DB-ALM Protocol n° 124 : Bovine Corneal Opacity and Permeability (BCOP) Assay - SOP of Microbiological Associates LTD., UK

Eye Irritation

This protocol represents a refined and optimised version of the DB-ALM Protocol N° 98, produced as an outcome of a prevalidation study on the replacement of the Draize rabbit eye irritation test. *(Prevalidation study protocol)*

Résumé

The protocol of the bovine corneal opacity and permeability (BCOP) assay developed by Gautheron *et al.*, (1992) (DB-ALM N° 98) 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 (Balls *et al.*, 1995) of the Draize rabbit eye irritation test (Draize *et al.*, 1944). 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 between- and within-laboratory variation (Anon., 1998).

This protocol represents a modified and optimised version of Gautheron's BCOP assay which participated in a subsequent study (BCOP assay Prevalidation Process; 1997-1998) (Southee and Curren, 1996; Southee 1998) to overcome the previously encountered shortcomings.

A refined and optimised version of BCOP assay in compliance with the respective OECD Test Guideline (OECD TG 437, 2009) is available in DB-ALM as Protocol N° 127: "Bovine Corneal Opacity and Permeability (BCOP) Assay", and a review document - as Method Summary: "bovine Corneal Opacity and Permeability (BCOP) Assay".

Experimental Description

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)

Experimental System(s):

CORNEA (BOVINE): Freshly isolated bovine cornea (intact, epithelium-removed, Descemet's membrane and endothelium-removed; stroma)

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, 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.

Data Analysis/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.

Test Compounds and Results Summary

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.

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 improvements such as the way of measuring permeability, the calculation of the results, the treatment and dilution of test compounds and the kind of positive controls used.

Status

Participation in Validation Studies:

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 (Southee and Curren, 1996; Southee, 1998). 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.

A modified version of this SOP has later on undergone further evaluation by the expert panel of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (ICCVAM, 2005). In 2006, ICCVAM issued a report endorsing the use of the BCOP, with certain limitations, as a screening test to identify substances as ocular corrosives and severe irritants in a tiered-testing strategy, using a weight-of-evidence approach, for regulatory hazard classification (ICCVAM, 2006).

Based on the positive outcome of the ICCVAM retrospective study (ICCVAM, 2006) **the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed (ESAC, 2007) 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** as determined by the US EPA (Category 1, US 1996), EU chemical substances classification category (R41, EU 2001 and 2008 2nd) and UN GHS (Category 1, UN 2007) within the context of a sequential testing strategy for eye irritation and corrosion (OECD Test Guideline 405, OECD 2012 and Method B.5 of Annex V of the Directive 67/548/EEC, EU 2004, which was later incorporated into the Annex to Commission Regulation 440/2008/EC, EU 2008 1st).

Regulatory Acceptance:

The OECD Test Guideline No 437: Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants was adopted in September 2009 (OECD TG 437, 2009).

The approved validation study protocol compliant with the respective OECD Test Guideline (OECD TG 437, 2009) is available as "Protocol N° 127" in the DB-ALM.

Last update: November 2009

PROCEDURE DETAILS, April 1997 *

Bovine Corneal Opacity and Permeability (BCOP) Assay - SOP of Microbiological Associates LTD., UK
DB-ALM Protocol n° 124

This protocol presents the standard operating procedure used in the study "BCOP assay prevalidation project" (1997).

* The US ICCVAM (2006), the EU ECVAM (ESAC, 2007) and OECD (2009) refer to this DB-ALM Protocol version in their statements, studies and/or regulation. Therefore, the herewith included Standard Operating Procedure will be maintained in the current format and content for documentation purposes.

Considering all the changes introduced during these studies conducted by the organisations indicated above which finally led to the regulatory acceptance of this method, a new protocol version has been prepared and is available as Protocol N° 127: "Bovine Corneal Opacity and Permeability (BCOP) Assay" in the DB-ALM.

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 (19G1½1,1 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 H_2O [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 OD490 and a sample of cMEM read. The spectrophotometer will be blanked on this solution prior to reading the transferred solutions. Any solutions giving an OD490 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 OD490 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 OD490 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.

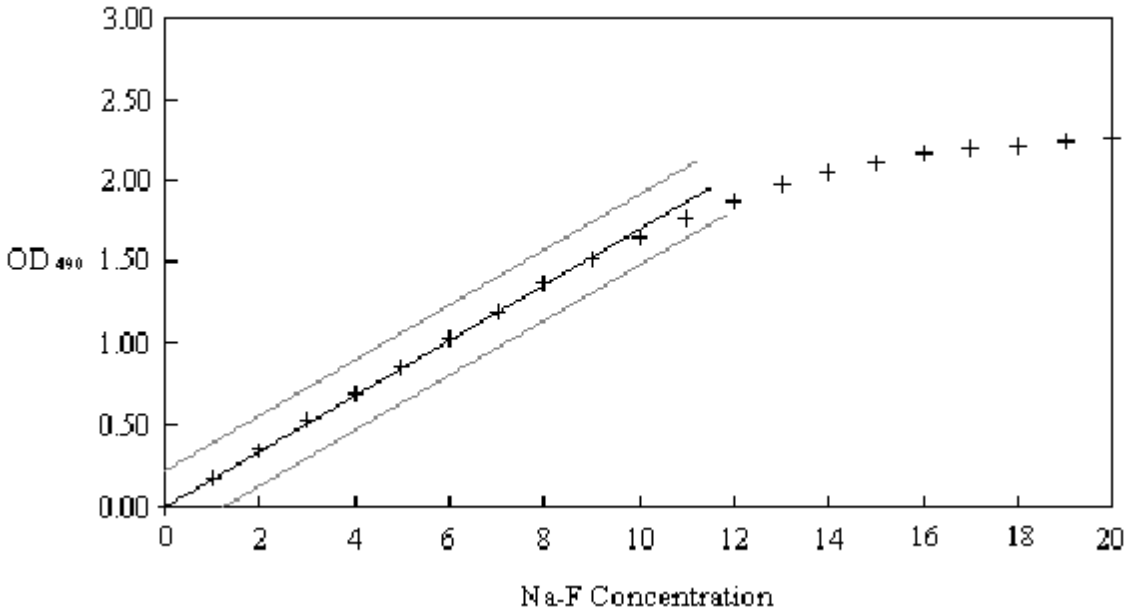
A series of 22 cuvettes will be prepared as described in Table 1. The OD determination is performed at 490 nm and results should closely follow those displayed in Figure 1.

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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