The Cytosensor Microphysiometer (CM) Toxicity Test
INVITTOX n° 130

Eye Irritation

The effects of a test compound on intracellular metabolism, as reflected by a decrease in the extracellular acidification rate, can be used as a measure of eye irritancy potential. This protocol represents a modified version of INVITTOX protocol No. 102 produced as an outcome of the successful ECVAM retrospective validation study.

Objective and Applications

TYPE OF TESTING	:	Screening, adjunct, partial replacement
LEVEL OF ASSESSMENT	:	Toxic potential, toxic potency
PURPOSE OF TESTING	:	Classification and labelling, ranking, safety
CONTEXT OF USE	:	Regulatory purpose: according to an outcome of the successful ECVAM retrospective validation study (ESAC, 2009), the Cytosensor Microphysiometer (CM) Toxicity Test is ready for consideration for regulatory use within a Top-Down and/or a Bottom-Up Approach of tiered eye irritation testing strategy (Scott *et al.*, 2010) to identify ocular corrosives and severe irritants and/or non-irritants for the chemical classes of compounds, as specified under the Applicability Domain. The CM Toxicity Test does not correctly identify moderate and mild ocular irritants, therefore it is not recommended as a full replacement method (ESAC, 2009).
APPLICABILITY DOMAIN	:	• Water-soluble chemicals (substances and mixtures) are used for a Top-Down Approach (ESAC, 2009). • Water-soluble surfactants and water-soluble surfactant-containing mixtures are used for a Bottom-Up Approach (ESAC, 2009).

Because of certain characteristics of the Cytosensor Microphysiometer (e.g. small tubing diameter), only solutions should be tested.

Rationale

The CM Toxicity Test is a cell function based *in vitro* assay, developed as an alternative to the Draize rabbit eye irritation test (Draize *et al.*, 1944; OECD TG 405, 2002).
The test system consists of a monolayer of adherent cells (mouse L929 fibroblasts), cultured on a transwell polycarbonate insert with a porous membrane, and a light-addressable potentiometric sensor detecting changes in pH (acidity). In the CM Toxicity Test, the potential ocular toxicity of chemical substances is evaluated according to the reduction in the metabolic rate induced by a test substance in the treated cultures of L929 cells. Change in metabolic rate is measured indirectly as a function of changes in extracellular acidification rate which decreases if cells receive a toxic insult (McConell *et al.*, 1992; Parce *et al.*, 1989)
The protocol herewith presented is the result of modifications of *INVITTOX* Protocol No. 102 based upon the COLIPA protocol (Brantom *et al.*, 1997; Harbell *et al.*, 1999) and IIVS protocol (SP200019 or COLIPA Protocol SP200014)introduced as an outcome of the successful retrospective validation of the Cytosensor Microphysiometer Test Method by ECVAM (ESAC, 2009).

A method summary data-sheet on the "Silicon Microphysiometer (SM) Assay or Cytosensor Microphysiometer (CM) Assay", laying down the general principles of this technique is available in the DB-ALM.

Experimental Description

Endpoint and Endpoint Measurement:

METABOLIC ACTIVITY: measured as the rate of acidification of the modified cell culture medium (w/ minimum buffer capacity) in an enclosed cell chamber with a light-addressable potentiometric sensor.

Endpoint Value:

MRD_{50}: metabolic rate decrement by 50% - the concentration of test material, as w/v% required to reduce the acidification rate to 50% of the starting baseline rate.

Experimental System(s):

L-929 FIBROBLASTS (mouse): commercially available murine cell line

Basic procedure

The Cytosensor Microphysiometer System measures the rate of extracellular acidification of populations of living cells maintained in low volume flow through chambers. The light addressable potentiometer forms the bottom of the flow through chamber and serves as a very sensitive and stable pH-meter. While medium is flowing through the chamber, the pH is stable since it is governed by the pH of the medium. When the flow of medium is stopped, the pH begins to drop in a linear manner over time due to excretion of acidic components by viable cells. The actual change in pH during this measurement is generally less than 0.2 pH units which is not harmful to the cells. Test samples, prepared as a dilution series, are introduced, in order of increasing concentration, to a predetermined number of cell containing transwells (one dilution series for each transwell) during the course of an experiment. Between sample introduction, the cells are washed with medium to remove the sample from the chamber. All acidification rate measurements are made on washed cells.
The operator introduces manually prepared test sample dilutions to the System by filling injection loops. Instrument control, such as flow rate and valve-switching, as well as data acquisition, analysis and storage, are handled by a computer and software that are part of the System.

After establishing a baseline acidification rate for each set of cells, and measuring the new rates subsequent to each sample addition, the concentration of test material, as w/v% required to reduce the acidification rate to 50%, is computed by interpolation between the rate data points spanning the 50% response level. This value is termed the MRD_{50} and is the endpoint for the test.

Data Analysis/Prediction Model

During the ECVAM retrospective validation study, water soluble chemicals that were characterised by MRD_{50} < 2 mg/ml were identified as ocular corrosives and severe irritants (EU R41, EU 2001 and 2008; GHS Category 1, UN 2009; US EPA Category I, US 1996).

Water-soluble surfactants and water-soluble surfactant-containing mixtures that were characterised by MRD_{50} > 10 mg/ml were identified as non-irritants (EU Not Classified, EU 2001 and 2008; GHS No Label, UN 2009). For non-irritants US EPA Category IV (US, 1996), MRD_{50} > 80 mg/ml was used.

Modifications of the Method

With respect to the *INVITTOX* protocol No. 102, the introduced protocol refinements mainly refer to:

- Increased duration of exposure time up to 810 s
- Introduced positive control (sodium lauryl sulfate (SLS) serial dilution prepared from a 10% stock in water)
- Defined prediction model
- Quality control criteria

Discussion

Initially, the Silicon Microphysiometer manufactured by Molecular Devices Corporation, Menlo Park, CA, was used as the main assay instrument, and cells were cultured on an indium-tin oxide coated surface of a cover slip (Bruner *et al.*, 1991; Bagley *et al.*, 1992; *INVITTOX* Protocol No. 97). Later, this instrument was replaced by the same manufacturer with a new instrument called the Cytosensor Microphysiometer, where the cells were cultured on a transwell polycarbonate insert with a porous membrane (Bagley *et al.*, 1992; Catroux *et al.*, 1993; Balls *et al.*, 1995, *INVITTOX* Protocol No. 102). It is anticipated that other Cytosensor Microphysiometer-like equipment and software may become available with either equivalent or better performance therefore the development of Performance Standards for the CM test method was recommended (ESAC, 2009).

Status

The Silicon Microphysiometer Toxicity Test *(INVITTOX* protocols No. 97 and 102) participated in the EC/HO International Validation Study on Alternatives to the Draize Eye Irritation Test (1992 - 1994) but did not meet the success criteria set by the management team of the study at that time (Balls *et al.*, 1995).

The herewith presented Standard Operating Procedure of the CM Toxicity Test is based on the *INVITTOX* protocol No. 102 modified as an outcome of the successful ECVAM retrospective validation study. **ESAC endorsed the scientific validity of the CM Toxicity Test for its use as an initial step within a Top-Down Approach of tiered eye irritation testing strategy (Scott *et al.*, 2010) to identify ocular corrosives and severe irritants (EU R41, EU 2001 and 2008; GHS Category 1, UN 2009; US EPA Category I, US 1996) from all other classes for the chemical applicability domain of water-soluble chemicals (substances and mixtures) as well as for its use as an initial step within a Bottom-Up Approach of tiered eye irritation testing strategy (Scott *et al.*, 2010) to identify non-irritants (EU Not Classified, EU 2001 and 2008; GHS No Label, UN 2009; US EPA Category IV, US 1996) from all other classes only for water-soluble surfactants and water-soluble surfactant-containing mixtures (ESAC, 2009).**

Since the CM Toxicity Test does not correctly identify moderate and mild ocular irritants (EU R36, EU 2001 and 2008; GHS Category 2A/B, UN 2009; US EPA Category II/III, US 1996), it is not recommended as a full replacement method (ESAC, 2009).

Proprietary and/or Confidentiality Issues

None reported

Abbreviations & Definitions

CM – Cytosensor Microphysiometer
COLIPA – European Cosmetic,Toiletry and Perfumery Association
ECVAM – European Centre for the Validation of Alternative Methods
EPA – US Environmental Protection Agency
ESAC – ECVAM Scientific Advisory Committee
EU – European Union
GHS – United Nations Globally Harmonized System of Classification and Labelling of Chemicals
IIVS – Institute for *In Vitro* Sciences
MMAS – Draize Modified Maximum Average Score
MRD_{50} – Metabolic Rate Decrement by 50% - the concentration of test material, as w/v% required to reduce the acidification rate to 50% of the starting baseline rate
OECD – Organization for Economic Co-operation and Development
SLS – sodium lauryl sulphate
TG – Test Guideline

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PROCEDURE DETAILS, 06 April 2011

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Note: This protocol represents a modified version of *INVITTOX* protocol No. 102 produced as an outcome of the successful ECVAM retrospective validation study.

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Health and Safety Issues

None reported.

Materials and Preparations

Cell or Test System

- L929 (mouse fibroblast) cell line

Equipment

Fixed Equipment

- Aspirator
- Balance
- Cytosensor Microphysiometer, manufactured by Molecular Devices Corporation (MDC), Menlo Park, California, USA
- Cytosoft computer program that collects data from Cytosensor and control its operation by MDC
- Freezer, liquid nitrogen container
- Hemocytometer or Coulter counter for cell counting
- Incubator, 37°C, 5% CO2, 90% humidity, for cell culture incubation
- Inverted microscope, to determine the confluence of cell cultures
- Laminar flow hood, for cell culture manipulations
- Pipettors
- Racks
- Refrigerator
- Spacers by MDC
- Statistical program (e.g. Excel), for calculation of MRD_{50}
- Water bath

Consumables

- 15 and 50 ml tubes
- 5 ml and 30 ml syringes
- Capsule cups, 12 mm, 3 µm pore size, to immobilize living cells in the sensor chamber
- Disposable beakers
- Pipette tips

Media, Reagents, Sera, others

- Ca^{2+} Mg^{2+} - free Hank's Balanced Salts Solution sodium bicarbonate-free (e.g. Quality Biological, Gaithersburg, MD, Cat# 119-065-101)
- DMEM (Dulbecco's Modified Eagle's Medium) with 4.5 mg/ml glucose (e.g. Quality Biological, Gaithersburg, MD, Cat# 112-013-101)
- DMEM (Dulbecco's Modified Eagle's Medium) serum-free, sodium bicarbonate-free with 4.5 mg/ml glucose (e.g. Quality Biological, Gaithersburg, MD, Cat# 112-129-101)
- Fetal bovine serum
- Gentamicin
- L-glutamine
- NaCl (sodium chloride)
- PBS (Ca^{2+} Mg^{2+} - free phosphate buffered saline)
- SLS (Sodium lauryl sulphate, e.g. Sigma)
- Sodium pyruvate
- Trypsin

Preparations

Media and Endpoint Assay Solutions

Working solutions	
Growth Medium	Dulbecco's Modified Eagle's Medium (DMEM)supplemented with: 1.0 mM sodium pyruvate 10% fetal bovine serum 2.0 mM L-glutamine
Seeding Medium	DMEM supplemented with: 1% fetal bovine serum 50 µg/ml gentamicin 2.0 mM L-glutamine
Low-Buffered Treatment Medium	Serum-free, Sodium Bicarbonate-free DMEM supplemented with: 50 µg/ml gentamicin 2.0 mM L-glutamine NaCl for consistent osmolarity (11.1 ml of 4 M NaCl solution per 1 L of medium)
Sodium lauryl sulfate (SLS) 10%	10% SLS in water (stock solution)
Trypsin 0.05%	0.05% Trypsin in Ca^{2+} Mg^{2+} - free Hank's Balanced Salts Solution

Test Compounds

Chemicals are dissolved in Low-Buffered Treatment Medium. The test compounds must be in a single phase solution/suspension at the highest dose used (generally 300 mg/ml) to prepare the required dilutions (see Endpoint Measurement(s)). If the substance can not form a single phase solution/suspension at concentration of 3.33 mg/ml, the test

sample should not be tested by the Cytosensor Microphysiometer using standard techniques.

Positive Control(s)

Sodium lauryl sulfate (SLS) prepared by serial dilution of a 10% stock solution in water (see Endpoint Measurement(s)). The positive control is included in each assay, and it should be tested like a test compound except that the dose range will be set based on historical data.

Negative Control(s)

At the beginning of each assay, at least four to five stable acidification rates are taken to determine the basal acidification rate, which is used as the internal negative control for each cell culture. Baseline rates are expected to fall between 50 and 200 µV/s after a stabilization period of approximately 1 hour. A capsule with cells in a chamber that fails to achieve these ranges should be replaced with another capsule which should then be allowed to stabilize for approximately 1 hour.

A solvent control is recommended when a solvent other than Low-Buffered Treatment Medium is used.

Method

Test System Procurement

- L929 cell line, stored in liquid nitrogen, was from ATCC, Manassas, VA (NCTC Clone 929).

Routine Culture Procedure

- Frozen L929 cells are thawed at 37°C in a water bath.
- Cells are seeded at the concentration of about $1.0x10^6$ cells in approximately 15 ml of Growth Medium in 75 cm^2 (T75) sterile tissue culture flasks.
- Cell culture is incubated in the humidified incubator maintained at 37 ± 1°C and 5 ± 1% CO_2 in air until an appropriate confluency is achieved.
- When cells are at or near the confluency (evaluated under inverted microscope), the Growth Medium is decanted and the cell layer is washed twice with approximately 10 ml of PBS for each 75 cm^2 of growth surface.
- The cells are trypsinised with approximately 3 ml of 0.05% trypsin for 15 to 30 seconds.
- The trypsin solution is aspirated and the cells are incubated at room temperature for approximately 2 to 5 minutes, until the cells begin to round.
- The cells are dislodged by tapping the flask.
- Approximately 5 ml of Growth Medium is added for each 75 cm^2 of growth surface and cells are dispersed by repeated pipetting.
- Cell suspension is seeded in 75 cm^2 sterile tissue culture flasks (T75) at a dilution factor of 1:4 or 1:8 and returned to the humidified incubator maintained at 37 ± 1°C and 5 ± 1% CO_2 in air.

Test Material Exposure Procedures

Preparation of cells grown on capsule cups

- When cells are at or near the confluency (evaluated under inverted microscope), the Growth Medium is decanted and the cell layer is washed twice with approximately 10 ml of PBS for each 75 cm^2 of growth surface.
- The cells are trypsinised with approximately 3 ml of 0.05% trypsin for 15 to 30 seconds.
- The trypsin solution is aspirated and the cells are incubated at room temperature for approximately 2 to 5 minutes, until the cells begin to round.
- The cells are dislodged by tapping the flask.

- Approximately 5 ml of Seeding Medium is added for each 75 cm^2 of growth surface and cells are dispersed by repeated pipetting.
- The cells are transferred by pipet to a conical centrifuge tube. If more than one flask is used, the contents of each are pooled.
- Cells are counted by hemocytomer or coulter counter.
- Cells are seeded with approximately $6.0x10^5$ cells per each capsule cup (0.5 ml of a $1.2x10^6$ cell suspension) with 1.5 ml of Seeding Medium added to each outside well.
- The capsule cups with cells are incubated in the humidified incubator maintained at 37 ± 1°C and 5 ± 1% CO_2 in air for 16 to 32 hours.
- At the time of use, cells should be < 80% confluent because fully confluent monolayer could interfere with accurate Cytosensor Microphysiometer readings.

Preparation of Cytosensor Microphysiometer

- 8 x 50 ml tubes containing at least 20 ml of Low-Buffered Treatment Medium are placed on the Cytosensor Microphysiometer and the injection loops are filled with Low-Buffered Treatment Medium using a 30 ml syringe.
- Using the "Front Panel" controls, the flow rate is set to High (50% of max) to fill the lines, and then set back to Idle (5%).
- Sterilant is aspirated from the sensor chambers and the chambers are washed by repeated filling with, and aspiration of, distilled water and then Low-Buffered Treatment Medium.

Exposure cycle of test compounds

- Prior to the start of the assay, the medium in capsule cups is switched to Low-Buffered Treatment Medium (37 ± 1°C) and the spacer is added to each capsule cup and gently tapped down to the bottom.
- Forceps is used to place the cell capsules into the sensor chambers, where gantries are lifted and plungers are raised one set at a time.
- When all the capsules are in place, the flow rate is set to High and obvious bubbles are cleared again.
- L929 cells contained in sensor chambers are exposed to each concentration of the test compound for 810 seconds.
- After the baseline data points have been taken (see Negative Control), the exposure cycle, consisting of three phases, is started.
- Each exposure cycle takes 20 minutes.
- First, the test compound at the lowest concentration is introduced into the sensor chamber for 13 minutes and 30 seconds. The nominal flow rate is set to 100 µl/min for the first minute, and 20 µl/min for the next 12 minutes and 30 seconds.
- The second phase – wash out phase lasts 6 minutes at the flow rate of 100 µl/min. The test compound is washed out of the sensor chamber during this phase.
- During the third phase, the flow is stopped for 25 seconds and the change in pH is measured.

Endpoint Measurement

The Cytosensor Microphysiometer System measures the rate of extracellular acidification of treated L929 cells maintained in an enclosed flow chamber.

Dose range finding assay

- A dose range finding assay is performed to establish an appropriate test article dose range for the definitive Cytosensor Microphysiometer toxicity test.
- Solutions at different concentrations are prepared in 5 ml glass test tubes by serial three-fold dilution, as below, in sterile, Low-Buffered Treatment Medium that has been warmed to approximately 37°C.

DILUTION #	CONCENTRATION	DILUTION
1	10 mg/ml	100 mg diluted to 10 ml (use weight, not volume even if it is a liquid)
2	3.33 mg/ml	3 ml of Dilution 1 plus 6 ml medium
3	1.11 mg/ml	3 ml of Dilution 2 plus 6 ml medium
4	0.370 mg/ml	3 ml of Dilution 3 plus 6 ml medium
5	0.123 mg/ml	3 ml of Dilution 4 plus 6 ml medium
6	0.0412 mg/ml	3 ml of Dilution 5 plus 6 ml medium
7	0.0137 mg/ml	3 ml of Dilution 6 plus 6 ml medium

- The exposure cycle should be repeated with increasing test compound concentrations until either the highest test compound concentration is reached or until the reduction of the metabolic rate to 50% of its basal rate (MRD_{50} value, see Data Analysis) has been surpassed.
- Each test compound concentration should be tested on a single set of cells.
- Data are exported to the Excel spreadsheet and if possible, an MRD_{50} value (see Data Analysis) should be calculated from the dose range finding assay.
- Positive control materials and solvent controls (for solvents other as Low-Buffered Treatment Medium) should be tested in the same manner. For the positive control, dilutions of the 10% SLS stock solution are made on a weight to volume basis using Low-buffered Treatment Medium and considering that the 10% solution is the 'neat'' test material. A dose range finding assay should be performed once on the positive control to set the appropriate ranges for the subsequent definitive trials.

Definitive assay

- The definitive assays are performed in the same manner as the dose range finding assay. The positive control is included in each assay.
- Seven concentrations of test compound spaced as three-fold dilutions are used for the definitive assay.
- Generally, three concentrations are chosen below the expected test compound concentration that results in the reduction of the metabolic rate to 50% of its basal rate (MRD_{50} value, see Data Analysis), one at approximately MRD_{50}, and three above the expected MRD_{50}.
- If a test compound fails to cause 50% toxicity in the Dose range finding assay, the maximum concentration used will generally be 270 to 300 mg/ml or less based on its ability to form single phase solution/suspension.
- The determination of the definitive MRD_{50} will be based upon the results of three definitive assays. If the MRD_{50} could be determined from the results of the Dose range finding assay, this value could be included as one of the three needed for determination of the definitive MRD_{50}.

Acceptance Criteria

Acceptance criteria are normally based on the performance of the positive control. The Cytosensor Microphysiometer toxicity test would be accepted if the positive control MRD_{50} value fell within 2 standard deviations of the historical range (0.0546 – 0.1140 mg/ml range was reported during ECVAM Validation study; in COLIPA and IIVS studies mean MRD_{50} value of 0.08 ± 0.01 mg/ml was reported).

Data Analysis

The acidification rates that occur after exposure of each test compound concentration are calculated by the Cytosoft software and compared to the mean basal acidification rate of the same cells prior to exposure to a test compound.
The data are exported to the Excel spreadsheet and the percent of control acidification rate is determined by comparing the dose response acidification rate to the basal acidification rate (Fig. 1):

$$\%\ \text{of control rate} = \frac{\text{acidification rate after test compound}}{\text{basal acidification rate}} \times 100$$

Fig. 1 Calculation of the percent of control acidification rate.

Percent of control acidification rates are plotted on the ordinate and the test compound concentration on the abscissa. The concentration of the test compound that results in a 50% reduction in acidification rate is interpolated from the obtained curve and referred to as the MRD_{50}.

Prediction Model

The following prediction model based on classification criteria that are described by the GHS (UN, 2009), the EU chemical substances classification system (EU, 2001 and 2008) and the US EPA classification system (US, 1996) was proposed during ECVAM retrospective validation study:

MRD_{50}	EU Classification Category	GHS Classification Category	EPA Classification Category
< 2 mg/ml	R41	1	I
>10 mg/ml	Not Classified	No Label	-
>80 mg/ml	-	-	IV

- Not relevant for the particular category.

The MRD_{50} cut-off value < 2 mg/ml was endorsed by ECVAM to be used for identification of ocular corrosives and severe irritants (EU R41, EU 2001 and 2008; GHS Category 1, UN 2009; US EPA Category I, US 1996) from all other classes for the chemical applicability domain of water-soluble substances and mixtures (ECVAM, 2009). Furthermore, the MRD_{50} cut-off value > 10 mg/ml (> 80 mg/ml for US EPA (Not Classified, US 1996)) was endorsed by ECVAM to be used for identification of non-irritants as determined by EU chemical substances classification (Not Classified, EU 2001 and 2008) and GHS (No Label, UN 2009) from all other classes only for water-soluble surfactants and water-soluble surfactant-containing mixtures (ESAC, 2009).

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