DB-ALM Protocol n° 71 : Fluorescein Leakage (FL) Test

Eye Irritation

Damage caused by the test compound to the tight junctions in a confluent MDCK monolayer is determined by the amount of topically applied fluorescein which leaks through the cell layer as an indication of the eye irritancy potential. This protocol is in compliance with the OECD Test Guideline No. 460: "Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants" and can be included in a tiered-testing strategy to classify substances as potential ocular corrosives or severe irritants without further testing in rabbits.

Objective & Application

TYPE OF TESTING	:	Replacement (partial) , Screening, Adjunct,
LEVEL OF ASSESSMENT	:	Toxic potential, Toxic potency
PURPOSE OF TESTING	:	Classification and labelling, Ranking

Context of Use:

The Fluorescein Leakage (FL) test method has been endorsed by OECD as Test Guideline No. 460: “Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants” (OECD, 2012). The test method can be used for regulatory and non-regulatory purpose. In the regulatory context of the OECD TG 460 and a sequential testing strategy for eye irritation and corrosion **the FL test method can be used in appropriate circumstances and with certain limitations as a screening test to identify Category 1 ocular corrosives and severe irritants**. Hazard Category 1 indicates a risk of severe and irreversible eye damage, defined, for the purpose of harmonized classification and labelling of chemical products, by the EU Regulation on Classification, Labelling and Packaging of Substances and Mixtures, UN Globally Harmonized System of Classification and Labelling of Chemicals and the US Environmental Protection Agency (EU-CLP, 2008; UN, 2009; US EPA, 1996). According to the sequential testing strategies for the acute eye irritation and corrosion, outlined in OECD TG 405 and EU Test Method B.5, a positive result can be accepted as indicative of an ocular corrosive or severe irritant response without any further testing. Substance giving a negative result should be tested with another validated *in vitro* or *ex vivo* ocular or skin corrosion test. Only when no alternative validated method is available or the results of the alternative tests are inconclusive, should further *in vivo* testing be performed in rabbits (OECD TG 405, 2012 and EU TRM B.5, 2008).

Applicability Domain:

The FL test method models ocular irritation caused by damage to the inter-cellular tight junctions and the cell membranes of the corneal and conjunctival epithelia. In the EC/HO validation study (Balls et al, 1995), the test method has been applied to a broad range of substances including alcohols, amines, carboxylic acids, esters, surfactants and surfactant-based formulations which can be dissolved or brought into stable emulsion in aqueous buffer or light mineral oil. The FL test method did not fulfil the validation study goal, which was to establish a full replacement to the Draize ocular sirritation test. However, the results obtained with diverse chemicals in this study were later used to identify a chemical subset, for which the test method has sufficient predictive capacity to be accepted for the classification purpose. **The FL test method has regulatory acceptance exclusively for water-soluble chemicals and mixtures**. OECD TG 460 advises caution when using the light mineral oil as a solvent, as the performance of the assay under such condition is little documented.Testing of coloured substances, insoluble solids and viscous materials with this test method is not recommended as they are difficult to remove after treatment. Other limitations apply to strong acids and bases, fixatives and highly volatile compounds, which are likely to kill the cells when applied within the dose range applied in the test.The FL test method is not recommended for the identification of chemicals which should be classified as mild/moderate irritants or of chemicals which should not be classified for ocular irritation (substances and mixtures) (i.e. GHS Cat. 2A/2B, no category; EU CLP Cat. 2, no category; US EPA Cat. II/III/IV)

Résumé

This method is based on that of Tchao (1988). The loss of trans-epithelial impermeability of a confluent monolayer of Madin Darby Canine Kidney (MDCK) cells through the effects of irritant materials is measured by the leakage of topically applied fluorescein across the cell monolayer.

The integrity of trans-epithelial permeability is a major function of an epithelium such as that found in the conjunctiva, the corneal epithelium and the skin. Trans-epithelial permeability is controlled by various inter-cellular junctions. The zonula occludens or tight junctions form a seal at the apical side of the epithelium and effectively prevent the passage of most molecules except water and some inorganic ions. The maculae adherens or desmosomes provide the strength in cell to cell connection as well as being permeability barriers.

In eye irritancy, an initial physiological event may be the loss of the impermeability of the corneal and conjunctival epithelium and the conjunctiva, allowing the irritant access to the underlying stroma (and possibly causing the corneal opacity). The tight junctions are disrupted and desmosomal junctions separate, leading to loss of cell contact and possible cell detachment.

Various epithelial cell types in culture, including the established MDCK cell line, also develop these junctions. Confluent cell monolayers, like the corneal epithelium, can form an impermeable barrier to most chemicals. This also

applies to the dye fluorescein when used in non-toxic concentrations and which is also used to assess the loss of corneal epithelium in humans. Using MDCK cells grown to confluence on porous filters, chemically induced loss of trans-epithelial impermeability is determined by measuring the "leakage" of topically applied fluorescein across the cellular layer. Thus, this assay models the disruption of the integrity of the corneal epithelial barrier by chemicals which may be potentially irritant to the eye.

A general bibliographic review of the "**Fluorescein Leakage Test (FLT)**" is also available in DB-ALM under **Method Summary** section and covers the methods development and various modifications that are outside the scope of this protocol.

Experimental Description

Endpoint and Endpoint Measurement:

CELL LAYER INTEGRITY: the integrity of the cell monolayer and its tight-junctions is assessed through the amount of fluorescein which passes through the cell layer, measured spectrophotometrically and expressed as % of the maximum leakage control

Endpoint Value:

FL_{20}: concentration of test chemical causing 20% of fluorescein leakage

Experimental System(s):

MADIN-DARBY CANINE KIDNEY (MDCK) CELL LINE: commercially available cells derived from dog (Cocker Spaniel) kidney

Basic Procedure

MDCK cells are cultured in the cell culture inserts, on the surface of a permeable membrane. Confluent cells are exposed for one minute to five fixed concentrations of test compound. For example: four increasing concentrations in the 1 – 100 mg/ml range and one highest dose taken from neat or saturated solution. See *Test Compounds* section of the **Procedure Details** for more information.

The amount of damage caused to the tight junctions is determined by the amount of fluorescein which leaks through a solvent treated confluent cell layer over a set period of time (30 min.). This is compared to two controls:

1. the amount of fluorescein which leaks through an untreated confluent monolayer, referred to as **0% leakage** or the blank control;
2. the amount of fluorescein which leaks through an insert on which there are no cells, referred to as **maximum leakage**.

The percentage of leakage and therefore amount of damage to the tight junctions is expressed, relative to these controls, for each of the set concentrations.

Data Analysis/Prediction Model

The FL_{20} value is used for predicting chemicals as ocular corrosives/severe irritants:

FL_{20} (mg/mL)	UN GHS C&L	EU CLP C&L	U.S. EPA C&L
≤100	Category 1	Category 1 (R41)	Category I

The chemicals with the FL_{20} value above the 100mg/ml cut-off need to be tested further, following the sequential testing strategies for the acute eye irritation and corrosion, outlined in the OECD TG 405 and EU Test Method B.5 (OECD, 2012; EU TRM B.5, 2008).

Test Compounds and Results Summary

In the EC/HO validation study (Balls et al., 1995), 59 compounds were tested in four laboratories, covering a wide range of organic and inorganic chemicals classified as:

- non-irritant (Not Classified)
- moderate irritant (Category 2)
- severe irritant (Category 1)

In the retrospective validation, initiated by EURL ECVAM (2008), the prediction model was defined and applied to the test results from EC/HO study with chemicals that had *in vivo* classification. A complete list of the evaluated chemicals is included in the OECD Streamlined summary document No. 180 (OECD, 2012). For the Category 1 chemicals (Corrosive/Severe ocular irritants), the performance under the Protocol 71 was as follows:

	Category 1 - EU CLP	Category 1 - UN GHS
Sensitivity	45.7%	43.8%
Specificity	92.9%	93.2%
Accuracy	77.9%	77.5%

Modifications of the Method

The original EC/HO validation study protocol DB-ALM No.71 was included in a retrospective validation study initiated by ECVAM. It was further optimized to address some of the shortcomings indentified in the peer review (EURL ECVAM, 2008):

- An additional washing step before the application of the test chemical was introduced
- A control step to detect an interaction between a test chemical and fluorescein, due to the chemical binding to the insert membrane (which may adversely interfere with the results of the test), was added
- Prediction model was developed (OECD, 2012)
- Set of proficiency chemicals was introduced to enable a standardized monitoring of the performance of the test components in the testing laboratory (OECD, 2012).

Acceptance Criteria and Proficiency Testing

- The mean fluorescence intensity of maximum fluorescein leakage samples should be higher than 4000 and fall within the linear range of the spectrofluorometer. The mean fluorescence intensity of the solvent alone 0% leakage sample should be equal or lower than 300, i.e. below 10% of the sample with the maximal leakage across the no-cell insert membrane.
- A test is considered acceptable if the positive control produced 20% to 40% damage to the cell layer (measured as % of fluorescein leakage).
- In the OECD TG 460 it is recommended that, prior to a routine use of the test, the test laboratory demonstrates its technical proficiency using the eight **Proficiency Chemicals**:

Chemical	CAS NR	Chemical Class [1]	Physical Form	*In Vivo* Classification [2]	*In Vitro* Classification [3]
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid	Category 1	Corrosive/ Severe Irritant
Promethazine hydrochloride	58-33-3	Amine/ Amidine, Heterocyclic, Organic sulphur compound	Solid	Category 1	Corrosive/ Severe Irritant
Sodium hydroxide (10%)	1310-73-2	Alkali	Liquid	Category 1	Corrosive/ Severe Irritant
Sodium lauryl sulfate (15%)	151-21-3	Carboxylic acid (salt)	Liquid	Category 1	Corrosive/ Severe Irritant
4-carboxy-benzaldehyde	619-66-9	Carboxylic acid, Aldehyde	Solid	Category 2 (A)	Non-corrosive/ Non-severe irritant
Ammonium nitrate	6484-52-2	Inorganic salt	Solid	Category 2 (A)	Non corrosive/ Non-severe irritant
Ethyl-2-methylaceto-acetate	609-14-3	Ketone, Ester	Liquid	Category 2 (B)	Non corrosive/ Non-severe irritant
Glycerol	56-81-5	Alcohol	Liquid	No Category	Non-corrosive/ Non-severe irritant

[1] Chemical classes are assigned to each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http://www.nlm.nih.gov/mesh)

[2]Based on results from the *in vivo* rabbit eye test (OECD TG 405, 2012) and using the UN GHS (UN, 2009) and EU CLP (EU, 2008).

[3]Based on results obtained with FL (DB-ALM Protocol No. 71).

Discussion

Fluorescein Leakage test with MDCK cells is a quantitative and relatively easy to perform. The test requires standard skills and equipment available in a cell culture laboratory.

The main limitation of the test is that is only applicable to the substances, which are soluble in the aqueous HBSS

medium.

The most critical components of the test:

- The handling and maintenance of the high quality of the cell culture: appropriate calcium concentration (1-1.8 mM) in the media (to preserve cell junction integrity), passage number, growth rate and confluence at the time of the exposure and of the endpoint measurement.
- The properties of the inserts and the supporting membrane as they may bind test substance or fluorescein and affect the accuracy of the test results.
- During the experiment maximum care must be taken not to damage the cell monolayer and filter membranes during the washing/aspiration cycles. At the end of the assay all inserts should be closely examined for damage.

The FL test can assess both acute toxic effects as well as the recovery of the cell monolayer from damage. The recovery is not yet assessed under the OECD TG 460, however there are other protocols available (e.g. DB-ALM No. 120), which are designed for that purpose.

Similar protocols for Fluorescein leakage can be applied to multi-layered tissue models, such as those currently being developed as 'skin equivalents'. An overview of other variants of the FL test (and prediction models) which have not reached regulatory acceptance yet, is included in the bibliographic review "Fluorescein Leakage Test (FLT)" – **Method Summary** in DB-ALM.

Fluorescein leakage, together with "Bovine Corneal Opacity and Permeability", "Isolated Chicken Eye" and the "Cytosensor Microphysiometer" tests are validated methods that can be used for Top - Down approach to predict corrosives/severe irritants. At this moment no *in vitro* method has been validated for the Bottom – Up approach to identify non-irritants, however there are ongoing validation studies that address this topic.

Status

Participation in Validation Studies:

This DB-ALM Protocol No. 71 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).

Later, this DB-ALM Protocol No.71 was included **in a retrospective validation study initiated by ECVAM** with a purpose to evaluate the scientific validity of possible tests to be used within the tiered eye irritation testing strategy (Scott *et al.*, 2009). **In 2009, the ECVAM Scientific Advisory Committee (ESAC) endorsed the scientific validity of the Fluorescein Leakage test method (DB-ALM Protocol No. 71) for its use as an initial step within a Top-Down Approach of tiered eye irritation testing strategy** (Scott *et al.*, 2009) **to identify ocular corrosives and severe irritants** (EU R41, GHS Category 1 and US EPA Category I) from all other classes for the chemical applicability domain of water-soluble chemicals (substances and mixtures) (ESAC, 2009). However, it was not recommended to be used within a Bottom-Up Approach (Scott *et al.*, 2009) of tiered eye irritation testing strategy (ESAC, 2009).

Regulatory Acceptance:

In October 2012 the "Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants" was adopted as OECD Test Guideline No. 460. The regulatory use of the test method is limited to the positive classification of the water-soluble chemicals (substances and mixtures) as ocular corrosives and severe irritants.

Health and Safety Issues

General Precautions

Unknown and coded test chemicals should be considered as potentially irritating and toxic and must be handled with maximum care.

Abbreviations and Definitions

Abbreviations:

CAS Nr. – Chemical Abstracts Service Registry Number

C&L – Classification and Labelling

CLP – (EU regulation for) Classification, Labelling and Packaging

DB-ALM – EURL ECVAM's DataBase service on Alternative Methods to animal experimentation

EC – European Commission

ECVAM - European Centre for the Validation of Alternative Methods. As of 2011, ECVAM has been established as the European Union Reference Laboratory for the Validation of Alternative Methods.

EPA – US Environmental Protection Agency

ESAC – ECVAM Scientific Advisory Committee

EU – European Union

EURL ECVAM – European Union Reference Laboratory for the Validation of Alternative Methods (formerly referred to as ECVAM)

FL- Fluorescein leakage

GHS – United Nations Globally Harmonized System of Classification and Labelling of Chemicals

HBSS - Hanks' Balanced Salt Solution

HO- Home Office

MDCK - Madin Darby Canine Kidney cells

OECD – Organization for Economic Co-operation and Development

R41 – Risk phrase: "Risk of serious damage to eyes"

TG – Test Guideline

Definitions:

Bottom-up approach – sequential testing strategy that begins with test methods that can accurately identify non-irritants.

Category 1 - irreversible effects on the eye

Top-down approach - sequential testing strategy that begins with test methods that can accurately identify severe irritants

Last update: February 2013

PROCEDURE DETAILS, *February 2013*

Fluorescein Leakage (FL) Test
DB-ALM Protocol n° 71

The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test.

Minor improvements were made after the retrospective validation study conducted by ECVAM and the subsequent adoption of the ***Fluorescein Leakage (FL) Test Method by OECD*** *as* ***TG 460****.*

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Materials and Preparations

Cell or Test System

Cell line MDCK (CB997), certified mycoplasma free.
These are obtainable from the European Collection of Animal Cell Cultures (ECACC):

PHLS Centre for Applied Microbiology and Research,
Porton Down, Salisbury SP4 0JG, Wilts, UK
ECACC Number: **84121903**

Equipment

Fixed Equipment

Incubator - 37°C, humidified, 5% CO_2/95% air.
Fluorescence measurement system (e.g. Cytofluor or equivalent), excitation 485 nm, emission 530 nm.

Consumables

Tissue culture flasks
24-well tissue culture plates
Cell culture inserts, for 24 well plates, Millicell-HA, 12mm, 0.45m pore size - Millipore Ltd

> ***Note:*** An equivalent such as Anocell 10 inserts (Whatman) can also be used. The adsorbent/binding properties of the test substance onto the insert and the test substance binding capacity of fluorescein may interfere with the result of the test. Therefore the inserts should be tested first for any significant interaction with the test substance and/or fluorescein.

Media, Reagents, Sera, others

> ***Note:*** All media and solutions used throughout the assay should contain 1.0-1.8 mM calcium, which is needed to ensure tight junction formation and integrity.

- Hank's balanced salt solution (HBSS), without phenol red, 500 ml, no. 041-04025M - Gibco Ltd or from powder, without phenol red, no. H1387 - Sigma Chemical Co. Ltd
- Trypsin-EDTA (0.05% w/v Trypsin, 0.02% w/v EDTA), 1X prepared in modified Puck's saline, 100ml, no. 043-05300H - Gibco Ltd
- Dulbecco's Modified Eagle's medium/Nutrient Mix F12 (1X concentrate with L-Glutamine, and containing 15mM HEPES), 500 ml, no. 041-01330M - Gibco Ltd
- Foetal calf serum (FCS), heat inactivated, 500 ml, no. 013-06290M - Gibco Ltd., UK
- Streptomycin sulphate, Sigma no. S6501 - Sigma Chemical Co. Ltd
- Benzylpenicillin (Penicillin G, sodium salt), Sigma no. PEN-NA - Sigma Chemical Co. Ltd
- Amphotericin B, solubilised (Fungizone), Sigma no. A 9528 - Sigma Chemical Co. Ltd
- Light mineral oil, Sigma no. M3516 - Sigma Chemical Co. Ltd
- Brij 35, Aldrich no. 85,836-6 - Aldrich Chemical Co. Ltd
- Fluorescein, disodium salt, water-soluble, Aldrich 16,630-8 - Aldrich Chemical Company Ltd, or Fluorescein, disodium salt, water-soluble, Sigma F6,377 - Sigma Chemical Co. Ltd

Preparations

Media and Endpoint Assay Solutions

All glassware, solutions etc. are sterile and all procedures are carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

Make up the following:

Dulbecco's supplemented medium

Composition per litre:
900 ml Dulbecco's MEM/Nutrient Mix F12
100 ml FCS
100 mg streptomycin sulphate
100,000 IU benzylpenicillin
1 mg amphotericin B

Store at 4°C. It is recommended that the medium be used within two weeks.

Fluorescein solution, 0.01% (w/v)

Dissolve 50 mg fluorescein in 500ml HBSS.

This solution may be stored at room temperature. It is recommended that the fluorescein solution is stored in the dark, since it may lose its fluorescence within one week under exposure to light.

Note: If using Anocell inserts, make up a 0.02% fluorescein solution.

Test Compounds

All compounds to be tested are made up in sterile HBSS from a stock solution, at 5 set concentrations: 1, 25, 100, 250 mg/ml, and a neat or a saturated solution. When testing a solid material a very high concentration, 750 mg/ml, should also be included. This concentration of material may have to be pipetted using a positive displacement pipette.

Note: Chemical binding to the insert membrane may occur for e.g. cationic chemicals, such as benzalkonium chloride, which can interact with the positively charged membranes (EURL-ECVAM, 2008). Residual presence of the chemical on the insert membrane may interfere with the test results. It may increase the chemical exposure period or physically reduce the leakage of fluorescein through the insert by binding of the dye. This effect can be tested by exposing the membrane alone to the top concentration of the chemical tested and then adding sodium-fluorescein dye at the normal concentration for the standard time (no cell control). If binding of the sodium-fluorescein dye occurs, the insert membrane appears yellow after the test material has been washed-off.

A fresh stock solution should be prepared for each experimental run and used within 30 minutes of preparation. Dilute both solid and liquid test materials on a weight per volume basis. Make up enough test material for 3 inserts per concentration tested.

Physical methods for aiding solubilization can cause degradation of the test chemical and so must be kept within certain guidelines:

(i) Vortexing the solvent with the chemical should be done for up to a maximum of 10 minutes.
(ii) Sonication can only be done for up to a maximum of 10 minutes, as prolonged sonication heats the solution which may cause degradation of the test material.

One test material is tested per each 24-well plate. If the toxicity is found to be between 25 and 100 mg/ml, the following concentrations should be tested: 1, 25, 50, 75, 100 mg/ml. However, if the toxicity is below 1 mg/ml, the following concentrations should be tested: 0.01, 0.1, 0.25, 1, and 10 mg/ml.

Note: Once a concentration range has been found which includes the FL_{20} value, the exact value is determined from three definitive experimental runs. A different flask of cells with the same range of concentrations should be tested twice more. This means that the FL_{20} is required for each definitive experimental run.

Use of mineral oil
Test the solubility of the chemical at 250mg/ml in HBSS prior to testing in the FL test. If the chemicals forms a stable suspension or emulsion, over 30 minutes, at this concentration HBSS can still be used as the solvent. However, if the material is found to be insoluble in the HBSS at this concentration light mineral oil can be considered as alternative solvent.

Positive Control(s)

100 mg/ml Brij 35. This concentration should give approximately 30% damage to the cell layer. Results are acceptable

if twenty to forty percent damage is seen, if not the results should be discarded.

Negative Control(s)

Phenol Red-Free HBSS

Method

Routine Culture Procedure

Cell maintenance and culture procedures

Stocks of MDCK cells can be stored in sterile screw-top vials in liquid nitrogen or -80°C freezer, following suspension in FCS containing 5% DMSO as a cryoprotective agent. The cells may be centrifuged for short periods, i.e. 5 minutes, at low speeds (approximately 100 x g.), prior to making up this suspension, the concentration of which is approximately 1 x 10^6 cells/ml. Cells are frozen down in 1 ml aliquots.

When required the cells should be rapidly thawed at 37°C in a water bath, suspended in fresh medium and centrifuged for 10 minutes at approximately 100 x g. Then the pellet of cells is resuspended in 25 ml of fresh medium and placed in a sterile tissue culture flask. Once the cells have attached over night, the medium should be replaced i.e. approximately 25 ml in a 75 cm^2 tissue culture flask.

When the cells are 70-80% confluent, they should be removed from the flask by trypsinisation:

Decant the medium and rinse the cultures twice with PBS at 37°C.
Add 1 ml of Trypsin-EDTA and incubate at 37°C for approx. 10 min.
Give the flask 2 or 3 sharp taps to detach the cells into a single suspension. If at this time no cells detach, the flask is left for a longer time until they do.
Add approximately 10 ml culture medium to prevent enzymatic damage.
Centrifuge the cells for 5 minutes at 1000 x g.
Resuspend the pellet in a known volume of fresh medium.

Dilute the cell suspension with medium at 37°C, to give a final concentration of 4 x 10^5 cells/ml, i.e. ~2 x 10^5 cells/insert.

Note: Cells should always be passaged from sub-confluent flasks. The cells to be used in the experiment should be within 3 to 30 passages from thawing.

When the cells are ready the assay is set up as follows (Day 0):

Aliquot 400µl of fresh medium into each well of a 24-well plate.
Carefully place an insert into each well.
Aliquot 400 µl cell suspension into 21 of the inserts.
Aliquot 400 µl culture medium alone into the remaining 3 inserts.
Agitate the plate gently to evenly spread the cell suspension.

Note: If the plates are not agitated gently the cells may rise up and spread up the sides of the insert.

The cells are grown to confluence over 96 hours. Replace medium in the wells and in the inserts 72 hours (Day 3) after the cells are initially seeded.

Test Material Exposure Procedures

Treatment of monolayer with test chemical

Once confluent, the cells are ready for treatment (Day 4). Plate lay-out:

M	M	M	B	B	B
P	P	P	T1	T1	T1
T2	T2	T2	T3	T3	T3
T4	T4	T4	T5	T5	T5

where:
M = maximum leakage of fluorescein through the membrane, with no cells.
B = blank, 0% leakage through the intact cell monolayer.
P = positive control on the tight junctions (100 mg/ml Brij 35).
T1-5 = concentrations of test compound (1 being the lowest).

Insert	Concentration (mg/ml)		
T1	1	1	0.01
T2	25	25	0.1

T3	100	50	0.25
T4	250	75	1
T5	neat or saturated solution	100	10

Make up test materials as previously outlined under *Test Compounds.*

Gently aspirate the medium from the inserts, without disturbing the monolayer.
Wash the cells twice with sterile, phenol red-free HBSS, pre-warmed to 37°C.
Add 200 µl of test compound (3 inserts per each concentration) to inserts T1-5.
Add 200 µl sterile HBSS to control inserts, i.e. 3 inserts labelled B which contain cells; and 3 inserts labelled M, which contain no cells.
Add 200 µl of the positive control, Brij 35 (100 mg/ml), to 3 inserts labelled P.
After 1 minute exposure, remove the test compound, positive control, etc., by aspiration.
Wash the cell monolayer twice with 400 µl sterile HBSS.

For each experimental run six chemicals may be tested by one person.
The time-table below illustrates a work plan for one person, testing 60 chemicals within a 5 week period:

TREATMENT TIME-TABLE

DAY		Protocol			
		EXPT. 1	EXPT. 2	EXPT. 3	EXPT. 4
	Monday	seed inserts			
1	Tuesday				
2	Wednesday				
3	Thursday	refeed MDCK monolayers			
4	Friday	treat monolayers with test chemical	seed inserts		
5	Saturday				
6	Sunday				
7	Monday		refeed MDCK monolayers	seed inserts	
8	Tuesday		treat monolayers with test chemical		
9	Wednesday				
10	Thursday			refeed MDCK monolayers	
11	Friday			treat monolayers with test chemical	seed inserts, etc...

Endpoint Measurement

Determination of permeability to fluorescein

The percentage of fluorescein which leaks through the cellular monolayer gives a quantitative indication of damage to the tight junctions.

Preapre a fresh 24-well plate, each well containing 400 µl warm HBSS.
Transfer washed inserts to the fresh plate.
Aliquot 400 µl fluorescein solution into each insert.
Leave at room temperature for 30 minutes.
Carefully remove inserts, using tweezers, from each well and visually check each filter for any damage which may have occurred during handling. Record any damage which may be seen.

At the end of the 30 minute incubation with fluorescein solution, and after removal of the insert, read the resulting fluorescein solution using a spectrofluorometer (excitation filter 485nm, emission filter 530nm).

From the fluorescence intensity values obtained, the percentage of fluorescein leakage can be determined for (the fixed concentrations) in comparison to the leakage of fluorescein across the filter of the insert alone (i.e. maximum leakage).

Note: The sensitivity of the spectrofluorometer is set so that there is the highest numerical difference between the maximum fluorescein leakage (insert with no cells) and the minimum fluorescein leakage (insert with untreated confluent cell monolayer). The maximum fluorescein leakage value should higher than 4000, but not greater than 9999 and within the linear response range of the instrument.

Acceptance Criteria

A test is considered acceptable if the positive control produced 20% to 40% damage to the cell layer, measured as % of fluorescein leakage (see **Data Analysis** below). In addition, the leakage across the blank solvent control should be less than 300 i.e. below 10% of that of the no-cells insert.

Data Analysis

Calculate:

- The mean maximum leakage fluorescence intensity = x
- The mean 0% leakage fluorescence intensity = y
  (this can also be considered as the background leakage or blank)
- 100% leakage (by subtracting the 0% leakage from the maximum leakage),
  i.e. z= x - y
- The percentage leakage for each fixed dose:
  Take the mean the fluorescence intensity readings, subtract the 0% leakage and divide by the 100% leakage.

%FL =[(m-y)/(z)] x 100%

where:

m = the mean fluorescence intensity of the concentration involved.
%FL = The percent of the fluorescein which leaks through the cell layer.

To calculate the concentration of chemical which causes 20% damage to the cell layer (FL_{20}):

$FL_{20} = [(A-B)/(C-B)] \times (M_C - M_B) + M_B$

where:

A = 20% (threshold damage expressed as % of fluorescein leakage)
B = % fluorescein leakage < A
C = % fluorescein leakage > A
M_C = Concentration (mg/ml) of C
M_B = Concentration (mg/ml) of B

If the toxicity is found between 250mg/ml and the undiluted sample, you should be aware, from the weight/volume calculation of the dilution, what the weight is of 1 ml of undiluted material. Hence the apparent concentration of 1 ml of undiluted material should be used in the calculation of the result of an FL_{20} value.

It should also be noted that the toxicity curves may not be linear or increasing throughout the full range of concentrations tested. For example, the percentage fluorescein leakage seen with undiluted test material could be lower than that for 100 or 250mg/ml - the FL_{20} might be 75 mg/ml with the 250 mg/ml and undiluted material giving 15% and 5% respectively.

If the FL_{20} is not reached at any concentration inclusive of the undiluted material, then the maximum percentage fluorescein leakage should be quoted along with the concentration of chemical causing this, for example, 100mg/ml (FL 18.5%), or undiluted sample with calculated 955mg/ml (FL 3.2%).

Control values
Because of the individuality of each spectrofluorometer it is suggested that a sensitivity is used which will give a maximum fluorescein leakage value of >4000.

Acceptance critera:
0% leakage, y = <300
100% leakage, z = 3500 - 6000

Reporting of results
The results should be calculated by the above formulae.

A reference should be made to the proficiency demonstrated by the testing laboratory and to the historical data obtained by the laboratory with regard to the controls and benchmark chemicals.

Any other significant effects observed should be reported alongside numerical data, such as e.g. incompatibilities between the test sample and the filter or o-ring of the filter insert.

EXAMPLE CALCULATION

Test chemical concentration (mg/ml)	Test chemical results % Fluorescein leakage

1	15
25	46
100	62
250	98

i) to calculate FL_{20} (mg/ml):

FL_{20}= [(20-15)/46-15)] x (25 - 1) + 1 = (15/31) x 24 + 1= **4.87 mg/ml**

i i) If the FL_{20} value is not reached by any concentration tested then the concentration (mg/ml) for the maximum percentage fluorescein leakage exhibited should be reported along with this FL value.

Prediction Model

It the FL_{20} value can be determined in the test, it can be used for the top-down classification of chemicals as ocular corrosives/severe irritants:

FL_{20} (mg/mL)	UN GHS C&L	EU CLP C&L	U.S. EPA C&L
100	Category 1	Category 1 (R41)	Category I

The chemicals with the FL_{20} value above the 100mg/ml cut-off need to be tested further, following the sequential testing strategies for the acute eye irritation and corrosion, outlined in the OECD TG 405 and EU Test Method B.5 (OECD, 2012; EU TRM B.5, 2008).

Annexes

Annex 1: FLUORESCEIN LEAKAGE SUMMARY

CELL CULTURE	
MDCK Cells	ECACC - 84121903
Culture medium	DMEM : Ham's F12 (1 : 1)
Serum	10% FCS (heat-inactivated)
Other	benzylpenicillin, streptomycin, amphotericin B
Filter insert	Millicell-HA, 12mm, 0.45µm (Millipore)
Treatment of insert prior to seeding with MDCK cells	no
Cell seeding density	4 x 10^5 cells/ml (400 µl per insert)
Days to confluence	96 hours
Comments on growth of monolayer	refeed after 72 hours
TESTING PROCEDURE	
Rinsing procedure prior to treatment of monolayer with test chemical	none
TEST CHEMICAL: Amount and dose range tested	200 µl of test chemical in HBSS or light mineral oil 1 mg/ml or 1 mg/ml or 0.01 mg/ml 25 mg/ml or 25 mg/ml or 0.1 mg/ml 100 mg/ml or 50 mg/ml or 0.25 mg/ml 250 mg/ml or 75 mg/ml or 1.0 mg/ml neat or 100 mg/ml or 10 mg/ml saturated solution *N.B. use 750 mg/ml if testing a solid*
	5 concentrations/chemical 3 inserts/concentration 1 chemical/plate
Exposure period to test chemical (minutes)	1/15
Temperature of plate at exposure	room temperature (approximately 23ºC)
REFERENCE CHEMICAL: Amount and dose range tested	100 mg/ml Brij 35
FLUORESCEIN LEAKAGE ASSAY	
Washing procedure before addition of fluorescein	2x with HBSS
Addition of fluorescein	inserts moved to 24-wp containing 400 µl HBSS; 400 µl 0.01% Na-fluorescein in HBSS added to insert
Incubation period with fluorescein (minutes)	30
Incubation temperature	room temperature
Determination of leakage	fluorescence intensity measured on spectrofluorometer with plate reading capacity, excitation 485nm, emission 530nm
Calculation and expression of results	% fluorescein leakage vs. chemical concentration concentration of test chemical causing 20% fluorescein leakage (FL_{20}) calculated or read from graph

Annex 2: DATA REPORTING SHEET - EXAMPLE

LABORATORY ID: _____
ASSAY: FLUORESCEIN LEAKAGE TEST

FINAL RESULTS (DEFINITIVE EXPERIMENTAL RUNS)
FL_{20} (mg/ml)

CHEMICAL NUMBER	DATE (dd/mm/yy)	ENDPOINT FL_{20} (mg/ml)	SOLVENT H or M	COMMENTS
	Mean +/- SEM			
	Mean +/- SEM			
	Mean +/- SEM			
	Mean +/- SEM			
	Mean +/- SEM			

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