permeability (e.g., opacimeter and spectrophotometer) to ensure linearity of measurements;
- Type of corneal holders used (e.g., model and specifications);
- Description of other equipment used;
- 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).

Criteria for an Acceptable Test

- Acceptable concurrent positive and negative control ranges based on historical data;
- If applicable, acceptable concurrent benchmark control ranges based on historical data.

Eyes Collection and Preparation

- 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, transport media and temperature conditions, any antibiotics used);
- Preparation & mounting of the bovine corneas including statements regarding their quality, suitability of corneal holders to the corneal diameter, temperature of corneal holders, and criteria for selection of corneas used for testing.

Test Procedure

- Number of replicates used;
- Identity of the negative and positive controls used (if applicable, also the solvent and benchmark controls);
- Test chemical concentration(s), application, exposure time and post-exposure incubation time used;
- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure;
- Description of decision criteria used.

Results

- Tabulation of data from individual test samples (*e.g.*, opacity and OD490 values and calculated IVIS or LIS for the test chemical and the positive, negative, and benchmark controls [if included], reported in tabular form, including data from replicate repeat experiments as appropriate, and means \pm the standard deviation for each experiment);
- Description of other effects observed, including staining of the cornea;
- The derived in vitro UN GHS classification, if applicable.

Discussion of the Results

Conclusion

LITERATURE

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ANNEX 1 - DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of “relevance.” The term is often used interchangeably with “concordance”, to mean the proportion of correct outcomes of a test method.

Benchmark substance: A substance used as a standard for comparison to a test chemical. A benchmark substance should have the following properties; (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects, and (v) known potency in the range of the desired response.

Bottom-Up Approach: step-wise approach used for a chemical suspected of not requiring classification for eye irritation or serious eye damage, which starts with the determination of chemicals not requiring classification (negative outcome) from other chemicals (positive outcome).

Cornea: The transparent part of the front of the eyeball that covers the iris and pupil and admits light to the interior.

Corneal opacity: Measurement of the extent of opaqueness of the cornea following exposure to a test chemical. Increased corneal opacity is indicative of damage to the cornea. Opacity can be evaluated subjectively as done in the Draize rabbit eye test, or objectively with an instrument such as an “opacimeter.”

Corneal permeability: Quantitative measurement of damage to the corneal epithelium by a determination of the amount of sodium fluorescein dye that passes through all corneal cell layers.

Eye irritation: Production of changes in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Interchangeable with “Reversible effects on the eye” and with “UN GHS Category 2” (4).

False negative rate: The proportion of all positive substances falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative substances that are falsely identified by a test method as positive. It is one indicator of test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

In Vitro Irritancy Score (IVIS): An empirically-derived formula used in the BCOP test method whereby the mean opacity is measured with OP-KIT and mean permeability values for each treatment group are combined into a single *in vitro* score for each treatment group. The *IVIS* = mean opacity value (read-out OP-KIT) + (15 x mean permeability value).

Irreversible effects on the eye: See “Serious eye damage”.

LLBO Irritancy Score (LIS): An empirically-derived formula used in the BCOP test method whereby the mean opacity is measured with LLBO and mean permeability values for each treatment group are combined into a single *in vitro* score for each treatment group. The *LIS* = mean opacity value read-out of LLBO in lux/7) + (15 x mean permeability value).

Mixture: A mixture or a solution composed of two or more substances in which they do not react (4)

Negative control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

Not Classified: Chemicals that are not classified for Eye irritation (UN GHS Category 2, 2A, or 2B) or Serious eye damage (UN GHS Category 1). Interchangeable with “UN GHS No Category”.

Opacitometer: An instrument used to measure “corneal opacity” by quantitatively evaluating light transmission through the cornea. There are different types of opacitometers on the market. The OP-KIT opacitometer, used in the validation of the BCOP, has two compartments, each with its own light source and photocell. One compartment is used for the treated cornea, while the other is used to calibrate and zero the instrument. Light from a halogen lamp is sent through a control compartment (empty chamber without windows or liquid) to a photocell and compared to the light sent through the experimental compartment, which houses the chamber containing the cornea, to a photocell. The difference in light transmission from the photocells is compared and a numeric opacity value is presented on a digital display. This type of opacitometer provides a centre-weighted reading of light transmission by measuring changes in voltage when the transmission of white light through the cornea alters. Another example is the Laser Light-Based Opacitometer (LLBO), which uses a helium-neon (HeNe) green laser (class IIa/3R) as light source instead of visible light (12)(21)(23)). The LLBO offers the advantage of analysing the complete corneal surface, and is therefore able to detect more efficiently opaque spots located around the periphery of the excised corneas. The different devices result in a different read-out and different threshold values that distinguish between the different irritation categories. The devices are interchangeable at no cost to data quality and reliability.

Positive control: A replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Reversible effects on the eye: See “Eye irritation”.

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.

Serious eye damage: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application. Interchangeable with “Irreversible effects on the eye” and with “UN GHS Category 1” (4).

Solvent/vehicle control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical-treated samples and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (4).

Surfactant: Also called surface-active agent, this is a substance, such as a detergent, that can reduce the surface tension of a liquid and thus allow it to foam or penetrate solids; it is also known as a wetting agent.

Surfactant-containing mixture: In the context of this Test Guideline, it is a mixture containing one or more surfactants at a final concentration of > 5%.

Top-Down Approach: step-wise approach used for a chemical suspected of causing serious eye damage, which starts with the determination of chemicals inducing serious eye damage (positive outcome) from other chemicals (negative outcome).

Test chemical: Chemical (substance or mixture) assessed in the test method.

Tiered testing strategy: A stepwise testing strategy where all existing information on a test chemical is reviewed, in a specified order, using a weight-of-evidence process at each tier to determine if sufficient information is available for a hazard classification decision, prior to progression to the next tier. If the irritancy potential of a test chemical can be assigned based on the existing information, no additional testing is required. If the irritancy potential of a test chemical cannot be assigned based on the existing information, a step-wise sequential animal testing procedure is performed until an unequivocal classification can be made.

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (4).

UN GHS Category 1: See “Serious eye damage”.

UN GHS Category 2: See “Eye irritation”.

UN GHS No Category: Chemicals that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B). Interchangeable with “Not Classified”.

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose.

Weight-of-evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a test chemical.

ANNEX 2 - PREDICTIVE CAPACITY OF THE BCOP TEST METHOD

Table 1: Predictive Capacity of BCOP OP-KIT for identifying chemicals inducing serious eye damage [UN GHS Cat. 1 vs Not Cat. 1 (Cat 2 + No Cat)] (1)(2)(3)

Classification	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
UN GHS	191	78.53	150/191	86.15	56/65	13.85	9/65	74.60	94/126	25.40	32/126

Table 2: Predictive Capacity of BCOP OP-KIT for identifying chemicals not requiring classification for eye irritation or serious eye damage (“non-irritants”) [UN GHS No Cat. vs Not No Cat. (Cat 1 + Cat 2)] (1)(2)(3)

Classification System	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
UN GHS	196	68.88	135/196	100	107/107	0	0/107	31.46	28/89	68.54	61/89

Table 3: Predictive Capacity of BCOP LLBO for identifying chemicals inducing serious eye damage [UN GHS Cat. 1 vs Not Cat. 1 (Cat 2 + No Cat)] (12)

Classification System	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		% ^a	No.	% ^a	No.	% ^a	No.	% ^a	No.	% ^a	No.
UN GHS	145	77.93	145	75.89	56	24.11	56	79.21	89	20.79	89

^a The proportion in the tables are based on weighted calculation. For each chemical, all results were taken into account and a correction factor was applied so that all chemicals had the same weight (weight of 1).

Table 4: Predictive Capacity of BCOP LLBO for identifying chemicals not requiring classification for eye irritation or serious eye damage (“non-irritants”) [UN GHS No Cat. vs Not No Cat. (Cat 1 + Cat 2) (12)

Classification System	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		% ^a	No.	% ^a	No.	% ^a	No.	% ^a	No.	% ^a	No.
UN GHS	145	82.76	145	93.75	104	6.25	104	54.88	41	45.12	41

^a The proportion in the tables are based on weighted calculation. For each chemical, all results were taken into account and a correction factor was applied so that all chemicals had the same weight (weight of 1).

ANNEX 3 - PROFICIENCY SUBSTANCES FOR THE BCOP TEST METHOD

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the eye hazard classification of the 13 substances recommended in Table 1. These substances were selected to represent the range of responses for eye hazards based on results in the *in vivo* rabbit eye test (TG 405) (26) and the UN GHS classification system (*i.e.*, Categories 1, 2A, 2B, or Not Classified) (4). Other selection criteria were that substances are commercially available, that there are high quality *in vivo* reference data available, and that there are high quality *in vitro* data available from the BCOP test method. Reference data are available in the Streamlined Summary Document (3) and in the ICCVAM Background Review Document for the BCOP test method (2)(27).

In situations where, for instance, a listed substance is unavailable or cannot be used for other justified reasons, another substance having the same physical form and same *in vivo* classification and for which adequate *in vivo* and *in vitro* reference data is available may be used (e.g. from the list of reference chemicals (2)(3) provided that the same selection criteria as described in Table 1 are applied. Using an alternative proficiency substance should be justified.

Table 1: Recommended substances for demonstrating technical proficiency with the BCOP Test Method

Chemical	CASRN	Chemical Class ¹	Physical Form	<i>In Vivo</i> Classification ²	BCOP Classification
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid	Category 1	Category 1
Chlorhexidine	55-56-1	Amine, Amidine	Solid	Category 1	Category 1
Benzoic acid	65-85-0	Carboxylic acid	Solid	Category 1	Category 1
Imidazole	288-32-4	Heterocyclic	Solid	Category 1	Category 1
Trichloroacetic acid (30%)	76-03-9	Carboxylic acid	Liquid	Category 1	Category 1
2,6-Dichlorobenzoyl chloride ⁴	4659-45-4	Acyl halide	Liquid	Category 2A	No stand-alone prediction can be made
Ethyl-2-methylacetoacetate	609-14-3	Ketone, Ester	Liquid	Category 2B	No stand-alone prediction can be made
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2 ³	No stand-alone prediction can be made
EDTA, di-potassium salt	25102-12-9	Amine, Carboxylic acid (salt)	Solid	Not Classified	Not Classified
Tween 20	9005-64-5	Ester, Polyether	Liquid	Not Classified	Not Classified
2-Mercaptopyrimidine	1450-85-7	Acyl halide	Solid	Not Classified	Not Classified
Phenylbutazone	50-33-9	Heterocyclic	Solid	Not Classified	Not Classified
Polyoxyethylene 23 lauryl ether (BRIJ-35) (10%)	9002-92-0	Alcohol	Liquid	Not Classified	Not Classified

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

¹Chemical classes were assigned to each test chemical 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>).

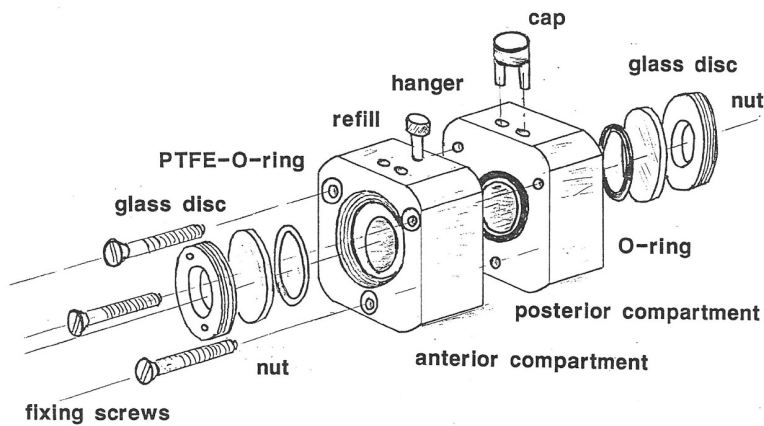
²Based on results from the *in vivo* rabbit eye test (OECD TG 405) (26) and using the UN GHS (4).

³Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e. 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The *in vivo* study included 3 animals. All endpoints apart from conjunctiva redness in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a conjunctiva redness score of 1 (at day 7) that fully recovered at day 10.

⁴2,6-Dichlorobenzoyl chloride can have variable results in different laboratories depending on the supplier and could be considered as suggested in this Annex 3, paragraph 2.

ANNEX 4 - THE BCOP CORNEAL HOLDER

The BCOP corneal holders are made of an inert material (e.g., polypropylene). The holders are comprised of two halves (an anterior and posterior chamber), and have two similar cylindrical internal chambers. Each chamber is designed to hold a volume of about 5 mL and terminates in a glass window, through which opacity measurements are recorded. Each of the inner chambers is 1.7 cm in diameter and 2.2 cm in depth¹. An o-ring located on the posterior chamber is used to prevent leaks. The corneas are placed endothelial side down on the o-ring of the posterior chambers and the anterior chambers are placed on the epithelial side of the corneas. The chambers are maintained in place by three stainless steel screws located on the outer edges of the chamber. The end of each chamber houses a glass window, which can be removed for easy access to the cornea. An o-ring is also located between the glass window and the chamber to prevent leaks. Two holes on the top of each chamber permit introduction and removal of medium and test compounds. They are closed with rubber caps during the treatment and incubation periods. The light transmission through corneal holders can potentially change as the effects of wear and tear or accumulation of specific chemical residues on the internal chamber bores or on the glass windows may affect light scatter or reflectance. The consequence could be increases or decreases in baseline light transmission (and conversely the baseline opacity readings) through the corneal holders, and may be evident as notable changes in the expected baseline initial corneal opacity measurements in individual chambers (i.e., the initial corneal opacity values in specific individual corneal holders may routinely differ by more than 2 or 3 opacity units from the expected baseline values). Each laboratory should consider establishing a program for evaluating for changes in the light transmission through the corneal holders, depending upon the nature of the chemistries tested and the frequency of use of the chambers. To establish baseline values, corneal holders may be checked before routine use by measuring the baseline opacity values (or light transmission) of chambers filled with complete medium, without corneas. The corneal holders are then periodically checked for changes in light transmission during periods of use. Each laboratory can establish the frequency for checking the corneal holders, based upon the chemistries tested, the frequency of use, and observations of changes in the baseline corneal opacity values. If notable changes in the light transmission through the corneal holders are observed, appropriate cleaning and/or polishing procedures of the interior surface of the cornea holders or replacement have to be considered.



¹The dimensions provided are based on a corneal holder that is used for cows ranging in age from 12 to 60 months old. In the event that animals 6 to 12 months are being used, the holder would instead need to be designed such that each chamber holds a volume of 4 mL, and each of the inner chambers is 1.5 cm in diameter and 2.2 cm in depth. With any newly designed corneal holder, it is very important that the ratio of exposed corneal surface area to posterior chamber volume should be the same as the ratio in the traditional corneal holder. This is necessary to assure that permeability values are correctly determined for the calculation of the IVIS or LIS by the proposed formula.

ANNEX 5 - THE OPACITOMETER

1.. The opacitometer is a light transmission measuring device. The standard device is the OP-KIT equipment from Electro Design (Riom, France) used in the validation of the BCOP test method, light from a halogen lamp is sent through a control compartment (empty chamber without windows or liquid) to a photocell and compared to the light sent through the experimental compartment, which houses the chamber containing the cornea, to a photocell. The difference in light transmission from the photocells is measured electronically as a change in voltage, compared, and a numeric opacity value is presented on a digital display. The opacity units are established. Another possible device is the Laser Light-Based Opacitometer (LLBO) from Peira Scientific Instruments, (Beerse, Belgium), which was shown to have similar performance as the OP-KIT (23)(12). The LLBO uses a helium-neon (HeNe) green laser (class IIa/3R) as light source instead of visible light. The operation wave-length is 543.5 nm producing a 10.0 mW coherent, random polarized monochromatic light beam (0.81 mm) in the green portion of the visible spectrum. The LLBO offers the advantage of analysing the complete corneal surface, and is therefore able to detect more efficiently opaque spots located around the periphery of the excised corneas. Other types of opacitometers with a different setup (e.g., not requiring the parallel measurements of the control and experimental compartments) may be used if proven to give similar results to the validated equipment.

2. The opacitometer should provide a linear response through a range of opacity readings covering the cut-offs used for the different classifications described by the Prediction Model (*i.e.*, up to the cut-off determining corrosiveness/severe irritancy). For the OP-KIT opacitometer (Riom, France), to ensure linear and accurate readings up to 75-80 opacity units, it is necessary to calibrate the opacitometer using a series of calibrators. Calibrators are placed into the calibration chamber (a corneal chamber designed to hold the calibrators) and read on the opacitometer. The calibration chamber is designed to hold the calibrators at approximately the same distance between the light and photocell that the corneas would be placed during the opacity measurements. Reference values and initial set point depend on the type of equipment used. Linearity of opacity measurements should be ensured by appropriate (instrument specific) procedures. For example, for the OP-KIT equipment from Electro Design (Riom, France), the opacitometer is first calibrated to 0 opacity units using the calibration chamber without a calibrator. Three different calibrators are then placed into the calibration chamber one by one and the opacities are measured. Calibrators 1, 2 and 3 should result in opacity readings equal to their set values of 75, 150, and 225 opacity units, respectively, $\pm 5\%$. In the case of the LLBO equipment from Peira Scientific Instruments (Beerse, Belgium), the opacitometer is switched on 30 minutes before use. It is an one-compartment/one-beam system. The unit of measurement is lux, the SI unit of illuminance. It is used in photometry as a measure of the apparent intensity of light hitting or passing through a surface, with wavelengths weighted according to the luminosity function, a standardized model of human brightness perception (23)(12)) Start values should be set to '2000 \pm 20 lux' with placement of an 'empty' cornea holder in the measuring box , and recording lux values from illuminance meter. Next, a verification has to be carried out prior to each series of measurement in order to test and guarantee that the device works faultlessly and delivers reliable measurements. The device is verified using four neutral density glass filters (OD 0.3; 0.6; 0.8 and 1.0), according to the manufacturer's specifications. After the verification the device is ready to measure opacity values of the (un)treated cornea. Replace 'empty' corneal holder by a cornea holder containing an (un)treated cornea, record lux value, replace by another cornea holder. After triplicate measures, check '2000 lux' reading using the 'empty' cornea holder and adjust if needed. Hereafter the device is ready to measure the next three cornea holders containing (un)treated corneas (21)(23)(12).

Table: Characteristics of OP-KIT and LLBO opacitometers (11)(12)(21)(23)

Visible light-based opacitometer (OP-KIT)	Laser light-based opacitometer (LLBO)
White (polychromatic) light	Laser (monochromatic) light
Two light sources of different lifetime (dual beam)	One light source (one beam)
Center-weighted reading	The whole cornea is analysed
Non-linear	Linear
-	The width of the light beam can be adjusted