Appendix A

Publicly Available Protocols for the BCOP Test Method

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Appendix A1

INVITTOX Protocol 98. The Bovine Corneal Opacity and Permeability Assay – Method of Gautheron

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THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY - METHOD OF GAUTHERON

The effects of a test compound on the opacity and permeability of a freshly collected bovine cornea can be used as a measure of eye irritancy potential.

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NOTE
*The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. It should be noted that this protocol might need to be modified in light of experience gained in the study. Additional information added in the course of producing this **INVITTOX** protocol, e.g. this note, is presented in italics.*

Critical Assessment

This technique has the advantage over the Draize test that both of the end-points used are objective and quantitative, in contrast to the subjective scoring used in the *in vivo* test. In contrast to cell-culture-based systems, the three-dimensional structure of the tissue is preserved, thus giving a closer approximation of the *in vivo* situation. The sacrifice of animals is not required, as slaughterhouse material is used. It does not require any special conditions or facilities for cell culture and is therefore inexpensive and relatively simple to implement.

The protocol includes a method to be used for the testing of solids, which may present some difficulties. Interference with opacity measurements may be caused by highly coloured test compounds which can stain the cornea.

General
This assay was developed in the Merck Sharp & Dohme Research Laboratories to assess the ocular irritancy potential of process intermediates. The test utilizes bovine corneas from eyes freshly collected in a local abattoir, and measures two end-points, namely opacity and permeability. The objective values obtained from both parameters are combined, and the *in vitro* irritancy scores are compared to a previously established scale of ocular irritancy. For in-house products, irritancy is classified into three broad categories: mild, moderate and severe.

Equipment

1. opacitometer, e.g. Electro-Design, RIOM, France
2. corneal holders (15) in polypropylene
3. dissection equipment (scissors, forceps, scalpels)
4. plastic containers for collection of eyes
5. electric screwdriver
6. vacuum pump
7. water-bath
8. spectrophotometer
9. mortar and pestle
10. common tissue culture and laboratory equipment

Marterials

1. Hank's balanced salt solution with Ca^{++}, Mg^{++}, (HBSS, Sigma H-1387), supplemented with 0.350 g/l sodium bicarbonate according to the supplier's recommendation.
2. Fetal bovine serum (FBS)
3. Eagle's Minimum Essential Medium (MEM, Sigma M-3024). This is routinely prepared from powder, supplemented with 2.2 g/l sodium bicarbonate and 0.292 g/l (2 mM) glutamine, according to the supplier's recommendations, and stored refrigerated (one-week stock). In experiments, the medium also contains 1% FBS, prepared daily, and is used at 32°C. In this protocol, MEM medium always refers to complete medium which should be preheated to 32°C before use.
4. Dulbecco's phosphate-buffered saline (DPBS Sigma-D5780).
5. Na-fluorescein (Sigma F-6377). The dye is used as a 0.4 or 0.5% solution in DPBS (4 or 5 mg/ml).
6. Saline, always refers to 0.9% NaCl in distilled water.

Bovine eyes
Eyes, excised by an abattoir employee, are collected in a plastic jar containing one litre of HBSS for approximately 25 eyes. Buffer storage and transportation of eyes to the laboratory are performed at room temperature. The eyes are generally used within two hours after killing the animals.

Procedure Details

1. Preparation of corneas
During dissection, great care should be taken to avoid damage to corneal surfaces (epithelial and endothelial). All eyes are carefully examined, and those presenting defects, such as neovascularization, pigmentation, opacity or scratches are discarded. Eye balls are first dissected free of surrounding tissues (lids, conjunctiva, ocular muscles and glands) and placed in a jar containing fresh HBSS. Selected corneas are dissected with a 2-3 mm rim of sclera for easier handling, and stored in a petri dish containing HBSS until use. Corneas are then mounted in holders, the endothelial side being placed onto the O-ring of the posterior part of the holder. The anterior part of the holder is placed on the cornea and held in place with three screws. Compartments are then filled (the posterior part first) with MEM medium and corneas are incubated for one hour in a water-bath at 32°C.

2. Basal opacity
Immediately after incubation, anterior and posterior compartments are refilled with fresh medium, and opacity is determined (the method to measure opacity is described below). It should be very close to zero, thus permitting the elimination of any damaged or folded corneas: the limits for selecting good corneas are below or equal to 3 and above or equal to -3.

3. Treatment
Medium is removed from the anterior compartment, using a needle (with the point cut to remove the liquid completely) attached to a vacuum pump, and replaced by the test compound or an appropriate vehicle. Two treatment protocols are used, depending on the physical state (liquid or solid) of the product evaluated:

Protocol 1 : 10 min. treatment - **for liquids and surfactants**
Protocol 2 : 240 min, treatment - **for all solids**

In both protocols, substances are prewarmed at 32°C for a few minutes before being applied to the cornea. This is particularly important for liquids since the treatment time is only 10 minutes. For solids, this step is sometimes difficult; they are prepared in a mortar and very insoluble substances might be very sticky, preventing their transfer into a tube. In this case, place the mortar into warm (32°C) water for a few minutes.

3.1. Protocol 1
Liquid substances are applied neat (0.750 ml). If dilutions are requested, the solvent can be saline, for water-miscible products. PEG-600 or Triacetin can be used for immiscible liquids; triacetin may be preferred when possible (i.e. if miscible with the test liquid), because PEG-600 may enhance the penetration of some substances.

Surfactants are usually applied at 10% in saline, or at the dilution provided, and 0.750 ml is applied onto each cornea. Other concentrations (in saline) can also be tested as required.

Because some compounds, for example certain organic solvents, may be aggressive to plastic, it is recommended that glass syringes be used for all chemicals. In order to apply compounds uniformly onto the corneas, slightly rotate the holder, maintaining the cornea in a horizontal position (holes should be closed with the caps provided). Corneas are incubated in a horizontal position for 10 minutes at 32°C in a water bath. The holders should be completely immersed in water to ensure a uniform temperature.

The test substance is then removed, and the epithelium is washed at least three times, until the medium is clear, with approximately 4 ml of MEM. The anterior compartment is refilled with medium, and opacity is measured. Corneas are again incubated at 32°C for a period of 2 hours. Both compartments are refilled with fresh medium and opacity is again determined. The values obtained at this time-point (120 min) are the only ones used in calculations.

3.2. Protocol 2
Solutions or suspensions of solid products are prepared at 20% (in practice, 1 g plus 5 ml saline), using a mortar and pestle for homogenous preparations (start grinding in the mortar with a small volume of liquid). A volume of 0.750

ml is applied onto the epithelium with an appropriate syringe and needle. For sticky suspensions, it may be necessary to use a needle with a large diameter, or even to unscrew the anterior glass for pasty substances. Corneas are placed in a horizontal position for 4 hours at 32°C. The holders should be completely immersed in a water bath. The test compound is then removed and the epithelium is washed at least three times, until the cornea is free of particles; gentle swirling movements of the holders are sometimes necessary. It is also possible to remove the anterior glass if product is still present in the chamber. Both compartments are refilled with fresh medium and the opacity measurement is performed immediately without any further incubation.

N.B. This is the general procedure testing substances. In the EC/UK Study, however, all liquids and surfactants were tested neat and all solids at 20% in saline.

3.3. Number of corneas used
The number of corneas used per experiment is generally 15, but more or less may be used depending on the availability of eyes, holders and the number of test compounds. Each experiment includes a control group treated with saline (or with triacetin or PEG-600 if one of these has been used as solvent), a positive control group treated with a reference substance (see section "Positive controls") and several (generally 3) groups of corneas treated with the test substance. Each group is composed of three corneas.

4. Opacity measurement
The opacitometer determines changes in light transmission passing through the corneas, and displays a numerical opacity value (arbitrary units).

4.1. Calibration
This operation is performed with no cornea in the Opacitometer (Electro-Design, RIOM, France), but using the calibration devices. The electrical zero (balance between photocells) is adjusted with the "balance" knob, and the apparatus is set to "75" with a standardized opaque sheet of polyester.

4.2. Measurement
The lateral glasses of the holders should be dried. Changes in corneal opacity are determined by comparison with "basal opacity" measured before treatment (t=0 opacity).

Each corneal holder is placed in the experimental (positive) compartment of the apparatus with *no holder* in the control (negative) compartment. Thus, the value obtained (for control or treated corneas) represents the absolute opacity value for a given cornea, but not the difference between a treated and a control cornea, as was determined in previous studies.

5. Permeability
This second step of the assay is performed immediately after the measurement of opacity. The medium is removed from the anterior compartment, and replaced by 1 ml of fluorescein solution (0.4% for liquids and surfactants, 0.5% for solids). Corneas are incubated in a horizontal position for 90 minutes, immersed in a water-bath at 32°C. Medium from the posterior chamber is then removed, and its optical density (O.D.) determined with a spectrophotometer at 490 nm.

6. Data calculation

6.1. Opacity
Opacity values measured at a given time-point (120 minutes in protocol 1, 240 minutes in protocol 2) are *first* corrected (individually for each cornea in the experiment) for basal opacity (t=0), i.e. "t=120 - t=0" and "t=240 - t=0". Then each individual cornea in the experimental groups, including the positive controls, is corrected for the mean value of saline-treated corneas (negative control) at this time-point. The values obtained are therefore the "corrected values" of opacity.

6.2. Permeability
The mean O.D. of saline-treated corneas is calculated, and then the individual value for each experimental cornea is corrected for this mean to give the "corrected value" of permeability.

6.3 In-vitro score
This score is calculated with corrected values of opacity and permeability *for each individual cornea*, using the formula:

score = opacity + (15 x O.D.)

For a given substance, the final in-vitro score will be the mean ± SD of three corneas.

7. Quality control

7.1. Basal opacity
Only corneas with a basal opacity of <= 3 and >= -3 are selected for the experiment.

7.2. Fluorescein solution
Stock fluorescein solutions prepared at 4 mg/ml or 5 mg/ml in DPBS should be diluted to 10 µg/ml in complete MEM (the medium used throughout the experiment to fill the posterior compartment), and O.D. determined before application to corneas. An acceptable range is between 1.610 and 1.910, otherwise the dilution should be performed again, and, if necessary, a new solution prepared. The actual value for each experiment has to be reported on the data sheets.

7.3. Positive controls
A group of three corneas treated with a known reference substance has to be included in each experiment. The substance selected will depend on the broad category of products to be evaluated:

a) BENZALKONIUM CHLORIDE (=BAK) (Sigma, # B-1383) 5% solution in saline, for surfactants
b) N,N,-DIMETHYLFORMAMIDE (=DMF) (Aldrich, # 15,481-4), 100%, for liquids.
c) IMIDAZOLE (=IMDZ) (Aldrich, # I-20-2), 20% in saline, for solids.

The limits (mean of three corneas) for a valid experiment are as follows:

Compound	Opacity	Permeability	Score
Benzalkonium chloride	> 60	> 3.000	> 110
N,N-Dimethylformamide	> 70	> 1.500	> 100
Imidazole	> 35	> 2.000	> 70

8. Data interpretation (optional)

Based on experience at MS&D with reference and in-house compounds, and on data from collaborative studies, the following classification system was established:

In-vitro score:

from	0	to	25	=	mild irritant
from	25.1	to	55	=	moderate irritant
from	55.1	to	80	=	severe irritant
>=	80			=	very severe irritant

Appendix A2

INVITTOX Protocol 124. Bovine Corneal Opacity and Permeability (BCOP) Assay – SOP of Microbiological Associates, United Kingdom

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BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) ASSAY - SOP OF MICROBIOLOGICAL ASSOCIATES LTD., UK

The effects of a test compound on the opacity and permeability of a freshly collected bovine cornea can be used as a measure of eye irritancy potential.

Background

This protocol is based on the SOP developed by Gautheron (*INVITTOX N°* 98), which participated in the EC/HO Validation Study and did not meet the criteria set by the management team of this study for its use as a replacement of the Draize rabbit eye irritation test (Balls *et al.,* 1995). A subsequent study (BCOP assay Prevalidation Process; 1997-1998) has been carried out to overcome the previously encountered shortcomings. The new and optimised protocol version is herewith included. The Microbiological Associates Ltd., in collaboration with other laboratories, has refined and optimised the original protocol developed by Gautheron with the aim to assess the effects of some of the variables in the assay in order to eliminate sources of variation, optimise the methodology and reduce inter and intralaboratory variation.

Experimental Description

Endpoint and Endpoint Detection	:	- Corneal opacity measured using an opacitometer. - Corneal permeability determined using sodium fluorescein and measured spectrophotometrically (increase in OD).
Test System	:	Freshly isolated bovine cornea (intact, epithelium-removed, Descemet's membrane and endothelium-removed; stroma)

Bovine eyes recovered from a slaughterhouse are inspected and undamaged corneas are dissected and mounted in specially constructed holders.
After a 1 hour incubation in media, the basal opacity of each cornea is recorded using an opacitometer.
Two methodologies have been developed to adapt the protocol to the physico-chemical nature of the test compound. The first method (A) is used to test non-surfactant liquids and surfactants. Liquids are tested neat and surfactants, liquid and solid, are diluted at 10%. Both are applied for 10 minutes.

Before reading the final opacity, the corneas are rinsed and incubated for 2 hours in refilled media to equilibrate.
The second method (B) is used with solids, tested at 20% (w/w) solution or suspension in 0.9% NaCl. After 4 hours incubation, the corneas are rinsed and the final opacity measured.
Then the permeability of each cornea is determined with a fluorescein solution after an incubation of 90 minutes. Method A uses a fluorescein concentration of 4 mg/ml and method B uses 5 mg/ml.

Test Compounds

Ten chemicals were selected for use in Phase III of the BCOP prevalidation process: 3 surfactants (anionic and non-ionic), 1 aromatic amine, 1 alcohol, 1 ester, 1 ether, 1 ketone, 1 inorganic chemical and 1 aldehyde.

Prediction Model

The two endpoints, corneal opacity and permeability, are combined to give a final in vitro score and related to the five categories of irritancy: non irritant, mild, moderate, severe, very severe (see section "Evaluation of Test Results" of the present SOP). These in vitro index scores were then compared with in vivo scores (Modified Maximum Average Scores) obtained in the Draize eye test and assigned to appropriate categories.

Modifications of the Method

With respect to the original protocol developed by Gautheron the protocol refinements, carried out during the recent prevalidation study, refer to reagents and procedure adopted; the way of measuring permeability, calculation of the results, the treatment and dilution of test compounds and the kind of positive controls used.

Status

This protocol has successfully been tested in the "BCOP assay Prevalidation Process (1997-1998)". The participating laboratories concluded that the process was effective in improving the reproducibility of the assay.
The refinements introduced into the protocol contributed to an

improvement in the intralaboratory variability of the assay. However, the assay was found to overestimate the irritancy of two chemicals and to underpredict the irritancy of the others of the 10 chemicals tested.

NOTE: General comments of the BCOP Method Summary apply. It can be obtained from ecvam.sis@jrc.it

Last update: August 1999

Procedure Details, April 1997*

BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) ASSAY - SOP OF MICROBIOLOGICAL ASSOCIATES LTD., UK

Note:This protocol presents the standard operating procedure used in the study "BCOP assay prevalidation project" (1997). It should be noted that this protocol might need to be modified in light of experience gained in the study.

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☐ The herewith included SOP has been sent to the person responsible for the method to update or confirm it. As soon as new information will become available this version will be updated.

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1. Procedure

1.1 SUMMARY

Bovine eyes obtained from the local slaughterhouse are inspected for scratches and defects etc. Undamaged corneas are dissected and mounted in specially constructed holders. After a 1 hour incubation in media, the basal opacity of each cornea is recorded using an opacitometer.

Two methodologies have been developed and are used depending on the physical / chemical nature of the test article. The nature of the test article to be tested will therefore determine the methodology employed.

Method A is used to test non surfactant liquids and surfactants. Liquids are tested neat and surfactants, both liquid and solid, are tested at a 10% dilution and applied to the cornea for 10 minutes. After the 10 minute incubation the corneas are rinsed, the holders refilled with media and the corneas incubated for a further 2 hours in media to equilibrate. The final opacity reading is taken.

Method B is used for the testing of solids which are tested as a 20% slurry for 4 hours. After a 4 hour incubation the corneas are rinsed and the final opacity measurement recorded.

The corneas are then exposed to a fluorescein solution, and the permeability of each cornea determined after an incubation of 90 minutes. Method A uses a fluorescein concentration of 4 mg/ml and Method B uses 5 mg/ml. An aliquot of the media from below the cornea is read in a spectrophotometer to determine the permeability of the cornea to the fluorescein solution. The opacity and permeability values are combined to obtain an in vitro score.

1.2 EQUIPMENT

- Opacitometer (see Appendix A)
- Cornea holders ~25
- Spectrophotometer (see Appendix B)
- Water bath 32ºC
- Vacuum pump
- Scalpel
- Scissors
- Forceps
- Electric Screwdriver
- Mortar & Pestle
- Positive displacement pipette
- Micro pipettes
- 5ml Syringes
- 30ml Syringes
- Needles (19G11⁄21,1 x 40)
- Cuvettes

1.3 MEDIA AND REAGENTS:

Media: Clear media without phenol red is to be used throughout the study

MEM without Phenol Red [Life Technologies; Cat No.51200]
or
Powdered MEM dissolved in sterile deionised H_2O
[Sigma; Cat No. M-3024]
with added sodium bicarbonate [Sigma; Cat No. S-5761]

L-glutamine [Gibco; Cat No.043-05030]

Foetal Bovine Serum (FBS) [PAA; Cat No.A15-652]

Preparation of complete MEM (cMEM):

> To MEM add 1% L-glutamine and 1% FBS (To be freshly prepared at the beginning of each assay)

Hank's Balanced Salt Solution W/O Phenol Red (HBSS)
[Life Technologies; Cat No. 14025-050]
or
Powdered HBSS dissolved in sterile deionised H2O
[Sigma; Cat No.H-1387]
Penicillin-Streptomycin (10000 IU/ml-10000 IU/ml) solution
[Life technologies; Cat No. 15140-114]

0.9% NaCl Solution [Sigma; Cat No. S-8776]
or
Deionised H_2O plus 0.9% NaCl (0.9g / 100 ml)
[Sigma; Cat No. S 7653]

Preparation of Stock Fluorescein solution; (see Appendix C)
cMEM plus Sodium Fluorescein [Sigma; Cat No. F-6377]

Ethanol [Sigma-Aldrich; Cat No. 27,074-1]

Benzalkonium Chloride [Sigma; Cat No. B1383]

Imidazole [Sigma-Aldrich; Cat No. I,20-2]

All chemicals and solutions to be disposed after 1 year of purchase or preparation unless an expiry date is stipulated on the original packaging.

2. Methodology

2.1 pH

An estimate of pH for each neat (liquid) test article or diluted test article (if diluted/suspended in 0.9% NaCl) will be determined and recorded using universal pH paper.

2.2 BOVINE EYES

Bovine eyes, excised by an abattoir employee, will be collected as soon after slaughter as possible. Care should be taken to avoid damaging the cornea during excision. Excised eyes will be contained and transported to the laboratory in HBSS containing 1% (v/v) Penicillin/Streptomycin Solution (enough to cover all eyes in the receptacle) at room temperature. The eyes will generally be used within 3 hours (±1 hour) after slaughter.

2.3 PREPARATION OF CORNEAS

All eyes will be carefully examined macroscopically for defects (opacity, scratches, pigmentation, etc) and those exhibiting defects will be discarded. The tissue surrounding the eyeball will be carefully pulled away and the cornea will be dissected such that approximately 2 to 3mm of sclera is present around the cornea. The isolated corneas will be stored in a petri dish containing HBSS plus 1% Penicillin/ streptomycin Solution until all corneas are dissected.

The corneas are mounted immediately in the corneal holders with the endothelial side against the O-ring of the posterior half of the holder. The cornea should be gently flattened over the O-ring and holder surface with a wetted, gloved finger to expel any air. The anterior half of the holder will then be positioned on top of the cornea and fixed in place with screws. Both compartments of the corneal holder will be filled with cMEM, using a 30ml syringe. The posterior compartment will always be filled first to return the cornea to its natural concave position. Care should be taken to make sure no air bubbles are present within the holders. The holders will be plugged and incubated for 1 hour±5 min at 32ºC±2ºC in a water bath.

2.4 TREATMENT GROUPS

Three corneas will be treated with each test article solution/suspension. Three corneas per assay will be treated with the positive control and three corneas with 0.9% NaCl as the negative control group.

One of two treatment methods (Method A or B) will be used depending on the physical nature and chemical characteristics (liquid or surfactant versus non-surfactant solid) of the test article. The controls used will depend on the method being used.

2.5 CONTROLS

Test Article Positive Control
Method A Liquid test articles ethanol
Surfactant test articles benzalkonium chloride (10%)

Method B Solid test articles imidazole (20%)

Negative Control 0.9% saline

2.6 TREATMENT OF CORNEAS

At the end of the one hour incubation period, the medium will be removed from both compartments using a suitable pipette tip or flat ended needle attached to a vacuum pump to ensure complete evacuation, and replaced with fresh cMEM. Again, care should be taken to make sure no air bubbles are present within the holders. The posterior compartment will be plugged and the anterior left unplugged for opacity determination.

2.7 OPACITY MEASUREMENT

The opacitometer will determine the light transmission through the centre of each mounted cornea. A numerical opacity value (arbitrary unit) will by displayed and recorded. The opacitometer will be calibrated

at the start of each experiment in each assay (see Appendix A) and the opacity of each of the corneas will be determined by reading each holder in the right hand chamber of a calibrated opacitometer.

Once the basal opacity of all corneas has been recorded, the mean value of all corneas can be taken and any corneas deviating from this by more than 3 units will be discarded. Sets of three corneas can be selected randomly for treatment with each test article, positive control compound and negative control.

Immediately prior to treatment the medium will be removed from the anterior compartment of the holder using a suitable pipette tip or flat ended needle attached to a vacuum pump, taking extra care to make sure all excess liquid has been removed. This will be replaced with the test article, positive control compound or negative control

2.7.1 Method A:

Non surfactant liquids and the positive control compound (ethanol) will be tested neat (100%). Known surfactants (either solids or liquids) and positive control (Benzalkonium Chloride) will be tested at a 10% (w/w) concentration in 0.9% NaCl.

Seven hundred and fifty µl of a test substance will be introduced into the anterior part of the holder using a suitable micro pipette, or if the test article is viscous, a suitable positive displacement pipette will be used. Control corneas will also be treated with 750µl of the negative control (0.9% NaCl) and with the positive control.

The anterior compartment will be plugged. The holder will be turned to a horizontal position and slightly rotated to ensure uniform covering of the test substance over the cornea, and will be incubated in a horizontal position at 32±2°C for 10 minutes (±30 seconds) in a water bath.

The test substance will then be removed and the epithelium will be washed at least 3 times (or until the wash medium is clear) with approximately 3 ml of cMEM using a syringe to add media. After each wash the medium will be removed using a pipette tip or flat ended needle attached to a vacuum pump. If the test article proves difficult to remove by this method, the front cover may be removed and the cornea carefully washed using a gentle stream of cMEM from a wash bottle.

The anterior compartment will then be refilled with cMEM using a syringe. Care should be taken to ensure that there are no air bubbles in the compartment. Once all air bubbles have been removed the anterior compartment is re-plugged, the corneas will then be incubated for 2 hours ±10 minutes at 32±2°C in the water bath.

At the completion of the 2 hrs incubation period, the media will be removed from the anterior and the posterior compartments using a pipette tip or flat ended needle attached to a vacuum pump and replaced with fresh cMEM, again making sure no air bubbles are present. The posterior compartment will be re-plugged, and the opacity of each cornea will be recorded. The values obtained at this measurement will be recorded and used in calculating the corneal opacity.

The corneas will be observed for opaque spots or other irregularities and these will be noted on the workbook and raw data forms.

2.7.2 Method B:

Solid materials and the positive control compound (imidazole) will be tested at 20% (w/w) solution or suspension in 0.9% NaCl. Homogeneous preparations can be prepared in a mortar and pestle by grinding the test article with a small amount of 0.9% NaCl and slowly adding the remaining amount.

Seven hundred and fifty µl of the test substance, negative control (0.9% NaCl) or positive control will be introduced into the anterior part of the holder using a suitable positive displacement pipette. The front cover may be removed to obtain even coverage of viscous solutions or pastes. The holder will be slightly rotated (with the corneas maintained in a horizontal position) to ensure uniform covering of the test substance over the cornea. Both compartments will be plugged and the corneas incubated in a horizontal position at 32±2ºC for 4 hours ± 5 minutes in a water bath.

After incubation, the test substance, negative control or positive control compound will be removed and the epithelium washed at least 3 times (or until the cornea is free of particles) with approximately 3 ml of cMEM each time using a syringe to add media and a vacuum to remove it. If the test article proves difficult to remove by this method, the front cover may be removed and the cornea gently washed with cMEM using a wash bottle.

The media in the anterior and the posterior compartments will then be removed and replaced with fresh cMEM, again making sure no air bubbles are present in the holder. The posterior compartment will be plugged and an opacity measurement performed immediately without any further incubation.

The corneas will be observed for opaque spots or other irregularities and these noted on the workbook and raw data forms.

2.8 PERMEABILITY DETERMINATIONS

When carrying out this assay for the first time, a calibration curve for the spectrophotometer to be used must be carried out. (see Appendix B).

Each assay also requires the preparation and reading of two samples of quality control solution (see Appendix C).

2.8.1 Method A:

After the final opacity measurement is performed, the medium will be removed from the anterior compartment using a suitable pipette tip or flat ended needle attached to a vacuum pump. One ml of a 4 mg/ml fluorescein solution (see Appendix C) will be added to the anterior compartment using a micro pipette.

2.8.2 Method B:

After the opacity measurement is performed, the medium will be removed from the anterior compartment using a suitable pipette tip or flat ended needle attached to a vacuum pump and replaced with 1ml of a 5 mg/ml fluorescein solution (see Appendix C).

2.8.3 Method A and B:

After the addition of the fluorescein solution to the anterior side of the holder, the compartment will be plugged and the corneas will be incubated in a horizontal position for 90 minutes ± 5 minutes at 32±2ºC in a water bath.

After incubation the medium in the posterior chamber will be mixed by drawing ~2.5ml gently up and down a 5ml syringe with a needle attached 3 times. An aliquot of the mixed medium from the posterior chamber will be removed using the syringe and needle, and transferred to a cuvette with a 1cm path length.

The spectrophotometer will be adjusted to read at OD_{490} and a sample of cMEM read. The spectrophotometer will be blanked on this solution prior to reading the transferred solutions. Any solutions giving an OD_{490} beyond the range of the spectrophotometer (see Appendix B) will be diluted 1:4 in cMEM.

2.9 HOLDER CLEANING

All holders should be stripped at the end of the assay by removing the screws, glass holder rings, glass and the centre O-ring. The separate parts should be washed, and preferably steeped in hot water containing a suitable detergent. Care should be taken to ensure all traces of Na-fluorescein are removed. All parts should then be rinsed in water to remove all detergent and allowed to dry.

3. Criteria for Determination of a Valid Test

The test will be accepted if the positive control causes an *In Vitro* Score that falls within two standard deviations of the current historical mean.

Ethanol: 36.0 to 56.0
Benzalkonium chloride: 98.8 to 209.2
Imidazole: 111.2 to 164.0

4. Evaluation of Test Results

The *In Vitro* Score is generated from the opacity and permeability measurements as described below. A suitable computer spreadsheet can be used to make the following calculations (See Appendix D).

4.1 OPACITY

The change in opacity value of each treated cornea or positive control and negative control corneas will be calculated by subtracting the initial basal opacity from the post treatment opacity reading, for each individual cornea.

The average change in opacity for the negative control corneas will be calculated and this value subtracted from the change in opacity of each treated cornea or positive control to obtain a corrected opacity.

The mean corrected opacity value of each treatment group will be calculated from the individual corrected opacity values of the treated corneas for each treatment condition.

4.2 PERMEABILITY

The corrected OD_{490} value (permeability) of each treated or positive control cornea will be calculated by subtracting the average negative control cornea value from the original permeability value for each

cornea.

The mean corrected permeability values of each treatment group will be calculated from the individual corrected permeability values of the treated corneas for each treatment condition.

4.3 *IN VITRO* SCORE CALCULATION

The following formula is used to determine the *In Vitro* Score:

In Vitro Score= Corrected Opacity Value+(15xCorrected OD_{490} Value)

The In Vitro Score will be calculated for each individual treatment and positive control cornea. The mean In Vitro Score value for each treatment group will be calculated from the individual In Vitro Score values.

4.4 DATA INTERPRETATION

The following classification system was established by Gautheron et al (1992) and refined by Vanparys et al 1994 for materials tested under standard conditions. Results from test situations should be compared to known materials tested under similar conditions.

Proposed Prediction Model

Draize *in vivo* Score	Draize Irritation Scale	*In Vitro* Score	Proposed *In Vitro* Irritation Scale
0 - 0.9	minimal	0 - 3	non eye irritant
1 - 25	minimal/slight	3.1-25	mild eye irritant
26 - 56	moderate	25.1-55	moderate eye irritant
57 - 84	marked	55.1-80	severe eye irritant
85 - 110	extreme	>80.1	very severe eye irritant

5. Regulatory Requirements/Good Laboratory Practice

This assay will be performed in compliance with the provisions of the

Good Laboratory Practice Regulations for Non clinical Laboratory Studies.

Appendix A

Calibration of Opacitometer

An opacitometer (formerly from Electro Design) can be obtained from STAG BIO at the following address:

STAG BIO
Rond Point La Pardieu 6
av. Michel Ange
BP 09F 63063
CLERMONT FD Cedex 01
FRANCE

The opacitometer will be calibrated at the beginning of every experiment on ever test day as follows:

- The unit will be switched on and allowed to warm up for at least 10 minutes prior to calibration.
- With both calibration blocks inserted into the reading chambers, the balance knob will be adjusted to give a reading of zero. Calibrator number 1 will be inserted into the right hand calibration block and a reading taken. Calibrator number 1 should be adjusted to read 75 with the calibration knob on the opacitometer.
- The other two calibrators can be checked in the right hand calibration block and should fall into the range of 145-155 (calibrator 2), 218-232 (calibrator 3).

Once calibrated, the unit should be left on for the duration of the test.

If the opacitometer does not read within these ranges, the unit should be recalibrated by the manufacturer, STAG BIO.

Protocol of BCOP only requires the use of the right hand chamber of the opacitometer for reading the opacity. A calibration block should be left in the left hand reading chamber of the opacitometer for the duration of the assay and the opacity of the treated corneas will be read in the right hand chamber only.

Appendix B

Spectrophotometer linearity

The linearity of the spectrophotometer to be used in these studies and its ability to replicate the readings obtained by other users of the BCOP must be determined. The following process is intended to identify any difference in individual spectrophotometers used in different laboratories.

The optical density (OD) of a series of dilutions of Na-fluorescein (NaF) solutions in cMEM should be recorded.

A (100X) stock solution of Na-fluorescein (NaF) is made by dissolving 0.2g NaF in 100ml cMEM; a second stock solution (1X) is then prepared by diluting 1ml of the first stock (100X) in 100ml of cMEM in a standard flask; a concentration of 20µg/ml is acheived.

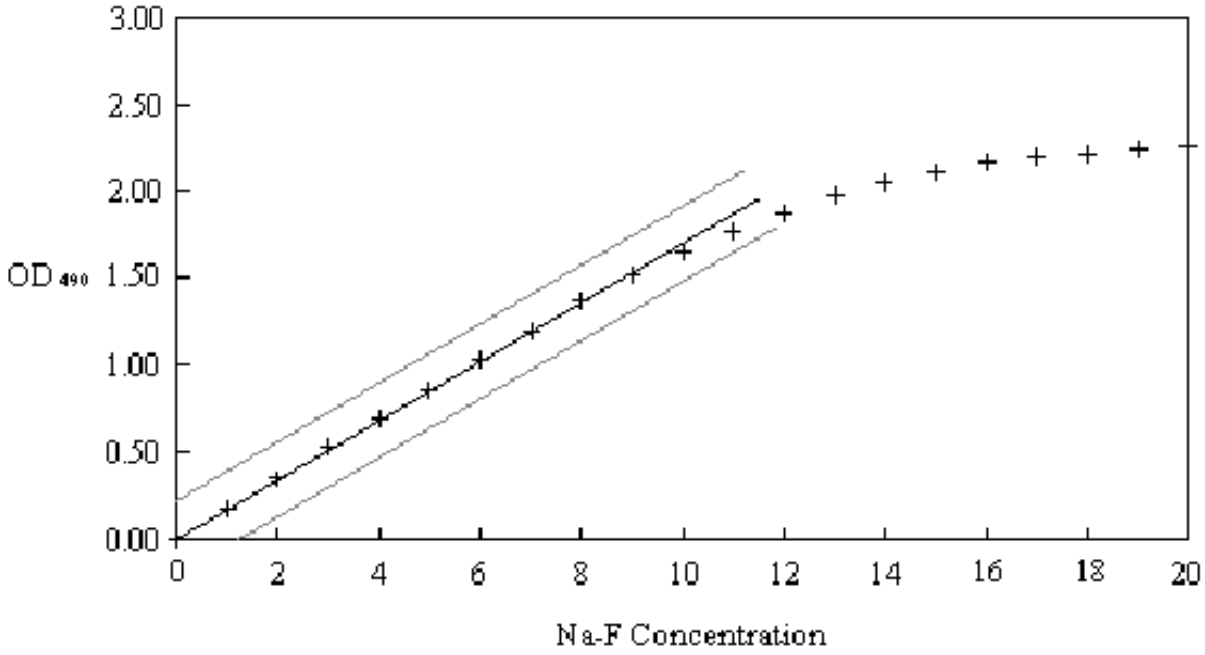
A series of 22 cuvettes will be prepared as described in Table 1. The OD determination is performed at 490 nm and results should closely follow those displayed in Figure 1.

Table 1: Preparation of the Standard Dilution Series of Na-fluorescein (NaF) in cMEM.

Cuvette No.	# µl cMEM	# µl stock 1x	Concentration (µg/ml)
1	0	2,000	20
2	100	1,900	19
3	200	1,800	18
4	300	1,700	17
5	400	1,600	16
6	500	1,500	15
7	600	1,400	14
8	700	1,300	13
9	800	1,200	12
10	900	1,100	11
11	1,000	1,000	10
12	1,100	900	9

13	1,200	800	8
14	1,300	700	7
15	1,400	600	6
16	1,500	500	5
17	1,600	400	4
18	1,700	300	3
19	1,800	200	2
20	1,900	100	1
blank 21&22	2,000	0	0

Figure 1: Example of a Calibration curve of a Spectrophotometer using a serial dilution of Na-F Solution in cMEM



A graph similar to that shown in Figure 1 should be prepared and used to determine the linear range of each spectrophotometer and thus determine the upper limit of absorbance. Solutions recording absorbance above the linear portion should be diluted further.

Figure 1 demonstrates spectrophotometer linearity below an OD490 of 1.80, hence if the OD490 > 1.80, a dilution factor of 1:4 will be required.

Appendix C

Preparation & Quality Control of Na-fluorescein Solution for use in the BCOP Assay

Method A;

Liquid/surfactant test compounds

A stock solution of Na-fluorescein (1g dissolved in cMEM 250ml) is prepared.
This is diluted 1/400 in cMEM in two steps;

Step 1: 950 µl cMEM + 50 µl Na-F stock;
Step 2: 50 µl of Step 1 solution + 950 µl cMEM dilution is performed.

The same process should be repeated to obtain two separate solutions for testing. The final solution from Step 2 is measured on the spectrophotometer after blanking on 1 ml of cMEM. The two values obtained are averaged and this reading must be between 1.71 and 1.91.

If the final dilution is within the specified range, the stock solution can be aliquoted into suitable vials and stored at -20oC ± 5oC in the dark until required for use. To improve the consistency between assays, vials can be thawed and diluted for use on the day of assay. Any prepared solution not required should be discarded.

Method B;

Solid test compounds

A stock solution of Na-fluorescein (1.25g dissolved in cMEM 250ml) is prepared.
This is diluted 1/500 in cMEM in two steps.

Step1: 950 µl cMEM + 50 µl Na-F stock;
Step2: 40 µl of Step 1 solution + 960 µl cMEM dilution is performed.

The same dilution sequence should be repeated to obtain two separate solutions for testing. The final solution from Step 2 is measured on the spectrophotometer after blanking on 1 ml of cMEM. The two values obtained are averaged and this reading must be between 1.71 and 1.91.

If the final dilution is within the specified range, the stock solution can be aliquoted into suitable vials and stored at -20oC ± 5oC in the dark until required for use. To improve the consistency between assays, vials can be thawed and diluted for use on the day of assay. Any prepared solution not required should be discarded..

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Appendix A3

Table of BCOP Protocols from the Reviewed Literature

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Table of BCOP Protocols from Reviewed Literature

REFERENCE	INVITTOX Protocol #124 (BCOP Prevalidation - SOP of Microbiological Associates Ltd., UK)	INVITTOX Protocol #98 (EC/HO Validation Study; Balls et al. 1995)	Bailey et al. (2004)	Bruner et al. (1998)	Cassidy and Stanton (1997)
TEST METHOD COMPONENT					
Collection of bovine eyes	Eyes excised by an abattoir employee and collected as soon as possible after slaughter	Eyes are excised by an abattoir employee and collected in a plastic jar that holds approximately 25 eyes		Eyes excised by an abattoir employee and collected as soon as possible after slaughter	Bovine eyes obtained from a local abattoir
Transport conditions	Eyes transported to the lab in a container with Hanks Balanced Salt Solution containing 1% (v/v) penicillin/streptomycin solution	Storage jar contains 1 L of Hanks Balanced Salt Solution with Ca++, Mg++, supplemented with 0.350 g/L sodium bicarbonate	Transported in a receptacle containing Hank's Balanced Salt Solution with Ca++ and Mg++, and with 100 IU/mL penicillin and 100 µg/mL streptomycin (HBSS)	Eyes transported to the lab in a container with Hanks Balanced Salt Solution containing 1% (v/v) penicillin/streptomycin solution	Not noted
Temperature	Transported at ambient temperature	Transported at ambient temperature	Transported on ice	Not noted	Not noted
Time after slaughter until use	3 (± 1) hours after slaughter	Within 2 hours after slaughter	Eyes arrive in the laboratory within 4-5 hours of removing first eyes in a batch from cattle	Eyes used within 12 hours after receipt at laboratory	Not noted
Cornea preparation	At lab, eyes carefully examined for defects; unacceptable eyes rejected	At lab, eyes carefully examined for defects; unacceptable eyes rejected	At lab eyes are examined carefully and those with defects such as neovascularization, pigmentation, opacity, or scratches are rejected for testing.	At lab, eyes carefully examined for defects; unacceptable eyes rejected	At lab, eyes carefully examined for defects; unacceptable eyes rejected
Description of cornea dissection	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea with care taken to avoid damage to corneal epithelium and endothelium	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that a rim of sclera surrounds cornea
Storage of isolated corneas until use	Isolated corneas stored in petri dish with HBSS 1% penicillin/streptomycin solution until all dissections completed	Isolated corneas stored in petri dish with HBSS until use	Isolated corneas stored in petri dish with HBSS/penicillin/streptomycin solution until mounted in holders	Rinsed in HBSS	Not described
Type of cornea holder used	Conventional cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Conventional cornea holder for opacitometer	Cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Stag Bio, Clermont, France	Specially designed holder for the assay with anterior (epithelial side) and posterior (endothelial side) chambers

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TEST METHOD COMPONENT					
Pretreatment incubation/equilibration in corneal holder	After positioning cornea in holder and fixing in place with screws, posterior then anterior compartments of the holder are filled with assay medium. Air bubbles should not be present in the chambers.	After positioning cornea in holder and fixing in place with screws, posterior then anterior compartments of the holder are filled with assay medium.	Holders and medium are prewarmed to 32°C before mounting corneas. Endothelial side of the cornea is placed against O-ring of posterior chamber. Anterior chamber is placed over the cornea and chambers are joined together. Posterior then anterior chambers are filled with assay medium, avoiding formation of air bubbles and minimizing shear forces on the corneal endothelium.	After positioning cornea in holder and fixing in place with screws, posterior then anterior compartments of the holder are filled with assay medium.	Corneas free of defects were mounted in holders. Both posterior and anterior holder compartments were filled with assay medium.
Duration	1 hour (±5min)	1 hour	1 hour	1 hour	1 hour
Temperature	32°C (±2°C)	32°C	32°C (±1°C) maintained in a forced air incubator	32°C (±1°C)	32°C
Medium used for incubation	Freshly prepared complete (c) MEM (MEM + 1% L-glutamine + fetal bovine serum; clear medium without phenol red is to be used)	Eagle's Minimum Essential Medium (MEM) supplemented with 2.2 g/L sodium bicarbonate and 0.292 g/L (2 mM) glutamine (stored refrigerated up to 7 days); 1% fetal bovine serum is added to MEM for experiments (prepared daily); complete (c)MEM is preheated to 32°C for experiments	Eagle's Minimum Essential Medium (MEM) without phenol red containing 1% fetal bovine serum (complete MEM)	Minimum essential medium (MEM) containing 1% fetal bovine serum	Complete minimum essential medium (MEM)
Basal (pretreatment) opacity measurement taken	An initial opacity measurement was made immediately after 1 hour equilibration period and replacement of incubation media with fresh complete MEM	An initial opacity measurement was made immediately after 1 hour equilibration period and replacement of incubation media with fresh complete MEM	After the 1 hour incubation period, the medium is removed from both chambers of each holder (anterior chamber first) and replaced with fresh complete MEM. Then an initial opacity reading is taken and recorded for each cornea.	An initial opacity measurement was made after equilibration period	An initial opacity measurement was made after equilibration period
Instrument used to measure opacity	Opacitometer, which determines light transmission through the center of each mounted cornea	Opacitometer, which determines light transmission through the center of each mounted cornea	Opacitometer (Spectro Designs OP-KIT), which determines light transmission through the center of each mounted cornea	Opacitometer	Spectro-Designs OP-KIT opacitometer
Instrument calibrated prior to test (y/n)	Yes	Not noted	Not noted	Not noted	Not noted
Criteria for acceptable corneas for testing after equilibration period	Basal opacity of all corneas in the test is recorded; mean opacity value is determined; corneas deviating from mean by >3 units are discarded	Basal opacity values should be between 3 and -3	Corneas that display an initial opacity reading greater than 10 units from the average opacity for all of the corneas are not used in the assay	Not noted	Not noted
Treatment groups used (No. of corneas used/test substance)	3 corneas per test article	3 corneas per treatment group	3 to 5 corneas per test article	5 corneas per treatment group (3 for permeability and 2 for histopathology)	5 corneas per treatment group
Controls	3 corneas for each control	3 corneas for each control	2 or 3 corneas	5 corneas for each control (3 for permeability and 2 for histopathology)	2 or 3 corneas used depending on the type of control

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TEST METHOD COMPONENT					
Positive control(s), if used	Varies for test substance. For liquids, the control is ethanol; for surfactants, benzalkonium chloride (10%); for solids, imidazole (20%)	Varies for test substance. For liquids, the control is N,N-dimethylformamide; for surfactants, benzalkonium chloride (5% in saline); for solids, imidazole (20% in saline)	***Liquids and surfactants***: undiluted ethanol; ***solid test articles:*** 20% (w/v) solution of imidazole in complete MEM (without phenol red)	Pre-treatment exposure of 5 corneas to 100% ethanol for 10 minutes; post-treatment exposure of 5 other corneas that went through 24 hour treatment regimen with complete MEM to 100% ethanol for 10 minutes	2 corneas were treated with ethanol
Negative/untreated control	0.9% saline	Saline	Corneas that have opacity values close to the average opacity for all corneas are chosen as the negative (or solvent) control corneas. The negative control is sterile, deionized water.	MEM	3 corneas with opacity readings close to the median opacity for all the corneas were treated with complete MEM
Other controls, if used		Triacetin or PEG-600 when used as the solvent for dilutions	When alternate solvents are used, such as saline or phosphate buffered saline, solvent controls are run through the assay		
Treatment of corneas	Just prior to treatment, the anterior chamber is completely emptied of cMEM using an appropriately sized pipette tip or needle attached to a vacuum pump	Just prior to treatment, the anterior chamber is completely emptied of cMEM using an appropriately sized pipette tip or needle attached to a vacuum pump		Corneas receive four consecutive 6 hour exposures to test article over 24 hours. Just prior to first treatment, the anterior chamber is emptied.	Just prior to treatment, the anterior chamber is completely emptied of complete MEM
Liquid substances	Test substances are added to anterior chamber of holder, which is turned to a horizontal position and rocked gently to ensure complete coverage of cornea	Test substances are prewarmed at 32°C for a few minutes then added to anterior chamber of holder	***Nonviscous and semiviscous liquids*** tested using "closed chamber method". ***Semiviscous and viscous liquids*** tested using "open chamber method".	Cosmetic formulations are tested by addition to the anterior chamber of the cornea holder	Liquid silicone polymers were tested by addition to the anterior chamber of the cornea holder
Concentration tested	100% (neat)	Usually 100% (neat); if dilutions are required, saline is used for water soluble substances and PEG-600 or triacetin are used for water insoluble substances	Generally tested at 100% (neat); dilutions performed as needed or requested	100% (neat)	100%
Amount tested	750 µL (test substances and controls)	750 µL (test substances and controls)	750 µL (test substances and controls)	750 µL (test substances and controls)	750 µL (test substances and controls)
Incubation time	10 minutes (±30 seconds)	10 minutes	Standard exposure time is 10 minutes; shorter or longer exposure times are also used	6 hours x 4 exposure periods for a total of 24 hours	10 minutes
Incubation temperature	32°C (±2°C) water bath	32°C water bath	32°C (±1°C) for exposure times > 3 minutes; ≤3 minutes incubated at room temperature	32°C (±1°C) water bath	32°C incubator

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TEST METHOD COMPONENT					
Rinsing procedure	Epithelium is washed 3 or more times with 3 mL of cMEM from a syringe or until the wash medium is clear	Epithelium is washed 3 or more times with 4 mL of cMEM from a syringe or until the wash medium is clear	Epithelium is washed 3 or more times with 2-3 mL of cMEM (with phenol red) from a syringe. Once the rinsing medium is clear, one last rinse of the epithelium is performed using fresh complete MEM (without phenol red).	At the end of each 6 hour incubation, the test article was washed from the cornea with MEM, fresh MEM was added to both chambers, and opacity was measured. Fresh test article was added to the front chamber after the first three exposure periods.	Epithelium is washed 3 or more times with complete MEM until test material is completely removed. The anterior compartment was refilled with complete MEM and relative opacity determined.
Post-treatment incubation (time, temp.)	cMEM is added to anterior chamber of holder and corneas are incubated for 2 hours (±10 minutes) in a 32°C (±2°C) water bath; fresh cMEM is added to both chambers and final opacity measurement is taken	cMEM is added to anterior chamber of holder and corneas are incubated for 2 hours in a 32°C water bath; fresh cMEM is added to both chambers and final opacity measurement is taken	The anterior chamber is refilled with fresh complete MEM. A post-treatment opacity reading is taken and recorded for each cornea. Visual observations are performed for each cornea. Holders are incubated in a vertical position at 32°C (±1°C) for up to 3 hours. For test articles with exposure times >10 minutes, the exposure time is subtracted from the 2-hour post-exposure incubation period. Other post-exposure incubation times may be used. If corneas are incubated for >4 hours, the incubation medium is supplemented with antibiotics, and changed every 6 hours.	Not performed	Corneas returned to incubator for approximately 2 hours, after which a second measure of relative opacity was taken (report does not state that fresh MEM is added before final opacity measurements)
Surfactants		Test substances are prewarmed at 32°C for a few minutes then added to anterior chamber of holder		Not applicable	Not applicable
Concentration tested	10% (w/w) in 0.9% saline	10% in saline; other concentrations (in saline) can be tested as required			
Amount tested	750 µL (test substance and controls)	750 µL (test substance and controls)			
Incubation time	10 minutes (±30 seconds)	10 minutes			
Incubation temperature	32°C (±2°C) water bath	32°C water bath			
Rinsing procedure	Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until the wash medium is clear	Epithelium is washed 3 or more times with 4 mL of cMEM from a syringe or until the wash medium is clear			
Post-treatment incubation (time, temp.)	cMEM is added to anterior chamber of holder and corneas are incubated for 2 hours (±10 minutes) in a 32°C (±2°C) water bath; fresh cMEM is added to both chambers and final opacity measurement is taken	cMEM is added to anterior chamber of holder and corneas are incubated for 2 hours in a 32°C water bath; fresh cMEM is added to both chambers and final opacity measurement is taken			
Solid substances		Test substances are prewarmed at 32°C for a few minutes then added to anterior chamber of holder		Not applicable	Not applicable

Table of BCOP Protocols from Reviewed Literature

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TEST METHOD COMPONENT					
Concentration tested	20% (w/w) solution or suspension in 0.9% NaCl	20% solution or suspension in saline (usually 1g test substance + 5 mL saline)	20% (w/w) solution or suspension in sterile deionized water, complete MEM, or saline (or other appropriate solvent)		
Amount tested	750 µL (test substance and controls)	750 µL (test substance and controls)	750 µL (test substance and controls)		
Incubation time	4 hours (±5 minutes)	4 hours	4 hours (±5 minutes)		
Incubation temperature	32°C (±2°C) water bath	32°C water bath (holders completely immersed)	32°C (±2°C) water bath		
Rinsing procedure	Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until all particles are removed; fresh cMEM is added to both chambers and final opacity measurement is taken	Epithelium is washed 3 or more times with cMEM until all particles are removed; fresh cMEM is added to both chambers and final opacity measurement is taken	Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until all particles are removed; fresh cMEM is added to both chambers and final opacity measurement is taken		
Post-treatment incubation (time, temp.)	Not performed	Not performed			
Endpoints assessed					
Corneal opacity					
Data collected for opacity	Numerical opacity value (arbitrary unit) displayed by opacitometer; opaque spots or other irregularities are noted	Numerical opacity value (arbitrary unit) displayed by opacitometer	Numerical opacity value (arbitrary unit) displayed by opacitometer	Opacity measurements were recorded directly from the output display of the opacitometer; each opacity measurement was made relative to an air blank	Opacity value not described, but likely a numerical opacity value with an arbitrary unit displayed by opacitometer
Permeability	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	3 of the 5 treated corneas are used for permeability measurements. After the final 24 hour opacity reading, MEM was removed from the front chamber.	After the final opacity reading, medium was removed from both chambers of the holder. The posterior chamber was refilled with complete MEM.
Amount and concentration of sodium fluorescein solution used	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber	1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber
Incubation time for fluorescein solution	90 minutes ±5 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes ±5 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally
Incubation temperature for fluorescein	32°C (±2°C) water bath	32°C water bath (holders completely immersed)	32°C (±1°C)	32°C (±1°C) water bath	32°C
Instrumentation used	Spectrophotometer set at 490 nm; cuvette with a 1 cm path length is used	Spectrophotometer set at 490 nm	Microplate reader	Beckman DU-640 spectrophotometer which is zeroed with a sample of MEM	Spectrophotometer set at 490 nm
Instrument calibrated (y/n)	Yes	Not noted	Yes	Not noted	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	INVITTOX Protocol #124 (BCOP Prevalidation - SOP of Microbiological Associates Ltd., UK)	INVITTOX Protocol #98 (EC/HO Validation Study; Balls et al. 1995)	Bailey et al. (2004)	Bruner et al. (1998)	Cassidy and Stanton (1997)
TEST METHOD COMPONENT					
Data collected for permeability	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm
Aliquot taken from posterior chamber for OD 490 nm reading	1 mL	Not noted	Most of medium is removed from posterior chamber, then mixed in a sample tube. A 360 µL aliquot is taken from the sample tube and transferred to a 96-well plate. Standard plate map provides 2 wells for each cornea in case a dilution is required.	1 mL	Not noted
Other observations			During the final, post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded. Special attention is taken to observe dissimilar opacity patterns, tissue peeling, or residual test article.	Histopathological examination of 2 corneas per treatment and control groups	Histological examination of all corneas
Evaluation of test results					
Corneal opacity					
Basal (pretreatment) opacity subtracted from opacity of each treated cornea?	Yes	Yes	Yes	Yes	Opacity changes for each cornea were calculated by subtracting the initial opacity value from the final opacity value
Opacity for each treated cornea corrected for average value of negative/solvent controls?	Yes	Yes	Yes	Yes	Yes
Mean corrected opacity value calculated for each treatment group?	Yes	No	Yes		Yes
Permeability					
OD value for each treated cornea corrected for average value of negative/solvent controls?	Yes	Yes	Yes	Yes	Yes
Mean corrected permeability value calculated for each treatment group?	Yes	No	Yes		Yes

Table of BCOP Protocols from Reviewed Literature

REFERENCE	INVITTOX Protocol #124 (BCOP Prevalidation - SOP of Microbiological Associates Ltd., UK)	INVITTOX Protocol #98 (EC/HO Validation Study; Balls et al. 1995)	Bailey et al. (2004)	Bruner et al. (1998)	Cassidy and Stanton (1997)
TEST METHOD COMPONENT					
Formula used to calculate *In Vitro* Score	*In vitro* score = corrected opacity value + (15 x corrected OD_{490} value); the *in vitro* score is calculated for each cornea and the mean *in vitro* score is calculated from the individual *in vitro* score values	*In vitro* score = corrected opacity value + (15 x corrected OD_{490} value); the *in vitro* score is calculated for each cornea and the mean *in vitro* score is calculated from the individual *in vitro* score values	*In vitro* score = mean corrected opacity value + (15 x mean corrected OD_{490} value)	*In vitro* score calculated only for ethanol controls = corrected opacity value + (15 x corrected OD_{490} value	*In vitro* score = mean opacity value + (15 x mean OD_{490} value)
***In vitro* classification of ocular irritancy**	BCOP score 0 -3 = nonirritant; 3.1 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 - 80 = severe; > 80.1 = very severe	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 - 80 = severe; > 80 = very severe	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate irritant; 55.1 and above = severe irritant.	Not discussed	BCOP score 0 - 25 = nonirritating to mild eye irritant; 25.1 - 55 moderate; ≥ 55.1 = severe
Criteria for an acceptable test	Test is accepted if positive control gives an *in vitro* score that falls within 2 SDs of the current historical mean: ethanol (36.0 - 56.0); benzalkonium chloride (98.8 - 209.2); imidazole (111.2 - 164.0)	Test is accepted if positive control values fall within following limits: benzalkonium chloride (opacity > 60, permeability >3.000, score > 110); N,N-dimethylformamide (opacity >70, permeability > 1.500, score > 100); imidazole (opacity >35, permeability > 2.000, score > 70)	Test is accepted if positive control gives an *in vitro* score that falls within 2 SDs of the current historical mean, which is updated every 3 months.	The acceptable range for the in vitro score for the ethanol positive control was 22.1 to 44.7 (historical mean ±SD)	The acceptable range for the in vitro score for the ethanol positive control was 33.7 to 69.6 (historical mean ± 2SD)
Conducted in compliance with GLPs	Yes	Not noted	Yes	Not noted	Not noted
Other useful information				Dose-response curves were presented in the publication for the formulations tested showing changes in opacity over 24 hours. Photomicrographs of some histological data also are presented.	Photomicrographs of some histological data are presented in the publication.

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Casterton et al. (1996)	Cerven and Moreno (1998)	Chamberlain et al. (1997) -- IRAG Evaluation (8 data sets)	Cooper et al. (2001)	Gautheron et al. (1994) (fresh and preserved corneas)
TEST METHOD COMPONENT					
Collection of bovine eyes	Bovine eyes are collected and stored in a plastic cooler containing Hanks' Balanced Salt Solution with Ca++ and Mg++	Bovine eyes were received from a local supplier	Bovine eyes were collected from a local slaughterhouse	Bovine eyes were obtained from a local abattoir where the eyes were excised	Bovine eyes were collected from a commercial abattoir in a plastic jar for about 25 eyes
Transport conditions	Not described	Eyes were transported to the laboratory in Hanks Balanced Salt Solution in a refrigerated container.	Eyes were immersed in pH-adjusted (7.2-7.4) Hanks salt solution within 2 hours after the animals were killed	Eyes transported in a container with Hanks balanced salt solution supplemented with penicillin/streptomycin	1 L of Hanks balanced salt solution (HBSS) with Ca++ and Mg++
Temperature	Ambient temperature	Not noted	Not noted	Transported to laboratory over ice packs	Ambient temperature
Time after slaughter until use	Immediately after receipt and no more than 3 hours after removal from carcass	Eyes were examined within 1 hour after receipt	Not noted	Not noted	Eyes were used within 2 hours of killing the animals
Cornea preparation	At lab, eyes carefully examined for defects; unacceptable eyes rejected	At lab, eyes carefully examined for defects; unacceptable eyes rejected	At lab, eyes carefully examined for defects; unacceptable eyes rejected	Corneas were grossly examined for damage and those exhibiting defects were discarded	At lab, eyes carefully examined for defects; unacceptable eyes rejected
Description of cornea dissection	Cornea dissected such that approximately 1 - 2 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea; iris and lens were removed	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea
Storage of isolated corneas until use	Isolated corneas stored in petri dish with Eagle's Minimum Essential Medium (MEM) until use	Not noted	Not noted	Isolated corneas stored in petri dish with HBSS until they were mounted in a corneal holder	*Fresh corneas:* isolated corneas stored in petri dish with HBSS until they were used. *Preserved corneas:* corneas were washed 3x, each for 15 minutes, in HBSS supplemented with antiobiotics (penicillin/streptomycin); after rinsing in normal HBSS, they were placed individually into wells of 6-well culture plates, each containing 12 mL preservative medium; plates were then placed in the refrigerator at 4-5°C until the next day; for use, the preserved corneas were removed from the refrigerator, left on the bench for 30 minutes at room temperature, and thereafter treated the same way as fresh corneas
Type of cornea holder used	Cornea holder with anterior and posterior chambers, and custom-fitted rack for spectrophotometer	Specially designed holders segmented into anterior and posterior chambers	Specially made holder with two 5 mL chambers that interface with the epithelial and endothelial surfaces of the cornea	Not noted	Not noted

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TEST METHOD COMPONENT					
Pretreatment incubation/equilibration in corneal holder	After positioning cornea in holder and fixing in place with screws, posterior then anterior compartments of the holder are filled with warmed MEM.	After positioning cornea in holder and fixing in place with screws, posterior then anterior compartments of the holder are filled with assay medium. Cornea was inspected afterwards to ensure it was still intact.	After mounting cornea in holder, both chambers were filled with medium.	Each cornea was mounted in a holder with the endothelial side against the O-ring of the posterior half of the holder; the anterior half of the holder was then positioned on top of the cornea and screws were tightened; posterior then anterior chambers were filled with medium	Corneas were mounted in holders, which were subsequently filled with medium
Duration	1 hour	At least 1 hour, but not longer than 2 hours	1 hour	1 hour	1 hour
Temperature	32°C water bath	32°C water bath	32°C water bath	32°C (±1°C)	32°C (±1°C)
Medium used for incubation	Eagle's MEM supplemented with sodium bicarbonate, L-glutamine, and fetal bovine serum; continually warmed at 32°C during use; free of phenol red	Minimal essential media supplemented with 1% fetal bovine serum (MEM).	Eagle's minimal essential medium (MEM) supplemented with 1% fetal bovine serum	Eagle's minimum essential medium (MEM) without phenol red, with 1% fetal bovine serum (complete MEM)	Minimum essential medium (MEM) supplemented with glutamine and sodium bicarbonate as indicated by the supplier; the pH was adjusted to 7.4 and the medium was freshly used or stored refrigerated (1 week stock); in daily experiments it was supplemented with 1% fetal bovine serum and used prewarmed at 32°C
Basal (pretreatment) opacity measurement taken	For initial absorbance readings, each cornea is read against a blank in the reference beam	An initial opacity measurement was made immediately after equilibration period and replacement of incubation media with fresh MEM	The report states that the first opacity measurement was taken after the cornea was exposed to test substance	An initial opacity measurement was made immediately after 1 hour equilibration period and replacement of incubation media with fresh complete MEM	Not noted
Instrument used to measure opacity	Cary 219 UV-VIS spectrophotometer set at 570 nm	OP-KIT opacitometer produced by Electro-Design Corp. of Riom, France	Specially-designed opacitometer to determine the difference in light transmission between treated and control corneas	Spectro Designs OP-KIT opacitometer (Stag Bio, Clermont, Ferrand, France)	Opacitometer (Electro-Design, Riom, France), which determines the difference in light transmission between a treated and a control cornea
Instrument calibrated prior to test (y/n)	Calibration not described; instrument is balanced on two blank holders (filled only with MEM)	Not noted	Not noted	Not noted	The instrument was previously calibrated with standardized opaque sheets of polyester
Criteria for acceptable corneas for testing after equilibration period	Corneas with absorbance values > 0.1 are removed from the study	Not noted	Not noted	Not noted	Not noted
Treatment groups used (No. of corneas used/test substance)	At least 4 corneas per test material	Five corneas	3 to 6 corneas for each treatment group	5 corneas per formulation tested	6 corneas per test substance
Controls	3 corneas	2 corneas (at each opacity reading, each treated cornea was scored in comparison with the 2 control corneas)	3 to 6 corneas for each control	3 corneas for negative control and 5 corneas for positive control	3 corneas

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TEST METHOD COMPONENT					
Positive control(s), if used	Not described		3 labs reported use of acetone as a positive control for liquids; one lab reported use of imidazole as a positive control for solids	Ethanol	Not noted
Negative/untreated control	3 corneas with the lowest absorbance values are selected as controls		Not described	3 corneas with opacity readings close to the median opacity for all the corneas were treated with complete MEM	3 corneas treated with MEM
Other controls, if used					
Treatment of corneas	The anterior chamber is aspirated of MEM	Just prior to treatment, the anterior chamber is completely emptied of MEM.			The medium was removed from both chambers of the holders using a needle attached to a vacuum pump or a syringe. The posterior chamber was refilled with fresh MEM
Liquid substances	Prewarmed (32°C) test material is added to anterior chamber; corneas incubated in a horizontal position to completely bath the corneal surface with test material	Test substances are added to anterior chamber of holder, which is turned to a horizontal position	Test substances are added to anterior chamber of holder, which is turned to a horizontal position	Shampoo formulations were tested	Test substances are added to anterior chamber of holder, which is turned to a horizontal position
Concentration tested	100%	100%	100%	100% and 10% (w/v) prepared in complete MEM	100%
Amount tested	1.00 mL	750 µL (test substances)	500 µL (test substances)	750 µL (test substances and controls)	750 µL (test substances and controls)
Incubation time	10 minutes	10 minutes (± 1 minute)	10 minutes (3 labs), 30 minutes (3 labs), or 60 minutes (1 lab); 1 lab used both 10 and 30 minute exposures; 1 lab did not report an exposure time	For most materials, incubation time was 10 minutes for undiluted materials and 60 minutes for 10% dilutions; in a separate study, 2 materials were tested undiluted for 10, 30, and 60 minutes AND as 10% dilutions for 10, 30, 60 and 120 minutes.	10 minutes
Incubation temperature	Room temperature	32°C water bath	32°C	32°C (±1°C) water bath	32°C

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TEST METHOD COMPONENT					
Rinsing procedure	Epithelium is washed 3 or more times with HBSS until the wash medium is clear. After last rinse, both chambers are aspirated and filled with fresh MEM.	Test substance removed from chamber by washing with MEM. Both chambers then refilled with fresh MEM. NOTE: For test materials containing alcohol, an additional opacity measurement was taken following the 10 minute exposure time and addition of fresh medium to both chambers	At the end of the exposure, the epithelial side was washed, the anterior compartment was refilled with MEM + 1% fetal bovine serum and a first opacity measurement taken	Epithelium is washed 3 or more times with complete MEM containing phenol red to ensure complete removal of test material; corneas given a final rinse with complete MEM without phenol red; anterior chamber was refilled with complete MEM and opacity determined	Epithelium is washed 3 or more times with 4 mL of MEM until the wash medium is clear. Anterior chamber was refilled with medium, and first opacity measurement taken.
Post-treatment incubation (time, temp.)	Corneas are incubated for 2 more hours in a 32°C water bath	Corneas are incubated for 2 hours in a 32°C water bath; the MEM was changed and opacity measured, comparing each of the 5 treated corneas to the 2 control corneas	Corneas are incubated for 2 more hours in a 32°C water bath, followed by a second opacity reading, which was the reported value	Corneas are incubated in a 32±1°C water bath until total incubation time reaches 120 minutes. Post-treatment incubation varies depending on initial exposure time from 110 minutes to 90 minutes to 60 minutes or no further incubation. A second opacity reading was taken for all corneas except for those with a 120 minute exposure time.	After treatment, corneas were incubated for 2 hours at 32°C; a second opacity measurement was taken, which was used for calculations
Surfactants	Not described	Not applicable	Although surfactants were tested by some labs, a specific protocol for surfactants was not included in report	Not tested	
Concentration tested					10% in MEM
Amount tested					750 µL (test substance and controls)
Incubation time					10 minutes
Incubation temperature					32°C
Rinsing procedure					Epithelium is washed 3 or more times with 4 mL of MEM until the wash medium is clear. Anterior chamber was refilled with medium, and first opacity measurement taken.
Post-treatment incubation (time, temp.)					After treatment, corneas were incubated for 2 hours at 32°C; a second opacity measurement was taken, which was used for calculations
Solid substances	Solids are applied directly to the corneal surface. The glass window of the anterior chamber of the corneal holder is removed to facilitate application of solids.			Not tested	

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REFERENCE	Casterton et al. (1996)	Cerven and Moreno (1998)	Chamberlain et al. (1997) -- IRAG Evaluation (8 data sets)	Cooper et al. (2001)	Gautheron et al. (1994) (fresh and preserved corneas)
TEST METHOD COMPONENT					
Concentration tested	100%	Solids dissolved in MEM at a 20% dilution	20% (200 mg/mL) in MEM + 1% fetal bovine serum; many compounds were tested as suspensions		Approximate 20% solution or suspension (200 mg + 1 mL) in MEM
Amount tested	Enough to cover the corneal thoroughly (about 1/8 teaspoon)	750 µL (test substance)	500 µL (test substance)		750 µL (test substance and controls)
Incubation time	1 hour		4 hours		4 hours
Incubation temperature	32°C water bath	32°C water bath	room temperature		32°C
Rinsing procedure	Epithelium is washed 3 or more times with HBSS until the wash medium is clear. After last rinse, both chambers are aspirated and filled with fresh MEM.	Test substance removed from chamber by washing with MEM. Both chambers then refilled with fresh MEM. Opacity was measured, comparing each of the 5 treated corneas to the 2 control corneas.	The epithelial side was washed, fresh medium was added, and opacity was measured		Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until all particles are removed; fresh cMEM is added to both chambers and opacity measurement is taken
Post-treatment incubation (time, temp.)	1 hour	Not performed	Not performed		Not performed
Endpoints assessed					
Corneal opacity					
Data collected for opacity	UV-VIS spectrophotometer absorbance readings at 570 nm	A pre-exposure determination of opacity was made for each control by measuring each against the blanks supplied with the opacitometer; a pre-exposure determination of opacity was made for each of the test corneas by measuring against each control cornea	Not described	The opacity values obtained at the second opacity measurement (except for the 120 minute exposure group) were used to calculate the corneal opacity	Numerical opacity value (arbitrary unit) displayed by opacitometer
Permeability	After the final absorbance readings, both chambers are aspirated and the posterior side is filled with fresh MEM.	Immediately following the 2 hour opacity measurement, the MEM was changed in the posterior chamber of both the control and test corneas.	Fresh medium is added to the posterior compartment	After the final opacity measurement, the medium was removed from both chambers of the holder. The posterior chamber was refilled with fresh complete MEM.	After the final opacity measurement, the medium was removed from both chambers of the holder. The posterior chamber was refilled with fresh MEM.
Amount and concentration of sodium fluorescein solution used	1 mL of fluorescein solution (0.4% in Dulbecco's phosphate buffered saline) was added to the anterior chamber	1.0 mL of 0.4% sodium fluorescein solution	1 mL of a 5 mg/mL solution of sodium fluorescein in Dulbecco's phosphate buffered saline was added to the anterior compartment	1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber	1 mL of a 0.4% fluorescein solution is used for liquids and surfactants; 1 mL of a 0.5% fluorescein solution is used for solids
Incubation time for fluorescein solution	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally
Incubation temperature for fluorescein	32°C water bath	32°C water bath	Not specified	32±1°C water bath	32°C
Instrumentation used	Dynatech MR5000 microplate reader	Spectronic 20 spectrophotometer	Spectrophotometer	Molecular Devices Vmax kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA, USA)	Spectrophotometer set at 490 nm
Instrument calibrated (y/n)	Not described	Not described	Not described	Not described	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Casterton et al. (1996)	Cerven and Moreno (1998)	Chamberlain et al. (1997) -- IRAG Evaluation (8 data sets)	Cooper et al. (2001)	Gautheron et al. (1994) (fresh and preserved corneas)
TEST METHOD COMPONENT					
Data collected for permeability	Optical density reading at 490 nm	Optical density reading at 450 nm	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm
Aliquot taken from posterior chamber for OD 490 nm reading	100 µL	Not described	Not specified	360 µL added to designated well of a 96-well plate	Not noted
Other observations				Corneal swelling (wet weight of 8 mm tissue punch) and histology	
Evaluation of test results					
Corneal opacity					
Basal (pretreatment) opacity subtracted from opacity of each treated cornea?	Pretreatment absorbance values for each treated cornea are subtracted from the corresponding 2 hour post-treatment absorbance values	A pre-exposure determination of opacity was made for each control by measuring each against the blanks supplied with the opacitometer; a pre-exposure determination of opacity was made for each of the test corneas by measuring against each control cornea	Not described	Yes	Basal opacity not performed
Opacity for each treated cornea corrected for average value of negative/solvent controls?	The absorbance for each treated cornea is corrected by the mean absorbance value for the three control corneas	The corrected mean opacity score was calculated, using the control and treated cornea opacity values as determined from the OP-KIT opacitometer	The difference in light transmission between treated and control corneas was determined with the opacitometer	The corrected opacity value of each cornea was calculated by subtracting the average change in opacity of the negative control corneas from that of each treated cornea	The difference in light transmission between treated and control corneas was determined with the opacitometer
Mean corrected opacity value calculated for each treatment group?	No	The corrected mean opacity score was calculated, using the control and treated cornea opacity values as determined from the OP-KIT opacitometer	The mean value of opacity ± SD was calculated for each substance	The mean opacity value of each treatment group was calculated by averaging the mean corrected opacity values of the treated corneas for each treatment group	For each substance evaluated, the mean value of opacity ± SD was calculated
Permeability					
OD value for each treated cornea corrected for average value of negative/solvent controls?	The instrument setup allows calculations to take into account both the blank and the control values; therefore, the resulting readings require no further correction	The corrected mean OD 450 nm score was calculated using the control and treated OD values	The amount of dye penetration through the control corneas was subtracted from the amount of dye penetration through treated corneas	The corrected OD_{490} was calculated by subtracting the mean OD_{490} value of the negative control corneas from the OD_{490} of each treated cornea	Not noted
Mean corrected permeability value calculated for each treatment group?	Instrument setup takes into account number of replicates per test material; the obtained value represents the mean corrected optical permeability that results from exposure of the corneal surface to a test material	The corrected mean OD 450 nm score was calculated using the control and treated OD values	The mean absorbance value ± SD was calculated for each substance	The mean OD_{490} value of each treatment group was calculated by averaging the corrected OD_{490} values of the treated corneas	The mean OD_{490} value of each treatment group was calculated by averaging the OD_{490} values of the treated corneas

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REFERENCE	Casterton et al. (1996)	Cerven and Moreno (1998)	Chamberlain et al. (1997) -- IRAG Evaluation (8 data sets)	Cooper et al. (2001)	Gautheron et al. (1994) (fresh and preserved corneas)
TEST METHOD COMPONENT					
Formula used to calculate *In Vitro* Score	The two endpoints, opacity and permeability, are evaluated separately.	*In vitro* score = corrected mean opacity value + (15 x mean corrected OD_{450} value)	For some submissions, *in vitro* score = opacity value + (15 x OD_{490} value). In other submissions, the opacity and permeability values are considered separately, with the irritancy classification based on the greater of the two values.	*In vitro* score = mean opacity value + (15 x mean OD_{490} value)	*In vitro* score = mean opacity value + (15 x mean OD_{490} value)
***In vitro* classification of ocular irritancy**	The irritation class is based on the endpoint that equates to the greater irritation potential: mild (opacity <0.400 or permeability <0.175); moderate (0.400 ≤ opacity < 1.300 or 0.175 ≤ permeability < 0.600); severe (opacity >1.300 or permeability >0.600)	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 and greater = severe	Not noted	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 and greater = severe (applied to both undiluted and diluted test materials)	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 and greater = severe
Criteria for an acceptable test	Not noted	Not noted	Not noted	Not described	Not noted
Conducted in compliance with GLPs	Not noted	Not noted	Not noted	Not described	Not noted
Other useful information			A generalized BCOP protocol was provided in the IRAG report for the eight BCOP data sets evaluated by the IRAG working group. Although some protocol differences were noted between the testing laboratories (e.g., exposure time and data analysis), some generalizations do not reflect a majority of other published protocols (e.g., amount of substance tested, use of assay medium, measuring basal corneal opacity prior to exposure period). Note that the generalized protocol description was not very detailed, and that individual protocols for each of the 8 data sets were not provided.		

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Collection of bovine eyes		Bovine eyes were obtained from a local abattoir where the eyes were excised	Bovine eyes were collected in a plastic container	Bovine eyes obtained from a local abattoir	Eyes collected from a local slaughterhouse
Transport conditions	Transported in a receptacle containing Hank's Balanced Salt Solution with Ca++ and Mg++, and with 100 IU/mL penicillin and 100 µg/mL streptomycin (HBSS)	Eyes transported in a container with Hanks balanced salt solution supplemented with penicillin/streptomycin	Eyes transported in Hanks balanced salt solution	Eyes transported to the laboratory in a saline solution (Hanks)	Eyes transported to the laboratory immersed in pH-adjusted (7.2 - 7.4) Hanks salt solution
Temperature	Transported on ice	Transported to laboratory over ice packs	Room temperature	Not noted	Not noted
Time after slaughter until use	Eyes arrive in the laboratory within 4-5 hours of removing first eyes in a batch from cattle	Not noted	Collection of eyes and transportation to testing laboratory was completed within 2 hours	Not noted	Eyes were collected and transported to the laboratory within 2 hours of killing the animals
Cornea preparation	At lab eyes are examined carefully and those with defects such as neovascularization, pigmentation, opacity, or scratches are rejected for testing.	Corneas were grossly examined for damage and those exhibiting defects were discarded	All eyes were carefully examined visually, or with a stereomicroscope, if needed, and eyes presenting defects were rejected	Eyes were carefully examined for their quality at the laboratory	At lab, eyes carefully examined for defects; unacceptable eyes rejected
Description of cornea dissection	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Iris and lens were removed, and cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Not noted	Selected corneas were dissected with a 2-3 mm rim of sclera attached; the iris and the lens were removed
Storage of isolated corneas until use	Isolated corneas stored in petri dish with HBSS/penicillin/streptomycin solution until mounted in holders	Isolated corneas stored in petri dish with HBSS until they were mounted in a corneal holder	Isolated corneas stored in petri dish with HBSS until they were mounted in a corneal holder	Not applicable. After dissection, corneas were quickly mounted in holders	Corneas were mounted in holders immediately after dissection
Type of cornea holder used	Cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Not noted	Conventional cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Composed of specially designed plastic chambers with two separate compartments	Holder consisted of two 5 mL chambers

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REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Pretreatment incubation/equilibration in corneal holder	Holders and medium are prewarmed to 32°C before mounting corneas. Endothelial side of the cornea is placed against O-ring of posterior chamber. Anterior chamber is placed over the cornea and chambers are joined together. Posterior then anterior chambers are filled with assay medium, avoiding formation of air bubbles and minimizing shear forces on the corneal endothelium.	Each cornea was mounted in a holder filled with medium	Each cornea was mounted in a holder with the endothelial side against the O-ring of the posterior half of the holder; the anterior half of the holder was then positioned on top of the cornea and screws were tightened; posterior then anterior chambers were filled with medium	Corneas were firmly clamped in between the anterior and posterior compartments	
Duration	1 hour	1 hour	1 hour	1 hour	1 hour
Temperature	32°C (±1°C) maintained in a forced air incubator	32°C (±1°C)	32°C (±1°C)	32°C	32°C
Medium used for incubation	Eagle's Minimum Essential Medium (MEM) without phenol red containing 1% fetal bovine serum (complete MEM)	Eagle's minimum essential medium (MEM) without phenol red, with 1% fetal bovine serum (complete MEM)	Prewarmed Eagle's minimum essential medium	Eagle's minimum essential medium supplemented with 1% fetal calf serum	Eagle's minimum essential medium (pH 7.2-7.4) supplemented with 1% fetal bovine serum
Basal (pretreatment) opacity measurement taken	After the 1 hour incubation period, the medium is removed from both chambers of each holder (anterior chamber first) and replaced with fresh complete MEM. Then an initial opacity reading is taken and recorded for each cornea.	An initial opacity measurement was made immediately after 1 hour equilibration period and replacement of incubation media with fresh complete MEM	An initial opacity measurement was made immediately after 1 hour equilibration period	Not noted	No
Instrument used to measure opacity	Opacitometer (Spectro Designs OP-KIT), which determines light transmission through the center of each mounted cornea	Spectro Designs OP-KIT opacitometer (Stag Bio, Clermont, Ferrand, France)	Opacitometer	Opacitometer	Specially-designed opacitometer; light passes simultaneously through a control and treated cornea held in separate chambers and the transmitted light is detected by photocells in each chamber
Instrument calibrated prior to test (y/n)	Not noted	Not noted	Not noted	Not noted	Instrument was calibrated but it's not clear if this was done prior to each test
Criteria for acceptable corneas for testing after equilibration period	Corneas that display an initial opacity reading greater than 10 units from the average opacity for all of the corneas are not used in the assay	Not noted	Not noted	Not noted	Not noted
Treatment groups used (No. of corneas used/test substance)	3 to 5 corneas per test article	5 corneas per formulation tested	3 corneas per formulation tested (cosmetics and personal care products)	Not noted	4 corneas per test compound
Controls	2 or 3 corneas	3 corneas for negative control and 2 corneas for positive control	3 corneas with the lowest opacity scores were selected as negative controls	Vehicle controls used, but specific number not noted	1 cornea for the "control" slot in the opacitometer

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Positive control(s), if used	Acetone was the concurrent positive control (10 minute exposure)	Ethanol			Not noted
Negative/untreated control	Corneas that have opacity values close to the average opacity for all corneas are chosen as the negative (or solvent) control corneas. The negative control is sterile, deionized water.	3 corneas with opacity readings close to the median opacity for all the corneas were treated with complete MEM	Eagle's MEM	Eagle's MEM	On each experimental day, two corneas were exposed to the vehicle and the one remaining the clearest was used as the control for the opacitometer
Other controls, if used					
Treatment of corneas			The MEM was removed from both compartments, anterior compartment first, and the posterior compartment refilled with fresh MEM.	After equilibration, fresh medium was added to the posterior compartment (endothelial side) and test material or vehicle was added to the anterior compartment (epithelial side)	To start the experiment, fresh MEM with 1% FBS was added to the posterior compartment
Liquid substances	***Nonviscous and semiviscous liquids*** tested using "closed chamber method". ***Semiviscous and viscous liquids*** tested using "open chamber method".		The test material was added to the anterior compartment	Cosmetic formulations were tested	Test substances were added to the anterior compartment (epithelium side)
Concentration tested	10% (w/v) solution	100% for conditioners; shampoos were tested at both 100% and 10% (w/v) prepared in complete MEM	100%	Not noted	100%
Amount tested	750 µL (test substances and controls)	750 µL (test substances and controls)	750 µL (test substances and control)	Not noted	500 µL
Incubation time	1 hour	Undiluted materials were incubated for 10 minutes and 10% dilutions were incubated for 60 minutes	10 minutes	10 minutes	30 minutes
Incubation temperature	32°C (±1°C)	32°C (±1°C)	32°C (±2°C) water bath	Not noted	Room temperature

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Rinsing procedure	Epithelium is washed 3 or more times with 2-3 mL of cMEM (with phenol red) from a syringe. Once the rinsing medium is clear, one last rinse of the epithelium is performed using fresh complete MEM (without phenol red).	Epithelium is washed 3 or more times with complete MEM containing phenol red to ensure complete removal of test material; corneas given a final rinse with complete MEM without phenol red; anterior chamber was refilled with complete MEM and opacity determined	The epithelium was washed at least three times, until the medium was clear, with MEM	The epithelial side was washed; no details provided	The epithelial side was washed; no details provided
Post-treatment incubation (time, temp.)	The anterior chamber is refilled with fresh complete MEM. A post-treatment opacity reading is taken and recorded for each cornea. Visual observations are performed for each cornea. Holders are incubated in a vertical position at 32°C (±1°C) for 1 hour.	After treatment, corneas were incubated for 2 hours at 32(±1)°C; a second opacity measurement was taken, which was used for calculations	The anterior compartment was refilled with MEM, and an initial opacity measurement taken. Corneas were incubated for 2 hours at 32(±1)°C, and a second opacity measurement was taken, which was used for calculations	The anterior compartment was refilled with fresh medium. Corneas were incubated for 2 hours, temperature not noted	The anterior compartment was refilled with MEM + 1%FBS and a first opacity reading was performed; corneas were incubated at 32°C for another 2 hours followed by a second opacity reading which was the reported value
Surfactants		Not tested	Not tested		
Concentration tested				Not noted	
Amount tested				Not noted	
Incubation time				10 minutes	
Incubation temperature				Not noted	
Rinsing procedure				The epithelial side was washed; no details provided	
Post-treatment incubation (time, temp.)				The anterior compartment was refilled with fresh medium. Corneas were incubated for 2 hours, temperature not noted	
Solid substances		Not tested		Not tested	

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Concentration tested			Concentration not noted, although solids were tested as solutions or suspensions		20% (200 mg/mL) in MEM + 1%FBS as solutions or suspensions
Amount tested			750 µL (test substance and vehicle control)		500 µL
Incubation time			4 hours		4 hours
Incubation temperature			32°C (±2°C) water bath		Room temperature
Rinsing procedure			Epithelium was washed at least three times with MEM until the medium was clear and and particulate free. Gentle swirling movements were necessary to remove particulates from the surface of the cornea. The posterior then the anterior chambers were refilled with fresh MEM, and a final opacity measurement taken.		Epithelial side was washed, but no details provided; opacity was measured
Post-treatment incubation (time, temp.)			Not performed		Not performed
Endpoints assessed					
Corneal opacity					
Data collected for opacity	Numerical opacity value (arbitrary unit) displayed by opacitometer	The opacity values obtained at the second opacity measurement (except for the 120 minute exposure group) were used to calculate the corneal opacity	Opacity was measured by placing each control cornea in the "control" compartment of the opacitometer. Each treated cornea was placed the "treated" compartment and the values displayed were recorded. The glass portion of each holder was dried prior to opacity measurement.	No details provided	The opacity reading is expressed as arbitrary units on a scale which is determined by calibrating the instrument with increasing thicknesses of a standard opaque material (provided by the manufacturer)
Permeability	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	After the final opacity measurement, the medium was removed from both chambers of the holder. The posterior chamber was refilled with fresh complete MEM.	Medium was removed from both chambers of each holder, anterior chamber first. Fresh MEM was added to the posterior chamber.	Medium was removed from both compartments. Fresh medium was added to the posterior compartment.	After the final opacity readings were completed, medium was removed from the holders. Fresh medium was added to the posterior compartment.
Amount and concentration of sodium fluorescein solution used	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	Sodium fluorescein solution was added to the anterior compartment; no details provided	1 mL of 5 mg/mL Na-fluorescein solution in Dulbecco's phosphate-buffered saline was added to the anterior compartment
Incubation time for fluorescein solution	90 minutes ± 5 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally	90 minutes	90 minutes; holder incubated horizontally
Incubation temperature for fluorescein	32°C (±1°C)	32±1°C water bath	32°C (±2°C) water bath	Not noted	Not noted
Instrumentation used	Microplate reader	Molecular Devices Vmax kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA, USA)	Optical density was measured spectrophotometrically in a plate reader using 200 µL MEM as a blank	Not specified	Measured spectrophotometrically at 490 nm (peak wavelength for Na-fluorescein absorbance)
Instrument calibrated (y/n)	Yes	Not described	Not noted	Not noted	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Data collected for permeability	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm	Absorbance values determined spectrophotometrically at 490 nm	Optical density reading at 490 nm
Aliquot taken from posterior chamber for OD 490 nm reading	Most of medium is removed from posterior chamber, then mixed in a sample tube. A 360 µL aliquot is taken from the sample tube and transferred to a 96-well plate. Standard plate map provides 2 wells for each cornea in case a dilution is required.	Not noted	200 µL	Not noted	Not noted
Other observations	During the final, post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded. Special attention is taken to observe dissimilar opacity patterns, tissue peeling, or residual test article.	Corneal swelling (dry weight of 8 mm tissue punch) and histology			
Evaluation of test results					
Corneal opacity					
Basal (pretreatment) opacity subtracted from opacity of each treated cornea?	Yes	Yes	Not noted	Not noted	Basal opacity not measured for each cornea
Opacity for each treated cornea corrected for average value of negative/solvent controls?	Yes	The corrected opacity value of each cornea was calculated by subtracting the average change in opacity of the negative control corneas from that of each treated cornea	The difference in light transmission between treated and control corneas was determined with the opacitometer	Not noted	Opacitometer determines the difference in light transmission between treated and control corneas
Mean corrected opacity value calculated for each treatment group?	Yes	The mean corrected opacity value of each treatment group was calculated	Not noted	Not noted	Mean opacity value ± SD was calculated for each treatment group
Permeability					
OD value for each treated cornea corrected for average value of negative/solvent controls?	Yes	The corrected OD_{490} was calculated by subtracting the mean OD_{490} value of the negative control corneas from the OD_{490} of each treated cornea	Not noted	Not noted	No
Mean corrected permeability value calculated for each treatment group?	Yes	The mean OD_{490} value of each treatment group was calculated	Not noted	Not noted	Mean OD value ± SD was calculated

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Gettings et al. (1996)	Jones et al. (2001)	Rachui et al. (1994)	Rougier et al. (1994)	Sina et al. (1995)
TEST METHOD COMPONENT					
Formula used to calculate *In Vitro* Score	*In vitro* score = mean corrected opacity value + (15 x mean corrected OD_{490} value)	*In vitro* score = mean opacity value + (15 x mean OD_{490} value)	*In vitro* score = opacity value + (15 x OD_{490} value)	*In vitro* score = opacity value + (15 x absorbance value)	It was not clearly stated that mean values were used in the formulas. *In vitro* score = opacity value + (15 x OD_{490} value). This formula was derived empirically during in-house and interlaboratory evaluation studies. Data generated for 36 compounds in a multilaboratory study were subjected to a multivariate analysis to determine the equation of best fit between the *in vivo* and *in vitro* data.
***In vitro* classification of ocular irritancy**	The surfactant-based formulations induced little opacity, so the permeability value was used to assign an *in vitro* classification (>0.600 = severe irritant)	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate; 55.1 and greater = substantial	For each test substance an average was taken of *in vitro* scores obtained for 3 corneas. BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 mild/moderate; 55.1 and greater = severe	Not noted; likely the same one used by Gautheron et al. (1994)	BCOP score 0 -15 = nonirritant/mild; >15 - 25 = mild eye irritant; >25 - 55 moderate; >55 = severe
Criteria for an acceptable test	Test is accepted if positive control gives an *in vitro* score that falls within 2 SDs of the current historical mean, which is updated every 3 months.	Not described	Not described	Not noted	Not noted
Conducted in compliance with GLPs	Yes	Not noted	Not noted		Not noted
Other useful information					

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Collection of bovine eyes			Bovine eyes were excised in the slaughterhouse shortly after slaughter.
Transport conditions	Transported in a receptacle containing Hank's Balanced Salt Solution with Ca++ and Mg++, and with 100 IU/mL penicillin and 100 µg/mL streptomycin (HBSS)	Transported in a receptacle containing Hank's Balanced Salt Solution with Ca++ and Mg++, and with 100 IU/mL penicillin and 100 µg/mL streptomycin (HBSS)	Eyes were transported immersed in Hanks' balanced salt solution
Temperature	Transported on ice	Transported on ice	Not noted
Time after slaughter until use	Eyes arrive in the laboratory within 4-5 hours of removing first eyes in a batch from cattle	Eyes arrive in the laboratory within 4-5 hours of removing first eyes in a batch from cattle	Not noted
Cornea preparation	At lab eyes are examined carefully and those with defects such as neovascularization, pigmentation, opacity, or scratches are rejected for testing.	At lab eyes are examined carefully and those with defects such as neovascularization, pigmentation, opacity, or scratches are rejected for testing.	
Description of cornea dissection	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Cornea dissected such that approximately 2 - 3 mm rim of sclera surrounds cornea	Corneas were dissected from eyes leaving a small sclera rim (about 2 mm), after which they were rinsed twice in HBSS before mounting in corneal holders
Storage of isolated corneas until use	Isolated corneas stored in petri dish with HBSS/penicillin/streptomycin solution until mounted in holders	Isolated corneas stored in petri dish with HBSS/penicillin/streptomycin solution until mounted in holders	Not noted
Type of cornea holder used	Cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Cornea holder for opacitometer with anterior (epithelial side) and posterior (endothelial side) chambers	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Pretreatment incubation/equilibration in corneal holder	Holders and medium are prewarmed to 32°C before mounting corneas. Endothelial side of the cornea is placed against O-ring of posterior chamber. Anterior chamber is placed over the cornea and chambers are joined together. Posterior then anterior chambers are filled with assay medium, avoiding formation of air bubbles and minimizing shear forces on the corneal endothelium.	Holders and medium are prewarmed to 32°C before mounting corneas. Endothelial side of the cornea is placed against O-ring of posterior chamber. Anterior chamber is placed over the cornea and chambers are joined together. Posterior then anterior chambers are filled with assay medium, avoiding formation of air bubbles and minimizing shear forces on the corneal endothelium.	
Duration	1 hour	1 hour	1 hour
Temperature	32°C (±1°C) maintained in a forced air incubator	32°C (±1°C) maintained in a forced air incubator	32°C
Medium used for incubation	Eagle's Minimum Essential Medium (MEM) without phenol red containing 1% fetal bovine serum (complete MEM)	Eagle's Minimum Essential Medium (MEM) without phenol red containing 1% fetal bovine serum (complete MEM)	Eagle's Minimal Essential Medium (MEM, Sigma) supplemented with serum and sodium hydrogen carbonate pH adjusted to 7.2 (complete MEM).
Basal (pretreatment) opacity measurement taken	After the 1 hour incubation period, the medium is removed from both chambers of each holder (anterior chamber first) and replaced with fresh complete MEM. Then an initial opacity reading is taken and recorded for each cornea.	After the 1 hour incubation period, the medium is removed from both chambers of each holder (anterior chamber first) and replaced with fresh complete MEM. Then an initial opacity reading is taken and recorded for each cornea.	An initial opacity measurement was made immediately after 1 hour equilibration period and replacement of incubation media with fresh complete MEM
Instrument used to measure opacity	Opacitometer (Spectro Designs OP-KIT), which determines light transmission through the center of each mounted cornea	Opacitometer (Spectro Designs OP-KIT), which determines light transmission through the center of each mounted cornea	OP-KIT, Electro Design, Riom, France
Instrument calibrated prior to test (y/n)	Not noted	Not noted	
Criteria for acceptable corneas for testing after equilibration period	Corneas that display an initial opacity reading greater than 10 units from the average opacity for all of the corneas are not used in the assay	Corneas that display an initial opacity reading greater than 10 units from the average opacity for all of the corneas are not used in the assay	Corneas were rejected if their background opacity grade was greater than 3
Treatment groups used (No. of corneas used/test substance)	3 to 5 corneas per test article	3 to 5 corneas per test article	6 corneas per test compound
Controls	2 or 3 corneas	2 or 3 corneas	3 corneas

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Positive control(s), if used	***Liquids and surfactants***: undiluted ethanol; ***solid test articles:*** 20% (w/v) solution of imidazole in complete MEM (without phenol red)	***Liquids and surfactants***: undiluted ethanol; ***solid test articles:*** 20% (w/v) solution of imidazole in complete MEM (without phenol red)	Not noted
Negative/untreated control	Corneas that have opacity values close to the average opacity for all corneas are chosen as the negative (or solvent) control corneas. The negative control is sterile, deionized water.	Corneas that have opacity values close to the average opacity for all corneas are chosen as the negative (or solvent) control corneas. The negative control is sterile, deionized water.	Complete MEM
Other controls, if used			
Treatment of corneas			Just prior to treatment, the anterior chamber is completely emptied of MEM
Liquid substances	***Nonviscous and semiviscous liquids*** tested using "closed chamber method". ***Semiviscous and viscous liquids*** tested using "open chamber method".	***Nonviscous and semiviscous liquids*** tested using "closed chamber method". ***Semiviscous and viscous liquids*** tested using "open chamber method".	Test substances were added to the anterior compartment (epithelium side)
Concentration tested	Generally tested at 100% (neat); dilutions performed as needed or requested	Generally tested at 100% (neat); dilutions performed as needed or requested	100%
Amount tested	750 µL (test substances and controls)	750 µL (test substances and controls)	750 µL
Incubation time	10 minutes (±30 seconds)	10 minutes (±30 seconds)	10 minutes
Incubation temperature	32°C (±1°C)	32°C (±1°C)	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Rinsing procedure	Epithelium is washed 3 or more times with 2-3 mL of cMEM (with phenol red) from a syringe. Once the rinsing medium is clear, one last rinse of the epithelium is performed using fresh complete MEM (without phenol red).	Epithelium is washed 3 or more times with 2-3 mL of cMEM (with phenol red) from a syringe. Once the rinsing medium is clear, one last rinse of the epithelium is performed using fresh complete MEM (without phenol red).	Not noted
Post-treatment incubation (time, temp.)	The anterior chamber is refilled with fresh complete MEM. A post-treatment opacity reading is taken and recorded for each cornea. Visual observations are performed for each cornea. Holders are incubated in a vertical position at 32°C (±1°C) for 2 hours.	The anterior chamber is refilled with fresh complete MEM. A post-treatment opacity reading is taken and recorded for each cornea. Visual observations are performed for each cornea. Holders are incubated in a vertical position at 32°C (±1°C) for 2 hours.	Corneas were incubated for 2 hours; however, no other details provided
Surfactants			Not tested
Concentration tested			
Amount tested			
Incubation time			
Incubation temperature			
Rinsing procedure			
Post-treatment incubation (time, temp.)			
Solid substances			

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Concentration tested	20% (w/w) solution or suspension in sterile deionized water, complete MEM, or saline (or other appropriate solvent)	20% (w/w) solution or suspension in sterile deionized water, complete MEM, or saline (or other appropriate solvent)	20% solutions or suspensions were prepared in complete MEM
Amount tested	750 µL (test substance and controls)	750 µL (test substance and controls)	750 µL
Incubation time	4 hours (±5 minutes)	4 hours (±5 minutes)	4 hours
Incubation temperature	32°C (±2°C) water bath	32°C (±2°C) water bath	Not noted
Rinsing procedure	Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until all particles are removed; fresh cMEM is added to both chambers and final opacity measurement is taken	Epithelium is washed 3 or more times with 3 mL of cMEM each time from a syringe or until all particles are removed; fresh cMEM is added to both chambers and final opacity measurement is taken	Not noted
Post-treatment incubation (time, temp.)			Not performed
Endpoints assessed			
Corneal opacity			
Data collected for opacity	Numerical opacity value (arbitrary unit) displayed by opacitometer	Numerical opacity value (arbitrary unit) displayed by opacitometer	Numerical opacity value (arbitrary unit) displayed by opacitometer
Permeability	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	Medium is removed from the anterior chamber, which is refilled with sodium fluorescein solution; amount of dye that reaches posterior chamber is evaluated as an indicator of increased permeability or damage to the cornea	Medium was removed from both chambers of the corneal holder and the posterior chamber was refilled with fresh complete MEM.
Amount and concentration of sodium fluorescein solution used	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	1 mL of a 4 mg/mL fluorescein solution is used for liquids and surfactants; 1 mL of a 5 mg/mL fluorescein solution is used for solids	0.4% or 0.5% sodium fluorescein solution for liquids or solids, respectively; dye diluted in Dulbecco's phosphate buffered saline (Sigma)
Incubation time for fluorescein solution	90 minutes ±5 minutes; holder is incubated horizontally	90 minutes ±5 minutes; holder is incubated horizontally	90 minutes; holder is incubated horizontally
Incubation temperature for fluorescein	32°C (±1°C)	32°C (±1°C)	Not noted
Instrumentation used	Microplate reader	Microplate reader	Cary 1 UV-visible spectrophotometer set at 490 nm
Instrument calibrated (y/n)	Yes	Yes	Not noted

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Data collected for permeability	Optical density reading at 490 nm	Optical density reading at 490 nm	Optical density reading at 490 nm
Aliquot taken from posterior chamber for OD 490 nm reading	Most of medium is removed from posterior chamber, then mixed in a sample tube. A 360 µL aliquot is taken from the sample tube and transferred to a 96-well plate. Standard plate map provides 2 wells for each cornea in case a dilution is required.	Most of medium is removed from posterior chamber, then mixed in a sample tube. A 360 µL aliquot is taken from the sample tube and transferred to a 96-well plate. Standard plate map provides 2 wells for each cornea in case a dilution is required.	Not noted
Other observations	During the final, post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded. Special attention is taken to observe dissimilar opacity patterns, tissue peeling, or residual test article.	During the final, post-treatment opacity reading, visual observations are performed for each cornea and, if necessary, are recorded. Special attention is taken to observe dissimilar opacity patterns, tissue peeling, or residual test article.	
Evaluation of test results			
Corneal opacity			
Basal (pretreatment) opacity subtracted from opacity of each treated cornea?	Yes	Yes	Initial opacity of each cornea was subtracted from the chemically induced value
Opacity for each treated cornea corrected for average value of negative/solvent controls?	Yes	Yes	Not noted
Mean corrected opacity value calculated for each treatment group?	Yes	Yes	Yes
Permeability			
OD value for each treated cornea corrected for average value of negative/solvent controls?	Yes	Yes	Not noted
Mean corrected permeability value calculated for each treatment group?	Yes	Yes	Yes

Table of BCOP Protocols from Reviewed Literature

REFERENCE	Swanson et al. (1995)	Swanson and Harbell (2000)	Vanparys et al. (1993)
TEST METHOD COMPONENT			
Formula used to calculate *In Vitro* Score	*In vitro* score = mean corrected opacity value + (15 x mean corrected OD_{490} value)	*In vitro* score = mean corrected opacity value + (15 x mean corrected OD_{490} value)	*In vitro* score = mean opacity value + (15 x mean OD_{490} value)
***In vitro* classification of ocular irritancy**	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate irritant; 55.1 and above = severe irritant.	BCOP score 0 - 25 = mild eye irritant; 25.1 - 55 moderate irritant; 55.1 and above = severe irritant.	BCOP score 0 -3 = nonirritant; 3.1 - 25 = mild eye irritant; 25.1 - 55 moderate; > 55 = severe
Criteria for an acceptable test	Test is accepted if positive control gives an *in vitro* score that falls within 2 SDs of the current historical mean, which is updated every 3 months.	Test is accepted if positive control gives an *in vitro* score that falls within 2 SDs of the current historical mean, which is updated every 3 months.	Not noted
Conducted in compliance with GLPs	Yes	Yes	Not noted
Other useful information			