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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants

INTRODUCTION

1. The Fluorescein Leakage (FL) test method is an *in vitro* test method that can be used under certain circumstances and with specific limitations to classify chemicals (substances and mixtures) as ocular corrosives and severe irritants, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (Category 1), the European Union (EU) Regulation on Classification, Labelling and Packaging of Substances and Mixtures (CLP) (Category 1), and the U.S. Environmental Protection Agency (EPA) (Category I) (1) (2) (3). For the purpose of this Test Guideline, severe irritants are defined as chemicals that cause tissue damage in the eye following test substance administration that is not reversible within 21 days or causes serious physical decay of vision, while ocular corrosives are chemicals that cause irreversible tissue damage to the eye. These chemicals are classified as UN GHS Category 1, EU CLP Category 1, or U.S. EPA Category I.

2. While the FL test method is not considered valid as a complete replacement for the *in vivo* rabbit eye test, the FL is recommended for use as part of a tiered testing strategy for regulatory classification and labelling. Thus, the FL is recommended as an initial step within a Top-Down approach to identify ocular corrosives/severe irritants, specifically for limited types of chemicals (*i.e.* water soluble substances and mixtures) (4)(5).

3. It is currently generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the *in vivo* eye test (TG 405 (6)) to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the *in vivo* eye test (5). The Top-Down approach (5) is designed to be used when, based on existing information, a chemical is expected to have high irritancy potential.

4. Based on the prediction model detailed in paragraph 35, the FL test method can identify substances within a limited applicability domain as ocular corrosives/severe irritants (UN GHS Category 1; EU CLP Category 1; U.S. EPA Category I) without any further testing. The same is assumed for mixtures although mixtures were not used in the validation. Therefore, the FL test method may be used to determine the eye irritancy/corrosivity of chemicals, following the

sequential testing strategy of TG 405 (6). However, a chemical that is not predicted as ocular corrosive or severe irritant with the FL test method would need to be tested in one or more additional test methods (*in vitro* and/or *in vivo*) that are capable of accurately identifying i) chemicals that are *in vitro* false negative ocular corrosives/severe irritants in the FL (UN GHS Category 1; EU CLP Category 1; U.S. EPA Category I); ii) chemicals that are not classified for eye corrosion/irritation (UN GHS No Category; EU CLP No Category; U.S. EPA Category IV); and/or iii) chemicals that are moderate/mild eye irritants (UN GHS Categories 2A and 2B; EU CLP Category 2; U.S. EPA Categories II and III).

5. The purpose of this Test Guideline is to describe the procedures used to evaluate the potential ocular corrosivity or severe irritancy of a test substance as measured by its ability to induce damage to an impermeable confluent epithelial monolayer. The integrity of trans-epithelial permeability is a major function of an epithelium such as that found in the conjunctiva and the cornea. Trans-epithelial permeability is controlled by various tight junctions. Increasing the permeability of the corneal epithelium *in vivo* has been shown to correlate with the level of inflammation and surface damage observed as eye irritation develops.

6. In the FL test method, toxic effects after a short exposure time to the test substance are measured by an increase in permeability of sodium fluorescein through the epithelial monolayer of Madin-Darby Canine Kidney (MDCK) cells cultured on permeable inserts. The amount of fluorescein leakage that occurs is proportional to the chemical-induced damage to the tight junctions, desmosomal junctions and cell membranes, and can be used to estimate the ocular toxicity potential of a test substance. Annex I provides a diagram of MDCK cells grown on an insert membrane for the FL test method.

7. Definitions are provided in Annex II.

INITIAL CONSIDERATIONS AND LIMITATIONS

8. This Test Guideline is based on the INVITTOX protocol No. 71 (7) that has been evaluated in an international validation study by the European Centre for the Validation of Alternative Methods (ECVAM) (8), in collaboration with the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM).

9. 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), as demonstrated by the validation study (4) (8).

10. The test method is only applicable to water soluble chemicals (substances and mixtures). The ocular severe irritation potential of chemicals that are water soluble and/or where the toxic effect is not affected by dilution is generally predicted accurately using the FL test method (8). To categorise a chemical as water soluble, under experimental conditions, it should be soluble in sterile calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, Hanks' Buffered Salt Solution (HBSS) at a concentration $\geq$ 250 mg/mL (one dose above the cut-off of 100 mg/mL). However, if the test substance is soluble below the concentration 100 mg/mL,

but already induces a FL induction of 20 % at that concentration (meaning FL_{20} < 100 mg/mL), it can still be classified as GHS Cat. 1 or EPA Cat. 1.

11. The identified limitations for this test method exclude strong acids and bases, cell fixatives and highly volatile chemicals from the applicability domain. These chemicals have mechanisms that are not measured by the FL test method, *e.g.* extensive coagulation, saponification or specific reactive chemistries. Other identified limitations for this method are based upon the results for the predictive capacity for coloured and viscous test substance (8). It is suggested that both types of chemicals are difficult to remove from the monolayer following the short exposure period and that predictivity of the test method could be improved if a higher number of washing steps was used. Solid chemicals suspended in liquid have the propensity to precipitate out and the final concentration to cells can be difficult to determine. When substances within these chemical and physical classes are excluded from the database, the accuracy of FL across the EU, EPA, and GHS classification systems is substantially improved (8).

12. Based on the purpose of this test method (*i.e.* to identify ocular corrosives/severe irritants only), false negative rates (see Paragraph 13) are not critical since such substances would be subsequently tested with other adequately validated *in vitro* tests or in rabbits, depending on regulatory requirements, using a sequential testing strategy in a weight of evidence approach (6) (see also paragraphs 3 and 4).

13. Other identified limitations of the FL test method are based on false negative and false positive rates. When used as an initial step within a Top-Down approach to identify water soluble ocular corrosive/severe irritant substances and mixtures (UN GHS Category 1; EU CLP Category 1; U.S. EPA Category I), the false positive rate for the FL test method ranged from 7% (7/103; UN GHS and EU CLP) to 9% (9/99; U.S. EPA) and the false negative rate ranged from 54% (15/28; U.S. EPA) to 56% (27/48; UN GHS and EU CLP) when compared to *in vivo* results. Chemical groups showing false positive and/or false negative results in the FL test method are not defined here.

14. Certain technical limitations are specific to the MDCK cell culture. The tight junctions that block the passage of the sodium-fluorescein dye through the monolayer are increasingly compromised with increasing cell passage number. Incomplete formation of the tight junctions results in increased FL in the non-treated control. Therefore, a defined permissible maximal leakage in the non-treated controls is important (see paragraph 38: 0% leakage). As with all *in vitro* assays there is the potential for the cells to become transformed over time, thus it is vital that passage number ranges for the assays are stated.

15. The current applicability domain might be increased in some cases, but only after analyzing an expanded data set of studied test substances, preferably acquired through testing (4). This Test Guideline will be updated accordingly as new information and data are considered.

16. For any laboratory initially establishing this assay, the proficiency chemicals provided in Annex III should be used. Laboratories can use these chemicals to demonstrate their technical competence in performing the FL test method prior to submitting FL assay data for regulatory hazard classification purposes.

PRINCIPLE OF THE TEST

17. The FL test method is a cytotoxicity and cell-function based *in vitro* assay that is performed on a confluent monolayer of MDCK CB997 tubular epithelial cells that are grown on semi-permeable inserts and model the non-proliferating state of the *in vivo* corneal epithelium. The MDCK cell line is well established and forms tight junctions and desmosomal junctions similar to those found on the apical side of conjunctival and corneal epithelia. Tight and desmosomal junctions *in vivo* prevent solutes and foreign materials penetrating the corneal epithelium. Loss of trans-epithelial impermeability, due to damaged tight junctions and desmosomal junctions, is one of the early events in chemical-induced ocular irritation.

18. The test substance is applied to the confluent layer of cells grown on the apical side of the insert. A short 1 min exposure is routinely used to reflect the normal clearance rate in human exposures. An advantage of the short exposure period is that water-based substances and mixtures can be tested neat, if they can be easily removed after the exposure period. This allows more direct comparisons of the results with the chemical effects in humans. The test substance is then removed and the non-toxic, highly fluorescent sodium-fluorescein dye is added to the apical side of the monolayer for 30 minutes. The damage caused by the test substance to the tight junctions is determined by the amount of fluorescein which leaks through the cell layer within a defined period of time.

19. The amount of sodium-fluorescein dye that passes through the monolayer and the insert membrane into a set volume of solution present in the well (to which the sodium-fluorescein dye leaks in) is determined by measuring spectrofluorometrically the fluorescein concentration in the well. The amount of fluorescein leakage (FL) is calculated with reference to fluoresence intensity (FI) readings from two controls: a blank control, and a maximum leakage control. 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 of the test substance. Then the FL_{20} (*i.e.* concentration that causes 20% FL relative to the value recorded for the untreated confluent monolayer and inserts without cells), is calculated. The FL_{20} (mg/mL) value is used in the prediction model for identification of ocular corrosives and severe irritants (see paragraph 35).

20. Recovery is an important part of a test substance's toxicity profile that is also assessed by the *in vivo* ocular irritation test. Preliminary analyses indicated that recovery data (up to 72 h following the chemical exposure) could potentially increase the predictive capacity of INVITTOX Protocol 71 but further evaluation is needed and would benefit from additional data, preferably acquired by further testing (7). This Test Guideline will be updated accordingly as new information and data are considered.

PROCEDURE

Preparation of the cellular monolayer

21. The monolayer of MDCK CB997 cells is prepared using sub-confluent cells growing in cell culture flasks in DMEM/Nutrient Mix F12 (1x concentrate with L-glutamine, 15 mM HEPES, calcium (at a concentration of 1.0-1.8 mM) and 10% heat-inactivated FCS/FBS). Importantly, all media/solutions used throughout the FL assay should contain calcium at a concentration between 1.8 mM (200 mg/L) and 1.0 mM (111 mg/L) to ensure tight junction formation and integrity. Cell passage number range should be controlled to ensure even and

reproducible tight junctions formation. Preferably, the cells should be within the passage range 3-30 from thawing because cells within this passage range have similar functionality, which aids assay results to be reproducible.

22. Prior to performing the FL test method, the cells are detached from the flask by trypsinisation, centrifuged and an appropriate amount of cells is seeded into the inserts placed in 24-well plates (see Annex I). Twelve mm diameter inserts with membrane of mixed cellulose esters, a thickness of 80-150 µm and a pore size of 0.45 µm, should be used to seed the cells. In the validation study, Millicell-HA 12 mm inserts were used. The properties of the insert and membrane type are important as these may affect cell growth and chemical binding. Certain types of chemicals may bind to the Millicell-HA insert membrane, which could affect the interpretation of results. Proficiency chemicals (see Annex III) should be used to demonstrate equivalency if other membranes are used.

23. Chemical binding to the insert membrane is more common for cationic chemicals, such as benzalkonium chloride, which are attracted to the positively charged membrane (8). Chemical binding to the insert membrane may increase the chemical exposure period, leading to an over-estimation of the toxic potential of the chemical, but can also physically reduce the leakage of fluorescein through the insert by binding of the dye to the cationic chemical bound to the insert membrane, leading to an under-estimation of the toxic potential of the chemical. This can be readily monitored 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. Thus, it is essential to know the binding properties of the test substance in order to be able to interpret the effect of the chemical on the cells.

24. Cell seeding on inserts should produce a confluent monolayer at the time of chemical exposure. 1.6×10^5 cells should be added per insert (400 µL of a cell suspension with a density of 4×10^5 cells / mL). Under these conditions, a confluent monolayer is usually obtained after 96 hours in culture. Inserts should be examined visually prior to seeding, so as to ensure that any damages recorded at the visual control described at paragraph 30 is due to handling.

25. The MDCK cell cultures should be kept in incubators in a humidified atmosphere, at 5% ± 1% CO_2 and 37 ± 1 ºC. The cells should be free of contamination by bacteria, viruses, mycoplasma and fungi.

Application of the Test and Control Chemicals

26. A fresh stock solution of test substance should be prepared for each experimental run and used within 30 minutes of preparation. Test substances should be prepared in calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, HBSS to avoid serum protein binding. Solubility of the chemical at 250 mg/mL in HBSS should be assessed prior to testing. If at this concentration the chemical forms a stable suspension or emulsion (*i.e.* maintains uniformity and does not settle or separate into more than one phase) over 30 minutes, HBSS can still be used as solvent. However, if the chemical is found to be insoluble in HBSS at this concentration, the use of other test methods instead of FL should be considered. The use of light mineral oil as a solvent, in cases where the chemical is found to be insoluble in HBSS, should be

considered with caution as there is not enough data available to conclude on the performance of the FL assay under such conditions.

27. All chemicals to be tested are prepared in sterile calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, HBSS from the stock solution, at five fixed concentrations diluted on a weight per volume basis: 1, 25, 100, 250 mg/mL and a neat or a saturated solution. When testing a solid chemical, a very high concentration of 750 mg/mL should be included. This concentration of chemical may have to be applied on the cells using a positive displacement pipette. If the toxicity is found to be between 25 and 100 mg/mL, the following additional concentrations should be tested twice: 1, 25, 50, 75, 100 mg/mL. The FL_{20} value should be derived from these concentrations provided the acceptance criteria were met.

28. The test substances are applied to the confluent cell monolayers after removal of the cell culture medium and washing twice with sterile, warm (37ºC), calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, HBSS. Previously, the filters have been visually checked for any pre-existing damages that could be falsely attributed to potential incompatibilities with test chemicals. At least three replicates should be used for each concentration of the test substance and for the controls in each run. After 1 min of exposure at room temperature, the test substance should be carefully removed by aspiration, the monolayer should be washed twice with sterile, warm (37ºC), calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, HBSS, and the fluorescein leakage should be immediately measured.

29. Concurrent negative (NC) and positive controls (PC) should be used in each run to demonstrate that monolayer integrity (NC) and sensitivity of the cells (PC) are within a defined historical acceptance range. The suggested PC chemical is Brij 35 (CAS No. 9002-92-0) at 100 mg/mL. This concentration should give approximately 30% fluorescein leakage (acceptable range 20-40% fluorescein leakage, *i.e*. damage to cell layer). The suggested NC chemical is calcium-containing (at a concentration of 1.0-1.8 mM), phenol red-free, HBSS (untreated, blank control). A maximum leakage control should also be included in each run to allow for the calculation of FL_{20} values. Maximum leakage is determined using a control insert without cells.

Determination of fluorescein permeability

30. Immediately after removal of the test and control substances, 400µL of 0.1 mg/mL sodium-fluorescein solution (0.01% (w/v) in calcium-containing [at a concentration of 1.0-1.8 mM], phenol red-free, HBSS) is added to the Millicell-HA inserts. The cultures are kept for 30 minutes at room temperature. At the end of the incubation with fluorescein, the inserts are carefully removed from each well. Visual check is performed on each filter and any damage which may have occurred during handling is recorded.

31. The amount of fluorescein that leaked through the monolayer and the insert is quantified in the solution which remained in the wells after removal of the inserts. Measurements are done in a spectrofluorometer at excitation and emission wavelengths of 485 nm and 530 nm, respectively. The sensitivity of the spectrofluorometer should be set so that there is the highest numerical difference between the maximum FL (insert with no cells) and the minimum FL (insert with confluent monolayer treated with NC). Because of the differences in the used spectrofluorometer, it is suggested that a sensitivity is used which will give fluorescence intensity > 4000 at the maximum fluorescein leakage control. The maximum FL value should not be

greater than 9999. The maximum fluorescence leakage intensity should fall within the linear range of the spectrofluorometer used.

Interpretation of results and Prediction model

32. The amount of FL is proportional to the chemical-induced damage to the tight junctions. The percentage of FL for each tested concentration of chemical is calculated from the FL values obtained for the test substance with reference to FL values from the NC (reading from the confluent monolayer of cells treated with the NC) and a maximum leakage control (reading for the amount of FL through an insert without cells).

The mean maximum leakage fluorescence intensity = x
The mean 0% leakage fluorescence intensity (NC) = y
The mean 100% leakage is obtained by subtracting the mean 0% leakage from the mean maximum leakage,
i.e. x - y = z

33. The percentage leakage for each fixed dose is obtained by subtracting the 0% leakage to the mean fluorescence intensity of the three replicate readings (m), and dividing this value by the 100% leakage, *i.e.* %FL = [(m-y) / z] x 100%, where:

m = the mean fluorescence intensity of the three replicate measurements for the concentration involved
% FL = the percent of the fluorescein which leaks through the cell layer

34. The following equation for the calculation of the chemical concentration causing 20% FL should be applied:

$$FL_D = [(A-B) / (C-B)] \times (M_C - M_B) + M_B$$

Where:
D = % of inhibition
A = % damage (20% fluorescein leakage)
B = % fluorescein leakage < A
C = % fluorescein leakage > A
M_C = Concentration (mg/mL) of C
M_B = Concentration (mg/mL) of B

35. The cut-off value of FL_{20} for predicting chemicals as ocular corrosives/severe irritants is given below:

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

C&L: classification and labelling

36. The FL test method is recommended only for the identification of water soluble ocular corrosives and severe irritants (UN GHS Category 1, EU CLP Category 1, U.S. EPA Category I) (see paragraphs 1 and 10).

37. In order to identify water soluble chemicals (substances and mixtures) (4) (7) (8) as "inducing serious eye damage" (UN GHS/EU CLP Category 1) or as an "ocular corrosive or severe irritant" (U.S. EPA Category I), the test substance should induce an FL_{20} value of $\leq$ 100 mg/mL.

Acceptance of results

38. The mean maximum fluorescein leakage value (x) should be higher than 4000 (see paragraph 31), the mean 0% leakage (y) should be equal or lower than 300, and the mean 100% leakage (z) should fall between 3700 and 6000.

39. A test is considered acceptable if the positive control produced 20% to 40% damage to the cell layer (measure as % fluorescein leakage).

DATA AND REPORTING

Data

40. For each run, data from individual replicate wells (*e.g.* fluorescence intensity values and calculated percentage FL data for each test substance, including classification) should be reported in tabular form. In addition, means ± SD of individual replicate measurements in each run should be reported.

Test Report

41. The test report should include the following information:

Test and Control Substances

- Chemical name(s) such as the structural name used by the Chemical Abstracts Service (CAS), followed by other names, if known;
- Chemical CAS number, if known;
- Purity and composition of the substance or mixture (in percentage(s) by weight), to the extent this information is available;
- Physical-chemical properties relevant to the conduct of the study (e.g. physical state, volatility, pH, stability, water solubility, chemical class);
- Treatment of the test/control substance prior to testing, if applicable (*e.g.* warming, grinding);
- Storage conditions;

Justification of the Test Method and Protocol Used

- Should include considerations regarding applicability domain and limitations of the test method;

Test Conditions

- Description of cell system used, including certificate of authenticity and the mycoplasma status of the cell line;
- Details of test procedure used;
- Test substance concentration(s) used;
- Duration of exposure to the test substance;
- Duration of incubation with fluorescein;
- Description of any modifications of the test procedure;
- Description of evaluation criteria used;
- Reference to historical data of the model (*e.g.* negative and positive controls, benchmark chemicals, if applicable);
- Information on the technical proficiency demonstrated by the laboratory;

Results

- Tabulation of data from individual test substances and controls for each run and each replicate measurement (including individual results, means and SDs);
- The derived classification(s) with reference to the prediction model and/or decision criteria used;
- Description of other effects observed;

Discussion of the Results

- Should include considerations regarding a non-conclusive outcome (paragraph 35: FL_{20} > 100 mg/mL) and further testing;

Conclusions

LITERATURE

1. UN (2009), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Third revised edition, New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1. Available at: [http://www.unece.org/trans/danger/publi/ghs/ghs_rev03/03files_e.html]
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3. U.S. EPA (1996), Label Review Manual: 2nd Edition, EPA737-B-96-001, Washington DC: U.S. Environmental Protection Agency.
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5. Scott, L. *et al.* (2010), A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-Up and Top-Down approaches, *Toxicol. In Vitro* 24, 1-9.
6. OECD (2002), *Test No. 405: Acute Eye Irritation/Corrosion*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264070646-en
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8. EC-ECVAM (2008), Fluorescein Leakage Assay Background Review Document as an Alternative Method for Eye Irritation Testing. Available under *Validation Study Documents*, Section *Eye Irritation* at: [http://ecvam.jrc.it/index.htm]
9. OECD (2005), *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, OECD Series on Testing and Assessment No. 34. OECD, Paris. Available at: [http://www.oecd.org/env/testguidelines]

ANNEX I

DIAGRAM OF MDCK CELLS GROWN ON AN INSERT MEMBRANE FOR THE FL TEST METHOD

A confluent layer of MDCK cells is grown on the semi-permeable membrane of an insert. The inserts are placed into the wells of 24 well plates.

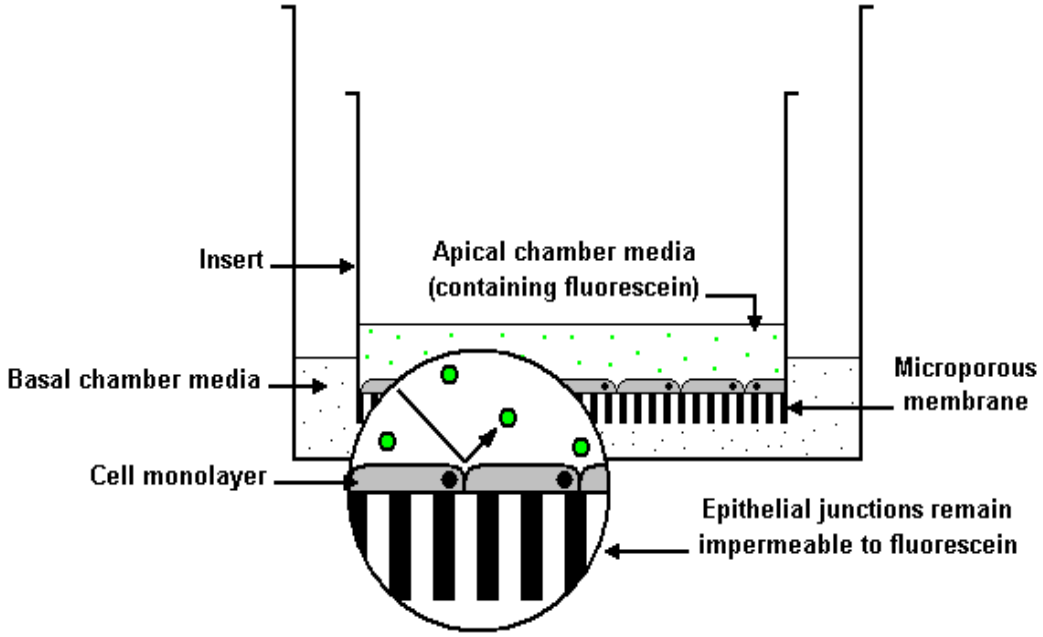

Figure taken from: Wilkinson, P.J. (2006), Development of an *in vitro* model to investigate repeat ocular exposure, Ph.D. Thesis, University of Nottingham, UK.

ANNEX II

DEFINITIONS

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

EPA Category I: Chemicals that produce corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days (4).

EU CLP (European Commission Regulation on the Classification, Labelling and Packaging of Substances and Mixtures): Implements in the European Union (EU) the UN GHS system for the classification of chemicals (substances and mixtures) (3).

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

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

FL_{20}: Can be estimated by the determination of the concentration at which the tested chemical causes 20% of the fluorescein leakage through the cell layer.

Fluorescein leakage: the amount of fluorescein which passes through the cell layer, measured spectrofluorometrically.

GHS (Globally Harmonized System of Classification and Labeling of Chemicals by the United Nation (UN)): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (2).

GHS Category 1: Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application.

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

Mixture: Used in the context of the UN GHS (2) as a mixture or solution composed of two or more substances in which they do not react.

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

Not-classified: Chemicals that are not classified as UN GHS Categories 1, 2A, or 2B; EU CLP Categories 1 or 2; or U.S. EPA Categories I, II, or III ocular irritants (2) (3) (4).

Ocular corrosive: (a) A chemical that causes irreversible tissue damage to the eye. (b) Chemicals that are classified as UN GHS Category 1; EU CLP Category 1; or U.S. EPA Category I ocular irritants (2) (3) (4).

Ocular irritant: (a) A chemical that produces a reversible change in the eye following application to the anterior surface of the eye; (b) Chemicals that are classified as UN GHS Categories 2A, or 2B; EU CLP Category 2; or U.S. EPA Categories II or III ocular irritants (2)(3)(4).

Ocular severe irritant: (a) A chemical that causes tissue damage in the eye following application to the anterior surface of the eye that is not reversible within 21 days of application or causes serious physical decay of vision. (b) Chemicals that are classified as UN GHS Category 1; EU CLP Category 1; or U.S. EPA Category I ocular irritants (2) (3) (4).

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

Proficiency Chemicals: A sub-set of the list of Reference Chemicals that can be used by a naïve laboratory to demonstrate proficiency with the validated reference test method.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (9).

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

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals.

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (9).

Serious eye damage: Is the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application**.**

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

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method.

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

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

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (9).

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

ANNEX III

PROFICIENCY CHEMICALS FOR THE FL TEST METHOD

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the ocular corrosivity classification of the 8 chemicals recommended in Table 1. These chemicals were selected to represent the range of responses for local eye irritation/corrosion, which is based on results in the *in vivo* rabbit eye test (TG 405) (*i.e.*, Categories 1, 2A, 2B, or No Category according to the UN GHS and EU CLP (1)(2)(6). However, considering the validated usefulness of the FL assay (*i.e.*, to identify ocular corrosives/severe irritants only), there are only two test outcomes for classification purposes (corrosive/severe irritant or non-corrosive/non-severe irritant) to demonstrate proficiency. Other selection criteria were that chemicals are commercially available, there are high quality *in vivo* reference data available, and there are high quality data from the FL test method. For this reason, the proficiency chemicals were selected from the "Fluorescein Leakage Assay Background Review Document as an Alternative Method for Eye Irritation Testing" (8), which was used for the retrospective validation of the FL test method.

Table 1: Recommended chemicals for demonstrating technical proficiency with FL

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)	Noncorrosive/ Non-severe irritant
Ethyl-2-methylaceto-acetate	609-14-3	Ketone, Ester	Liquid	Category 2(B)	Noncorrosive/ Non-severe irritant
Glycerol	56-81-5	Alcohol	Liquid	No Category	Noncorrosive/ Non-severe irritant

Abbreviations: CAS NR = Chemical Abstracts Service Registry Number

[1]Chemical classes were 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) and using the UN GHS and EU CLP (1)(2)(6).

[3]Based on results obtained with FL (INVITTOX Protocol No. 71).