

## Bovine Corneal Opacity and Permeability (BCOP) Assay

### *INVITTOX* n° 127

#### Eye Irritation

*This protocol represents a refined and optimised version of INVITTOX protocol N° 124 in compliance with the respective OECD Test Guideline No. 437 for its use as part of a tiered-testing strategy to classify substances as ocular corrosives or severe irritants without further testing in rabbits.*

#### Objective and Applications

TYPE OF TESTING	: Screening, adjunct, replacement
LEVEL OF ASSESSMENT	: Toxic potential, toxic potency
PURPOSE OF TESTING	: Classification and labelling, ranking
CONTEXT OF USE	: The BCOP assay shall be used in appropriate circumstances and with certain limitations as a screening test to identify potential ocular corrosives and severe irritants as determined by US EPA (Category 1, US 1996), EU chemical substances classification (R41, EU 2001 and 2008 2 <sup>nd</sup> ) and UN GHS (Category 1, UN 2007)(ESAC, 2007; OECD TG 437, 2009) in the context of a sequential testing strategy for eye irritation and corrosion (OECD TG 405, 2002 and Method B.5, EU 2004 and 2008 1 <sup>st</sup> ), as part of a weight-of-evidence approach.  According to these guidelines, a positive result could be accepted as indicative of an ocular corrosive or severe irritant response without further testing, whereas substances giving negative results should be subsequently tested in another validated non-animal eye irritation protocol or in rabbits according to a sequential testing strategy.
APPLICABILITY DOMAIN	: All types of substances (including formulations) could be used (ICCVAM, 2006).  The limitation of the assay is the high false positive rates for alcohols and ketones and the high false negative rate for solids. The exclusion of these compounds improves the accuracy of the BCOP assay (ICCVAM, 2006).

#### Rationale

The BCOP assay is an organotypic assay, developed as an alternative to the Draize rabbit eye irritation test (Draize *et al.*, 1944). In this assay the quantitative measurements of changes in corneal opacity and permeability with an opacitometer and a visible light spectrophotometer are used to evaluate the potential ocular irritancy of a test substance (ICCVAM, 2006).

The BCOP assay originally developed by Gautheron *et al.*, (1992) participated in the EC/HO Validation Study but did not meet the criteria set by the management team of the study at that time (Balls *et al.*, 1995). This protocol is available in the DB-ALM as "*INVITTOX* N° 98".

The subsequent modification and optimisation of this protocol by Microbiological Associates Ltd. has led to its successful evaluation in BCOP assay Prevalidation Process in 1997-1998 (Southee and Curren, 1996; Southee 1998). This protocol is available in the DB-ALM as "*INVITTOX* N° 124"...

The protocol herewith presented (*INVITTOX* N° 127) is the result of a further review of available data undertaken by ICCVAM in 2005 and endorsed by ECVAM in 2007 that finally led to its regulatory acceptance (OECD TG 437, 2009).

A review document of the BCOP assay is available as "Method Summary" in the DB-ALM.

## Experimental Description

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### Endpoint and Endpoint Measurement:

CORNEAL OPACITY: corneal opacity measured using an opacitometer.

CORNEAL PERMEABILITY: corneal permeability determined using sodium fluorescein and measured spectrophotometrically (increase in OD<sub>490</sub>).

### Endpoint Value:

*IN VITRO* IRRITATION SCORE: an *in vitro* irritation score (IVIS) which equals mean opacity value + (15 × mean permeability OD<sub>490</sub> value) is used to evaluate the irritation potential of a test material. An IVIS ≥ 55.1 would be predictive of a corrosive or severe eye irritant.

### Experimental System(s):

CORNEA (bovine): Freshly isolated bovine cornea.

*Note: use of cornea from calves (6 – 12 months old) is also foreseen in the OECD Test Guideline (OECD TG 437, 2009).*

### Basic procedure

Bovine eyes recovered from a slaughterhouse are inspected and undamaged corneas are dissected and mounted in specially constructed holders. After 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, which may be liquid or solid, are diluted at 10% weight/volume (w/v). Both are applied for 10 minutes. Before reading the final opacity, the corneas are rinsed and incubated for 2 hours in fresh media to equilibrate.

The second method (B) is used with solids, tested at 20% (w/v) solution or suspension in 0.9% NaCl or deionised H<sub>2</sub>O. 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. In Method A a fluorescein concentration of 4 mg/mL is used and in Method B - 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* irritation score.

## Data Analysis/Prediction Model

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

A substance that induces an IVIS ≥ 55.1 is defined as corrosive or severe irritant (ICCVAM, 2006, OECD TG 437, 2009).

## Modifications of the Method

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In the BCOP assay, eyes from cattle (12 – 60 months old) have traditionally been used. However the possibility to use eyes from calves (6 – 12 months old) is foreseen in the OECD Test Guideline (OECD TG 437, 2009).

With respect to *INVITTOX* protocol N°124, the protocol refinements mainly refer to procedure improvements such as phenol red indicator in washing medium, direct application of undiluted solids using an open chamber method, the prediction model and positive controls used.

## Status

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### Participation in Validation Studies:

The herewith presented Standard Operating Procedure of the BCOP assay is based both on the *INVITTOX* *INVITTOX* protocol N°124 and on a protocol from the Institute for In Vitro Sciences (IIVS) that has **retrospectively been evaluated by ICCVAM (2005) and endorsed for its use, with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants within a tiered-testing strategy (ICCVAM, 2006).**

Based on the positive outcome of the ICCVAM retrospective study (ICCVAM, 2006), **the ESAC unanimously endorsed the BCOP test method for the use in appropriate circumstances and with certain limitations as a screening test to identify substances as ocular corrosives and severe irritants** in the context of a sequential testing strategy for eye irritation and corrosion (TG 405, OECD 2002 and Method B.5, EU 2004, 2008) as part of a weight-of-evidence approach (ESAC, 2007).

### Regulatory Acceptance:

**The OECD Test Guideline for the BCOP assay (OECD TG 437, 2009) has been adopted on the 7<sup>th</sup> of September 2009 (OECD, 2009).**

## Proprietary and/or Confidentiality Issues

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None reported.

## Abbreviations & Definitions

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BCOP – Bovine Corneal Opacity and Permeability assay  
ECVAM – European Centre for the Validation of Alternative Methods  
EPA – US Environmental Protection Agency  
ESAC – ECVAM Scientific Advisory Committee  
EU – European Union  
ICCVAM – US Interagency Coordinating Committee on the Validation of Alternative Methods  
IVIS – *in vitro* irritation score  
OECD – Organization for Economic Co-operation and Development  
TG – Test Guideline  
UN GHS – United Nations Globally Harmonized System of Classification and Labelling of Chemicals  
w/v – weight per volume

*Last update: October 2009*

## PROCEDURE DETAILS, October 2009

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### Bovine Corneal Opacity and Permeability (BCOP) Assay *INVITTOX* n° 127

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#### Health and Safety Issues

All procedures with bovine eyes, corneas and contaminated media should follow the testing facility's applicable policies and procedures for handling animal-derived materials. Universal laboratory safety precautions for are recommended.

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#### Materials and Preparations

##### Cell or Test System

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.

##### Equipment

###### *Fixed Equipment*

- Opacitometer (see Appendix A)
- Cornea holders sufficient for controls and test articles
- Spectrophotometer or microplate reader (see Appendix B)
- Incubator at 32°C or water bath at 32°C
- 10 mL syringes or vacuum pump for aspirating media
- Scalpel
- Scissors
- Forceps
- Electric Screwdriver
- Mortar & Pestle
- Positive displacement pipette
- Micro pipettes

### *Consumables*

- 5 or 10 mL syringes
- 30 mL Syringes
- Needles (19 G, 1 x 40, or 1.5 inch)
- Petri dishes
- 96-well plates (polystyrene) or cuvettes of an appropriate size for spectrophotometer

### **Media, Reagents, Sera, others**

- Complete MEM (cMEM) without phenol red: Minimal Essential Medium (MEM) (Eagle's) without Phenol Red [e.g., Life Technologies; Cat No. 51200 ] containing 1% Fetal Bovine Serum (FBS) [e.g., PAA; Cat No.A15-652] and 1% L-glutamine [e.g., Gibco; Cat No.043-05030]
- Complete MEM (cMEM) with phenol red: Minimal Essential Medium (MEM) (Eagle's) with Phenol Red containing 1% Foetal Bovine Serum (FBS) [e.g., PAA; Cat No.A15-652] and 1% L-glutamine [e.g., Gibco; Cat No.043-05030]
- Hanks' Balanced Salt Solution (1X) without phenol Red (HBSS) [e.g., Life Technologies; Cat No. 14025-050], containing penicillin (100 IU/mL) and 100 µg/mL streptomycin [e.g., Life technologies; Cat No. 15140-114]
- 0.9% NaCl Solution [e.g., Sigma; Cat No. S-8776]
- Sodium Fluorescein solution [e.g., Sigma; Cat No. F-6377]: (4 mg/mL for liquid test articles, or 5 mg/mL for solid test articles) diluted in Dulbecco's Phosphate Buffered Saline
- Sodium hydroxide
- Ethanol, 200 proof
- Dimethylformamide
- Imidazole [e.g., Sigma-Aldrich; Cat No. I,20-2]

### **Preparations**

#### *Media and Endpoint Assay Solutions*

Complete MEM solutions may be stored at 2-8 °C for up to two weeks. HBSS may be stored at 2-8 °C for up to two weeks until the manufacturer's suggested expiry. The fluorescein solutions may be stored at 2-8 °C, protected from exposure to light, for up to three months.

#### *Test Compounds*

An estimate of pH for each neat (liquid) test article or diluted test article (if diluted/suspended in 0.9% NaCl or deionised H<sub>2</sub>O) will be determined and recorded using universal pH paper.

Method A. Non surfactant liquids will be tested neat (100%). Known surfactants (either solids or liquids) will be tested at a 10% (w/v) concentration in 0.9% NaCl or deionised H<sub>2</sub>O.

Method B. Solid materials and the positive control compound (imidazole) will be tested at 20% (w/v) solution or suspension in 0.9% NaCl or deionised H<sub>2</sub>O. For example, homogeneous preparations can be prepared by vortexing or using a mortar and pestle.

With justification direct application of undiluted solids is allowed using an open chamber method (OECD TG 437, 2009).

#### *Positive Control(s)*

Method A Liquid test articles - 10% (w/v) sodium hydroxide or pure ethanol  
Surfactant test articles – 10% (w/v) dimethylformamide or pure ethanol

Method B Solid test articles - 20% (w/v) imidazole

#### *Negative Control(s)*

0.9% NaCl or deionised H<sub>2</sub>O

## Method

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### Test System Procurement

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. Eyes from cattle 12-60 months old are typically used, however the use of corneas from young animals (6-12 months old) is permissible. In the latter case, the report of age and/or weight of animals used is encouraged (OECD TG 437, 2009).

Excised eyes will be contained and transported to the laboratory in HBSS containing penicillin/streptomycin solution (enough to cover all eyes in the receptacle) at room temperature, or on wet ice (to minimize deterioration or bacterial contamination). The eyes will generally be used within 6 to 8 hours after slaughter.

### Routine Culture Procedure

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 3 mm of sclera is present around the cornea. The isolated corneas will be stored in a petri dish containing HBSS containing penicillin/streptomycin solution until all corneas are dissected.

### Test Material Exposure Procedures

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 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 10 mL or 30 mL 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 at least 1 hour at 32°C ±1°C in a water bath or an incubator.

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 or syringe 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. Baseline opacity readings are taken for each cornea. These initial opacity readings are used in the calculation of the final net opacity change for the treated corneas.

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 or deionised H<sub>2</sub>O 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.

### Endpoint Measurement

#### OPACITY MEASUREMENT

The opacitometer will determine the light transmission through the centre of each mounted cornea. A numerical opacity value (arbitrary unit) will be displayed and recorded. The opacitometer will be calibrated at the start of each experiment in each assay (see Appendix A). The opacity of each of the corneas will be determined by placing the "air" control holder in the left compartment of the opacitometer, and reading the opacity of each corneal holder in the right hand compartment.

Any corneas that show macroscopic tissue damage or an initial opacity >7 opacity units are discarded (OECD TG 437, 2009). 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.

#### Method A:

Non surfactant liquids will be tested neat (100%). Known surfactants (either solids or liquids) will be tested at a 10% (w/v) concentration in 0.9% NaCl or deionised H<sub>2</sub>O.

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 750 µl of the negative control (0.9% NaCl or deionised H<sub>2</sub>O) and with the appropriate 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 ± 1°C for 10 minutes (± 30 seconds) in a water bath or in an incubator.

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 containing phenol red (to determine the effectiveness of rinsing acidic or alkaline materials) 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 or syringe. If the test article proves difficult to remove by this method, the front glass window (open chamber) may be removed and the cornea carefully washed using a gentle stream of cMEM from a wash bottle or syringe. cMEM without phenol red is used for the final rinse.

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, and the corneas will then be incubated for 2 hours ± 10 minutes at 32 ± 1°C in the water bath or incubator.

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 compartments will be plugged, and a post-treatment opacity of each cornea recorded. The values obtained at this measurement will be recorded and used in calculating the change in 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.

#### Method B:

Solid materials and the positive control compound (imidazole) will be tested at 20% (w/v) solution or suspension in 0.9% NaCl or deionised H<sub>2</sub>O. Homogeneous suspensions will be prepared as described in the Preparations section.

In certain circumstances and with proper justification, solids may also be tested neat by direct application onto the corneal surface using the open chamber method (OECD TG 437, 2009).

750 µL of the test article, negative control (0.9% NaCl or deionised H<sub>2</sub>O) or positive control will be introduced into the anterior part of the holder using a suitable positive displacement pipette. The front glass window (open chamber) 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 ± 1°C for 4 hours ± 5 minutes in a water bath or an incubator.

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 or syringe to remove it. If the test article proves difficult to remove by this method, the front glass window may be removed and the cornea gently washed with cMEM using a wash bottle or syringe.

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 compartments 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.

## 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).

### 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.

### 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).

### 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  $\pm$  5 minutes at  $32 \pm 1^\circ\text{C}$  in a water bath or incubator.

After incubation the medium in the posterior chamber will be entirely removed and transferred into labelled tubes and mixed. An aliquot of the mixed medium from the posterior chamber will be transferred to a cuvette with a 1 cm path length (or 360  $\mu\text{L}$  transferred to a 96-well plate).

The spectrophotometer or plate reader will be adjusted to read at OD<sub>490</sub> 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<sub>490</sub> beyond the range of the spectrophotometer (see Appendix B) will be diluted in cMEM to obtain readings within the linear range.

### 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.

## **Acceptance Criteria**

The test will be accepted if the positive control causes an *in vitro* irritation score that falls within two standard deviations of the current historical mean of the testing facility.

## **Data Analysis**

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The *in vitro* irritation score is generated from the opacity and permeability measurements as described below. A suitable computer spreadsheet can be used to make the calculations.

### 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.

### PERMEABILITY

Mean OD<sub>490</sub> for the blanks will be subtracted from the OD<sub>490</sub> for each well to determine the corrected OD<sub>490</sub> values. The corrected OD<sub>490</sub> 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. Any dilutions made to bring OD<sub>490</sub> into linear range will have the diluted OD<sub>490</sub> multiplied by the dilution factor.

#### IN VITRO IRRITATION SCORE CALCULATION

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

$$\text{In Vitro Irritation Score} = \text{Corrected Opacity Value} + (15 \times \text{Corrected OD}_{490} \text{ Value})$$

The *In Vitro* Irritation Score will be calculated for each individual treatment and positive control group.

#### Prediction Model

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The following prediction model (PM) is a modification of the PM suggested by Gautheron, *et al.* (1994). Results from test situations should be compared to known materials tested under similar conditions. This classification system is the most consistent with recent regulatory reviews of the assay performance.

<b><i>In Vitro</i> Irritation Score</b>	<b><i>In Vitro</i> Irritation Scale</b>
0 - 3	non eye irritant
3.1-25	mild eye irritant
25.1-55	moderate eye irritant
≥55.1	severe/corrosive eye irritant

*In Vitro* Irritation Score (IVIS) ≥ 55.1 is used to define a corrosive or severe irritant (OECD TG 437, 2009).

## Annexes

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### Appendix A

#### Calibration of Opacitometer

An opacitometer (formerly from Electro Design, and STAG BIO) can be obtained from MC2 at the following address:

MC2  
19 Rue Patrick DEPAILLER  
La Pardieu  
63063  
CLERMONT FERRAND CEDEX 01  
FRANCE

The opacitometer will be calibrated at the beginning of every experiment on every 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, MC2.

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.

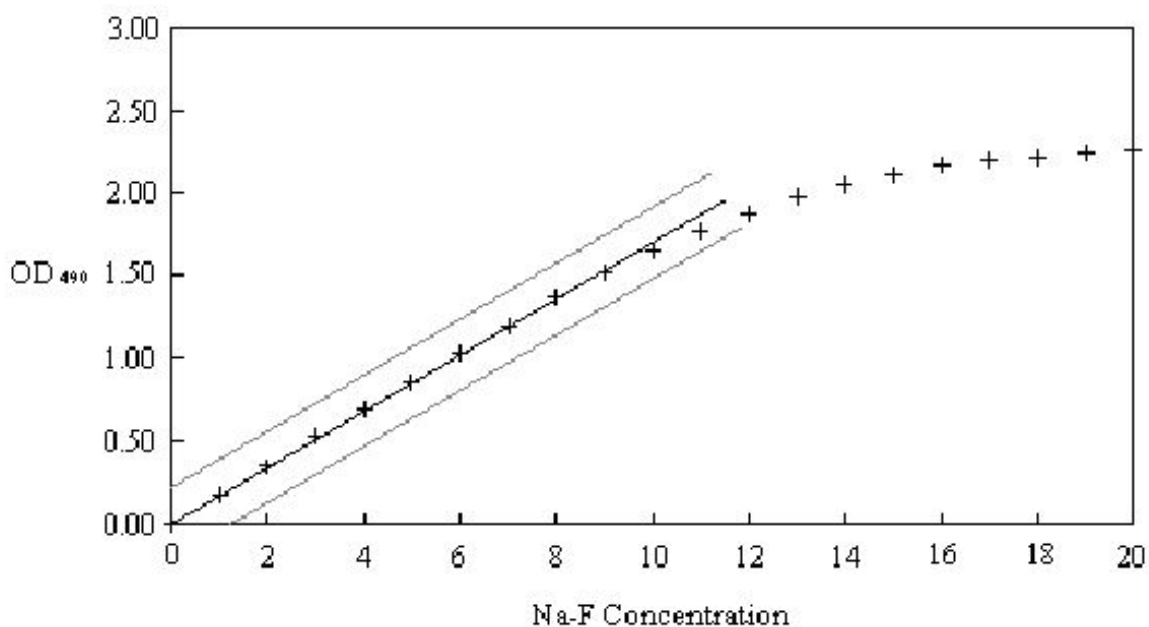
A (100X) stock solution of Na-fluorescein (NaF) is made by dissolving 0.2g NaF in 100 mL cMEM; a second stock solution (1X) is then prepared by diluting 1 mL of the first stock (100X) in 100 mL of cMEM in a standard flask; a concentration of 20 µg/mL is achieved.

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.

<b>Cuvette No.</b>	<b># µL cMEM</b>	<b># µL stock 1x</b>	<b>Concentration (µg/mL)</b>
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 OD<sub>490</sub> of 1.80, hence if the OD<sub>490</sub> > 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 (1 g dissolved in cMEM 250 mL) is prepared. This is diluted 1/400 in cMEM in two steps;

Step 1: 950  $\mu$ L cMEM + 50  $\mu$ L Na-F stock;

Step 2: 50  $\mu$ L of Step 1 solution + 950  $\mu$ L 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  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$  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 (1.25 g dissolved in cMEM 250 mL) is prepared. This is diluted 1/500 in cMEM in two steps.

Step1: 950  $\mu$ L cMEM + 50  $\mu$ L Na-F stock;

Step2: 40  $\mu$ L of Step 1 solution + 960  $\mu$ L 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  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$  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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