DB-ALM Protocol n° 191 : SkinEthic™ HCE Eye Irritation Test Solid (EITS)

Eye Irritation

The SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test Solid (EITS) is an in vitro assay used to assess the acute ocular irritation potential of solid chemicals.

Résumé

The purpose of the SkinEthic™ HCE EIT method is to assess the eye irritation potential of chemicals using the Reconstructed Human Corneal Epithelium model.

The SkinEthic™ HCE EIT method is used to assess the serious eye damage/eye irritation potential of liquid (EITL: Eye Irritation Testing of Liquids) and solid test chemicals (EITS: Eye Irritation Testing of Solids).

Two protocols depending on the physical state of the testing chemicals have been developed and they are both available from DB-ALM: the current protocol is for solid chemicals (EITS), whereas the SkinEthic™ HCE EITL is for liquid/viscous chemicals DB-ALM Protocol No. 190).

The SkinEthic™ HCE EITS, hereafter described, has been validated in a study coordinated by L'Oreal (Alépée et al., 2016a), and subsequently independent peer reviewed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Scientific Advisory Committee (ESAC opinion No. 2016-02).

This test method, accepted in the OECD Test Guideline No. 492 (OECD, 2017a), is recommended to identify solid chemicals that do not require classification for eye irritation or serious eye damage according to United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS).

However, SkinEthic™ HCE EIT, and this EITS protocol, is not intended to differentiate on its own between serious eye damage and eye irritation and this differentiation will need to be addressed by another tier of a test strategy in the context of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2017c).

On the basis of the data currently available, the SkinEthic™ HCE EITS protocol has been shown to be applicable to a wide range of solid chemicals, covering a large variety of chemical types, chemical classes, functional groups, molecular weights, LogPs, chemical structures, colored and/or direct MTT reducers, etc. (Alépée et al., 2016a). This test method allows the hazard identification of mono and multi-component test solid chemicals. Gasses and aerosols cannot be also evaluated with the current protocol.

Liquid/viscous chemicals cannot be evaluated with the current protocol but their assessment can be performed using SkinEthic™ HCE EITL assay (Alépée et al., 2016b) which is described in the DB-ALM Protocol No. 190.

Experimental Description

Endpoint and Endpoint Measurement:

Cell viability determination is based on cellular mitochondrial dehydrogenase activity, measured by tetrazolium salt MTT reduction [(3,4,5-dimethyl triazole 2-yl) 2,5-diphenyltetrazoliumbromide] (Mossman, 1983).

Viable cells of the tissue construct reduce the vital dye MTT into a blue MTT formazan precipitate, which is then extracted from the tissue using isopropanol (or a similar solvent).

The extracted MTT formazan may be quantified using either a standard absorbance (Optical Density (OD)) measurement or a High/ Ultra-high Performance Liquid Chromatography (HPLC/UPLC) spectrophotometry procedure (Alépée et al., 2015; Alépée et al., 2016a).

Endpoint Value:
The reduction of cell viability in test chemicals treated tissues is compared to treated tissues with negative control (100% viability) and expressed as a %. The MTT-formazan reduction % in viability is used to predict the eye hazard potential of the test chemical.

**Experimental System(s):**

Human Corneal Epithelium model (SkinEthic™ HCE):
When cultured at the air-liquid interface in a chemically defined medium on a permeable synthetic membrane insert, the transformed human corneal epithelial cell line forms a corneal epithelial tissue. Obtained tissues are ultra-structurally (tissue morphology and thickness) similar to the corneal epithelium of the human eye (Nguyen et al., 2003). As in vivo epithelium, the SkinEthic™ HCE model is characterized by the presence of intermediate filaments, mature hemi-desmosomes and desmosomes, and specific cytokeratins. The 0.5 cm² multilayered epithelium contains at least 4 cell layers, including columnar cells and wing cells.

**Discussion**

**Ethical issues.** The test is based on an in vitro system, no ethical issues are related.

**Special equipment.** No specific equipment is needed (only classical laboratory devices are required to perform the test method).

**Amount of training required.** Two training days are necessary to establish the test method in a naïve laboratory. It includes a practical training in which (i) the main steps of the protocol are emphasized (ii) a demonstration of the method are observed and (iii) then performed by the trainers. It also includes depth discussions about the detailed protocol.

**Duration of the test.** 3 days are required to perform a run: A run begins on Day 1 with tissue conditioning, on Day 2 with cell treatment with chemicals, and on Day 3 with data acquisition. A trained experimenter can perform at least 13 test chemicals in a run.

**Costs.** Testing costs are available upon request to the testing facilities (e.g. Contract Research Organization).

**Status**

**Participation in Validation Studies:**

The SkinEthic™ HCE method for testing solid chemicals (EITS protocol) has been evaluated in a multicentre validation study coordinated by L’Oréal (Alépée et al., 2016a).

The SkinEthic™ HCE EITS protocol was proved to be transferable to laboratories considered to be naïve in the conduct of the assays and also to be reproducible within- and between laboratories. The level of reproducibility in terms of concordance of predictions that can be expected from SkinEthic™ HCE EITS from data on 60 solid chemicals is in the order of 95% within laboratories and 96.7% between laboratories, respectively (Alépée et al., 2016a). Considering the data obtained in the validation study (Alépée et al., 2016a), the SkinEthic™ HCE EITS has an overall accuracy of 81% (based on 95 solid chemicals), sensitivity of 91% (based on 42 in vivo known classified chemicals), false negative rate of 9% (based on 42 chemicals), specificity of 74% (based on 53 in vivo known No category chemicals) and false positive rate of 26% (based on 53 chemicals), when compared to reference in vivo rabbit eye test data (OECD TG 405) classified according to the UN GHS classification system (OECD, 2017d).

Following the validation study, the EURL ECVAM Scientific Advisory Committee (ESAC) has independently peer reviewed the Skin Ethic™ HCE EIT method (ESAC Opinion, 2016) which can used for testing of liquid (see DB-ALM Protocol No. 190) and solid chemicals as described in this current protocol.

From the validation study (Alépée et al., 2016a) and the independent peer review (ESAC Opinion, 2016), it was concluded that the SkinEthic™ HCE EIT method and more specifically the EITS protocol is able to correctly identify solid chemicals (both substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS without further testing, within a testing strategy such as the Bottom-Up/Top-Down approach suggested by Scott et al. e.g., as an initial step in a Bottom-Up approach or as one of the last steps in a Top-Down approach (Scott et al. 2010; UN, 2015; OECD, 2017c).
However, the SkinEthic™ HCE EIT method is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation). This differentiation should be considered in combination with other sources of information in the context of an IATA (OECD, 2017c).

**Regulatory Acceptance:**

The SkinEthic™ HCE EIT method using solid (EITS protocol, e.g. the current DB-ALM Protocol No.191) and liquid (EITL protocol, DB-ALM Protocol No.190) testing chemicals is recommended as scientifically valid according to OECD Test Guideline (TG) 492 which was adopted on 9th October 2017 (OECD, 2017a, OECD, 2017b).

**Proprietary and/or Confidentiality Issues**

The Reconstructed Human Tissue SkinEthic™ HCE technology, associated to production of model and media are proprietary to Episkin, France.

No intellectual property rights are associated with the present test method

**Health and Safety Issues**

**General Precautions**

*Normal handling procedures for biological materials* should be followed:

- It is recommended to wear glasses and gloves during handling.
- After use, the epithelium, the material, and all media in contact with the tissue should be decontaminated (for example, by using a 10% solution of bleach in appropriate containers), prior to elimination.

*Safety instructions for working with test chemicals:*

- Test chemicals should be handled following material safety datasheet. Store the test chemicals in ventilated safety cupboards. Respect specific storage conditions if necessary (special temperature, protected from light, etc.) according to the material safety datasheet guidelines.
- Unknown test chemicals with no or incomplete safety handling information should be considered as irritating and toxic and must be handled with maximum care in accordance with test chemical safety guidelines.

**MSDS Information**

*Safety precautions:*

MTT (R68, R36, R37, R38 / H315, H319, H335, H341)  
Isopropanol (R11, R36, R67 / H225, H319, H336)  
Methyl Acetate (H225, H319, H336)  

*Work in ventilated cabinets:* to prevent accidental contact wear protective gloves, and if necessary safety glasses.

**Abbreviations and Definitions**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>No pred</td>
<td>No prediction can be made</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EITL</td>
<td>Eye Irritation Test Liquid</td>
</tr>
<tr>
<td>EITS</td>
<td>Eye Irritation Test Solid</td>
</tr>
<tr>
<td>EU CLP</td>
<td>European Classification Labelling and Packaging Regulation</td>
</tr>
<tr>
<td>HCE</td>
<td>Human Corneal Epithelium</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>hr/hrs</td>
<td>Hour/hours</td>
</tr>
<tr>
<td>IP</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>KU</td>
<td>Negative control killed treated tissue</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower Limit Of Quantification</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NC</td>
<td>Not Classified</td>
</tr>
<tr>
<td>NgC</td>
<td>Negative Control</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NSC_killed</td>
<td>Non Specific Color in killed tissues—killed tissue without MTT incubation</td>
</tr>
<tr>
<td>NSC_living</td>
<td>Non Specific Color in living tissues—living tissue without MTT incubation</td>
</tr>
<tr>
<td>NSMTT</td>
<td>Non Specific MTT reduction in killed tissue—killed tissue with MTT incubation</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS without Ca^{2+} &amp; Mg^{2+}</td>
</tr>
<tr>
<td>PC</td>
<td>Positive Control</td>
</tr>
<tr>
<td>Qualified Test</td>
<td>The test of a test chemical is qualified (qualified test) if it meets the acceptance criteria for the test within a qualified run. Otherwise, the test is considered as Non-Qualified (invalid).</td>
</tr>
<tr>
<td>Run</td>
<td>Set of up to 2 series by experimenter conducted with the same tissue batch within the same day.</td>
</tr>
<tr>
<td>RhT</td>
<td>Reconstructed human Tissue</td>
</tr>
<tr>
<td>RT</td>
<td>RoomTemperature</td>
</tr>
<tr>
<td>Series</td>
<td>a set of up to 13 test chemicals plus Negative Control (NgC) and Positive Control (PC) all concurrently tested on at least 2 tissues replicates.</td>
</tr>
<tr>
<td>Test</td>
<td>A test chemical tested on at least two tissue replicates when the cytotoxic effect is quantitatively measured by using the MTT assay. A reported technical issue before the viability measurement is not considered as a “Test” for the test chemical.</td>
</tr>
<tr>
<td>TT</td>
<td>TestTreatment</td>
</tr>
<tr>
<td>ULOQ</td>
<td>Upper Limit Of Quantification</td>
</tr>
<tr>
<td>UN GHS</td>
<td>United Nations Globally Harmonized System</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-high Performance Liquid Chromatography</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
</tr>
</tbody>
</table>

_Last update: 16 October 2017_
SkinEthic™ HCE Eye Irritation Test Solid (EITS)
DB-ALM Protocol n° 191

Quick flow chart

The experimental procedures for the in vitro SkinEthic™ HCE EITS protocol are briefly outlined here below. A detailed description of the different steps is available in the following sections of this protocol.

Receipt: Transfer epithelium from agarose to maintenance medium in 6-well plate (1 mL Tissue maintenance medium/well)

↓

Culture inserts equilibration period: Incubate at least overnight
(37±2°C, 5±1% CO₂, ≥ 90% humidity)

↓

Tissue conditioning: Transfer tissues to fresh maintenance medium in the 6-well plate
1 plate/test items (2 tissues/plate)
(used the same plate for Receipt, Treatment, Post-incubation periods)
Incubate at least 30 min (37±2°C, 5±1% CO₂, ≥ 90% humidity)

↓

Treatment: 2 tissues each with 30±2 µL PBS without Ca²⁺ & Mg²⁺ (Negative Control)
or 30±2 µL methyl acetate (Positive Control)
or 30 µL PBS without Ca²⁺ & Mg²⁺ (PBS-)+ 30±2 mg test chemical

↓

Treatment Period: Incubate for 4 hours ± 5 min (37±2°C, 5±1% CO₂, ≥ 90% humidity)

↓

Rinse with PBS- (25 mL: 2 mL/ jet)

↓

Post-soak Immersion: Immerse tissues in 4 mL fresh maintenance medium, in 12-well plates

↓

Post-soak Period: Incubate for 30±2 min at room temperature (RT)

↓

Transfer tissues to fresh maintenance medium in the same 6-well plates

↓

Post-incubation Period: Incubate for 18 hrs ± 30 min (37±2°C, 5±1% CO₂, ≥ 90% humidity)

↓

Viability: Transfer tissues into MTT solution (1 mg/mL) in 24-well plates (300µL MTT/well)

↓

Incubate tissues for 3 hours ± 15 min (37±2°C, 5±1% CO₂, ≥ 90% humidity)

↓

Extraction: Place the inserts on 1.5 mL isopropanol in 12-well plates

↓

Extract formazan at least 2 hours at RT

↓

Remove the insert and homogenize formazan extract

↓

Read OD with microplate spectrophotometer at 570±30 nm
and/or analyse the extract samples by HPLC/UPLC spectrophotometry
Contact Details

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Materials and Preparations

Cell or Test System

The SkinEthic™ HCE tissues are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm²) with the necessary culture media (maintenance medium). The SkinEthic™ medium and SkinEthic™ HCE tissue model are provided by Episkin (France) with related technical documentation (www.episkin.com).

<table>
<thead>
<tr>
<th>Description of the kit</th>
<th>Comment</th>
<th>Storage conditions</th>
<th>Shelf life</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkinEthic™ HCE units (0.5 cm²)</td>
<td>Tissues are shipped on semi solid agar’s medium in order to maintain good shipment conditions for the tissues</td>
<td>RT</td>
<td>see technical data sheet</td>
</tr>
<tr>
<td>SkinEthic™ Maintenance Medium</td>
<td>Culture medium for incubations</td>
<td>Fridge</td>
<td>see technical data sheet</td>
</tr>
</tbody>
</table>

Store the SkinEthic™ HCE tissues at room temperature until their transfer into SkinEthic™ maintenance medium. Store the SkinEthic™ maintenance medium in the fridge and pre-warm only at room temperature (RT).
## Equipment

### Fixed Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiological safety cabinet (laminar flow hood)</td>
<td>safe work under sterile conditions</td>
</tr>
<tr>
<td>Non-sterile ventilated cabinet</td>
<td>safe work with test chemicals, applications, washes</td>
</tr>
<tr>
<td>Cell incubator 37±2°C, 5±1% CO₂, ≥ 90% humidity</td>
<td>tissues incubations</td>
</tr>
<tr>
<td>Plate reader (96 well) with a 570± 30 nm wavelength</td>
<td>Optical Density readings (MMT formazan)</td>
</tr>
<tr>
<td>Laboratory balance (accuracy 0.1 mg)</td>
<td>test chemicals weighing</td>
</tr>
<tr>
<td>Shaker plates</td>
<td>shaking before reading (formazan extraction sample)</td>
</tr>
<tr>
<td>HPLC/UPLC-UV/Visible spectrophotometry</td>
<td>Performance Liquid Chromatography readings (MMT formazan)</td>
</tr>
</tbody>
</table>

### Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 sterile bottle</td>
<td>reconstituting MTT reagent stock solution</td>
</tr>
<tr>
<td>1 sterile bottle</td>
<td>diluting MTT in assay medium</td>
</tr>
<tr>
<td>1 glass funnel</td>
<td>dropping wash fluids in the bottle</td>
</tr>
<tr>
<td>Wash bottle (500 mL)</td>
<td>collecting wash fluids</td>
</tr>
<tr>
<td>Adjustable multi-step pipette, 25 mL</td>
<td>distributing 1 mL maintenance medium and PBS</td>
</tr>
<tr>
<td>Adjustable multi-step pipettes, 5 mL</td>
<td>distributing maintenance medium, MTT, Isopropanol and PBS</td>
</tr>
<tr>
<td>Adjustable micro-pipette – 0 to 200 µL</td>
<td>pipetting 200 µL formazan extracts</td>
</tr>
<tr>
<td>Adjustable positive displacement micro-pipette 0- 50 µl</td>
<td>application of 30 µL</td>
</tr>
<tr>
<td>Stop-watches/Timers</td>
<td>controlling contact and step times</td>
</tr>
<tr>
<td>Small clean blunt-edged forceps</td>
<td>handling tissue inserts</td>
</tr>
<tr>
<td>Spatula</td>
<td>weighing powder</td>
</tr>
<tr>
<td>Small glass weight boat</td>
<td>weighing powder</td>
</tr>
<tr>
<td>Mortar and pestle</td>
<td>grinding granular</td>
</tr>
<tr>
<td>96-well plates</td>
<td>reading Optical Density</td>
</tr>
<tr>
<td>12-well plates</td>
<td>post soak and IP extraction</td>
</tr>
<tr>
<td>24-well plates</td>
<td>MTT incubation</td>
</tr>
<tr>
<td>6-well sterile plates</td>
<td>transfer tissue inserts upon receipt treatment, post incubation</td>
</tr>
<tr>
<td>“Parafilm&quot;</td>
<td>covering plates during formazan extraction</td>
</tr>
<tr>
<td>cotton tip swabs</td>
<td>drying the tissue surface</td>
</tr>
</tbody>
</table>
Nylon mesh Ø=7.5 mm
(sefar Nitex 03-150/38 or equivalent) for sticky or powder difficult to apply

HPLC/UPLC vial
HPLC/UPLC reverse phase column

<table>
<thead>
<tr>
<th>Media, Reagents, Sera, others</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Item</strong></td>
</tr>
<tr>
<td>Isopropanol (CASRN 67-63-0)</td>
</tr>
<tr>
<td>MTT reagent (3-4,5-dimethyl thiazole 2-yl)2,5-diphenyltetrazolium bromide(CASRN 298-93-1, Sigma M2128 or equivalent)</td>
</tr>
<tr>
<td>Dulbecco’s D-PBS without Ca²⁺ &amp; Mg²⁺ GIBCO 14190-144 or equivalent (PBS⁻)</td>
</tr>
<tr>
<td>Tissue maintenance medium (SkinEthic™)</td>
</tr>
<tr>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>Solvents HPLC/UPLC grade</td>
</tr>
<tr>
<td>Formazan (CASRN 57360-69-7); purity &gt;97% (Sigma 88417 or equivalent)</td>
</tr>
<tr>
<td>Methyl acetate (CASRN 79-20-9); purity &gt;99% (Sigma 45999 or equivalent)</td>
</tr>
</tbody>
</table>

**Preparations**

*Media and Endpoint Assay Solutions*

**MTT stock solution preparation**
- Prepare a 5 mg/mL MTT solution in PBS-.
- Thoroughly mix this stock solution during 15±2 minutes at RT.
- Keep in the fridge (2 to 8°C) protected from light up to 16 days.

**MTT ready to use solution preparation**
- Pre warm maintenance medium at RT
- Dilute MTT stock solution preparation 1/5 with maintenance medium (1v+4v, final concentration: 1 mg/mL)
- Keep at RT, protect from light until use (do not exceed 3 hours storage)

*Note:* MTT solution is light sensitive. Protect it from light using foil.

The culture medium (maintenance medium) is delivered with the SkinEthic HCE tissues; it is store in the fridge. All these solutions and media are prepared or open under a safety cupboard.

**Test Compounds**

*Preparation of test chemicals*

Solid test chemicals (even granular powder) should be crushed to a very fine powder, if necessary, using a mortar and a pestle.

*Application of test chemicals*

The test chemical (± color, ± MTT reducer) is topically applied onto HCE tissues. For detailed experimental setup see the section "Test Material Exposure Procedures" on p.16.

**Prior to routine use of the SkinEthic™ HCE EITS protocol for regulatory purposes,** as recommended in OECD Test Guidelines 492, laboratories should demonstrate technical proficiency by correctly predicting the seven proficiency solid chemicals (see Table 1 in the following page).
### Table 1. List of proficiency solid chemicals

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>CAS RN</th>
<th>Organic Functional Group ¹</th>
<th>Physical State</th>
<th>Viability (%) ²</th>
<th>Prediction</th>
<th>MTT Reducer</th>
<th>Colour Interfer.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vivo Category 1</strong> ³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,5-Dimethyl-2,5-hexanediol</td>
<td>110-03-2</td>
<td>Alcohol</td>
<td>S</td>
<td>0.2±0.1</td>
<td>No prediction can be made</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Sodium oxalate</td>
<td>62-76-0</td>
<td>Oxocarboxylic acid</td>
<td>S</td>
<td>5.3±4.1</td>
<td>No prediction can be made</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>In Vivo Category 2A</strong> ³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>532-32-1</td>
<td>Aryl; Carboxylic acid</td>
<td>S</td>
<td>0.6±0.1</td>
<td>No prediction can be made</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>In Vivo Category 2B</strong> ³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2-Dimethyl-3-methylene bicyclo [2.2.1] heptane</td>
<td>79-92-5</td>
<td>Alkane, branched with tertiary carbon; Alkene; Bicycloheptane; Bridged-ring carbocycles; Cycloalkane</td>
<td>S</td>
<td>15.8±1.1</td>
<td>No prediction can be made</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>In Vivo No Category</strong> ³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea</td>
<td>101-20-2</td>
<td>Aromatic heterocyclic halide; Aryl halide; Urea derivatives</td>
<td>S</td>
<td>101.9±6.6</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)</td>
<td>103597-45-1</td>
<td>Alkane branched with quaternary carbon; Fused carbocyclic aromatic; Fused saturated heterocycles; Precursors quinoid compounds; tert-Butyl</td>
<td>S</td>
<td>97.7±5.6</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Potassium tetrafluoroborate</td>
<td>14075-53-7</td>
<td>Inorganic Salt</td>
<td>S</td>
<td>92.9±5.1</td>
<td>No Cat</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

**Abbreviations:**

CASRN = Chemical Abstracts Service Registry Number; UN GHS = United Nations Globally Harmonized System of Classification and Labelling of Chemicals; Colour interf. = colour interference with the standard absorbance (Optical Density (OD)) measurement of MTT formazan; S=solid.

¹ Organic functional group assigned according to an OECD Toolbox 3.1 nested analysis ([https://www.qsartoolbox.org/](https://www.qsartoolbox.org/)).

² Based on results obtained with SkinEthic™ HCE EITS in the validation study (Alépéé et al,2016a).

³ Based on results from the in vivo rabbit eye test (OECD TG 405) and using the UN GHS.
Positive Control(s)
Methyl acetate is used as Positive Control (PC).

Negative Control(s)
Phosphate Buffer solution without Ca\(^{2+}\) & Mg\(^{2+}\) (PBS -) is used as Negative Control (NgC).

**Note.** The negative and positive controls correspond to the quality control named viability in OECD TG 492 that must be performed for each run by the user. This data is not provided by the tissue supplier.

The following paragraphs describe the procedures to check direct MTT reduction and/or color interfering of testing chemicals prior to experiments. An illustrative flowchart providing guidance and summarising the steps to follow to identify and handle direct MTT-reducers and/or color interfering chemicals is provided as Annex 4 on page 30.

### Checking for direct MTT reduction of test chemicals (Annex 4)

Relative conversion of MTT by the tissue is the parameter evaluated in this assay, therefore it is necessary to assess the non-specific reduction of MTT by the test chemicals used. **Prior to experiments, test chemicals** should be put in contact with the MTT solution as described below.

- **When Optical Density (OD) is chosen as endpoint for viability assessment**

  This verification might be performed once before starting the experiment (ideally the week before the study).

  To identify this possible interference, each test chemical is checked for its ability to reduce MTT without tissue (step 1). In case of identified MTT interaction, proceed to step 2.

  **Step 1:**
  - Fill tubes or a 24-well plate with 30±2 mg of the test chemical to be evaluated or water for control.
  - Add 300 µL of MTT solution (1 mg/mL) and mix.
  - Incubate the mixture for 3 hours ± 15 minutes at 37±2°C, 5±1% CO\(_2\), ≥ 90% humidity protected from light (test conditions).

  If the MTT solution color turns blue or purple, the test chemical interacts with the MTT (see picture on the left).

  It is then necessary to evaluate during the future studies the part of OD due to the non-specific reduction of the MTT (i.e. by using killed epithelium tissues) to define the %NSMTT (Non-Specific reduction of the MTT) value.

  A: control  
  B: test chemical 1: no interaction  
  C: test chemical 2: slight interaction  
  D: test chemical 3: strong interaction

  **Step 2:**
  - Use killed tissues that possess no metabolic activity but can absorb and bind the test chemical like viable tissues (see page 16 for more details).
  - Each MTT interacting test chemical is applied onto at least two killed tissues using this EITS protocol. In addition to that, at least two killed tissues are treated with PBS- as control (negative control killed tissue, KU).
  - For details see Figure 1 (condition 2), p.13.

The evaluation of direct MTT reduction of test chemical (steps 1 and 2 described above) is performed only on one occasion (one single run even if additional runs are required to classify the test chemical).

- The Evaluation of Test chemical and MTT direct interaction has to be documented using the documentation sheet in Annex 1, p.27.
- Report systematically the part of OD due to the non-specific reduction of the MTT (to define the %NSMTT value for a MTT-reducing test chemical) for a test chemical before calculating the final viability (see specific calculation under “Data Analysis” section, p.21).
• **When HPLC/UPLC-spectrophotometry is chosen as endpoint for viability assessment**

The same procedure as for OD measurement (see paragraph above on this page) is followed to evaluate the direct MTT reduction of test chemicals.

### Checking for color test chemicals only (Annex 4)

• **When OD is chosen as endpoint**

Colored test chemicals or test chemicals able to develop a color after contact with the tissue can generate a remaining Non Specific Color (%NSC<sub>living</sub>). Therefore, each test chemical has to be checked for its colorant properties. Indeed, test chemicals that appear red, blue, black and green by absorbing light should be potentially considered as intrinsic colorants.

- □ Adapted controls should always be included for colored solid test chemicals.
- □ For uncolored solid test chemicals, this possible interference should first be checked (step 1) before deciding to include adapted controls (step 2).

Specific controls must be used in these cases consisting of test chemical-treated tissues that followed all the steps of the method except the MTT incubation. %NSC<sub>living</sub> is determined after isopropanol extraction and OD reading in similar conditions (see specific calculation under “Data Analysis” section, from p.21)

![Diagram](image)

**Note:** Orange is an example. A coloring test chemical can have of course another color.

**Step 1:**
- Fill Eppendorf tubes with 10±2 mg of the test chemical to be evaluated.
- Add 90 µL of water.
- Vortex the solution for a few seconds.
- Incubate the solution for 30±2 min at RT.
- Perform a direct visual observation (see picture on the left).

When a colored solution is observed, the tissue staining ability of the test chemical should be checked (step 2), otherwise no adapted controls are required.

It is then necessary to evaluate during the study(ies) the part of OD due to the non-specific color (i.e. by using living epithelium tissues without MTT conversion test) to define the %NSC<sub>living</sub> value (see step 2 below).

**Step 2:**
- The Non-Specific Color (%NSC<sub>living</sub>) is quantified by using at least 2 living tissues per chemical. For details Figure 1 (condition 3), p.13.
- Coloring test chemical controls are treated and handled like normal treated tissues except that they do not get into contact with the MTT solution as they are incubated in maintenance medium.

**A:** Control  
**B:** Test chemical 1: no color  
**C:** Slight coloration of an orange Test chemical  
**D:** Strong coloration of an orange Test chemical

The visual possible interference should be checked once (step 1). In case the test chemical has a potential to color the tissue, possible interference (adapted controls, step 2) should be checked in parallel to the evaluation of a test chemical.

© EURL ECVAM DB-ALM: Protocol
An independent %NSC\textsubscript{living} control needs to be conducted for each test performed (concurrently to every testing; i.e. for each time in each run).

- The Evaluation of Test chemical and color direct interaction has to be documented using the documentation sheet in Annex 2, p.28.
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration (to define the %NSC\textsubscript{living} value), for a test coloring chemical before calculating the final % viability (see specific calculation under "Data Analysis" section, from p.21).

- **When HPLC/UPLC-spectrophotometry is chosen as endpoint:**

  No pre-check or control are necessary.

**Checking for color test chemicals with possible MTT direct interaction (Annex 4)**

- **When OD is chosen as endpoint:**

  The test chemical intrinsic color can, in some cases, interfere with the MTT formazan extraction readings. Blue, dark purple and black test chemical may be directly tested on colorant controls without additional checking test due to their high probabilities to interfere with the blue MTT (formazan salt).

  In that case, if the color of the test chemical interferes with the MTT pre-check, an additional adapted control is needed. Each coloring test chemical is applied onto at least two killed tissues and incubated in maintenance medium instead of MTT solution to determine the Non-Specific Color on killed tissues (%NSC\textsubscript{killed}) (see Figure 1, condition 4 on p.13).

  The evaluation of %NSC\textsubscript{killed} is performed only on one occasion (one single run even if additional runs are required to classify the test chemical).

  - Results are documented using the documentation sheets available as Annex 1, p.27 and Annex 2, p.28.
  - Report systematically and concurrently to every testing the part of OD due to the non-specific coloration on killed tissues (to define the %NSC\textsubscript{killed} value for a coloring MTT-reducer test chemical) before calculating the final % viability (see specific calculation under "Data Analysis" section, from p.21)

- **When HPLC/UPLC-spectrophotometry is chosen as endpoint:**

  Colored test chemicals or test chemicals that become colored in contact with water or isopropanol that interfere too strongly with the MTT-reduction assay may still be assessed using HPLC/UPLC-spectrophotometry instead of standard absorbance (OD).

  As this analytical method allows the separation between MTT formazan and test chemical, NSC controls (%NSC\textsubscript{living} or %NSC\textsubscript{killed}) are never required.

  Based on this separation capacity of HPLC/UPLC system, two distinct peaks could be generated. In case of overlapping pattern, an alternative separation method should be considered. Evaluation of direct MTT reduction will be performed to define the %NSMTT (at least two killed tissues with MTT incubation). See Figure 2, p.14.
Figure 1. Summary of adapted controls choice depending of test chemical physical properties (when OD method is chosen)

Case by case test conditions for OD reading

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
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<tbody>
<tr>
<td>MTT interaction</td>
<td>Coloration interference</td>
<td>Test conditions</td>
<td>Final Corrected Viability</td>
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<td>%TT - %NSMTT</td>
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<td>1 + 3</td>
<td>%TT - %NSliving</td>
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<td>1 + 2 + 3 + 4</td>
<td>%TT - %NSMTT - %NSliving - %NSkilled</td>
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</table>

Results for test chemicals producing %NSMTT and/or %NSCliving and/or %NSCkilled ≥ 50% of the negative control should be taken with caution.
Figure 2. Summary of adapted controls choice depending of test chemical physical properties (when HPLC/UPLC-spectrophotometry method is chosen)

Case by case test conditions for HPLC/UPLC-spectrophotometry endpoint

<table>
<thead>
<tr>
<th></th>
<th>MTT interaction</th>
<th>Coloration interference</th>
<th>Test conditions</th>
<th>Final Corrected Viability</th>
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<tr>
<td>Case 1</td>
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<td>- or +</td>
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<td>%TT</td>
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<tr>
<td>Case 2</td>
<td>+</td>
<td>- or +</td>
<td>1 + 2</td>
<td>%TT - %NSMTT</td>
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</table>

Results for test chemicals producing %NSMTT ≥ 50% of the negative control should be taken with caution.
Method

The Good Laboratory Practices with adherence to laboratory testing standards should be applied upon the need.

Test System Procurement

The SkinEthic™ HCE tissues are produced by Episkin (France) (www.episkin.com) and they are provided with the necessary maintenance medium. Each SkinEthic™ HCE tissue is controlled by the manufacturer. The human-derived HCE cells are free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the reconstructed tissue is checked by the supplier for absence of contamination by fungi and bacteria.
For the SkinEthic™ medium and SkinEthic™ HCE tissue model refer to the Technical Data and Certificate of Analysis provided by the supplier. Once received examine all kit components for integrity. If there is a question, a concern or something unusual, call + 33 (0) 4 37 28 22 00, sales@episkin.com.

Routine Culture Procedure

After reception and before proceeding to treatment steps (as described under the section "Test material exposure procedures" on p.16) SkinEthic™ HCE tissues are prepared as follows.

Reception

- Upon receipt of the epithelium kits, pre-warm maintenance medium at RT (if necessary) and transfer each epithelium from their transport packaging plate to 6-well plates containing 1 mL maintenance medium per well. Note: The maintenance medium should be pre-warmed only at room temperature (and not at 37°C).
- Verify the absence of air bubbles below tissues and incubate them at least overnight at 37±2 °C, 5±1% CO₂, ≥ 90% humidity until treatment steps. Note: One plate is used for each test chemical, 2 wells per plate.

Tissue conditioning

- Pre-warm the maintenance medium at RT.
- Before test chemical treatment, label the plate lid with the appropriate test chemical information.
- Dispense 1 mL of pre-warmed maintenance medium into the second and the third column of the 6-wells plate: the second column is for 4 hrs application’ step and the third column for the 18 hrs post incubation step (see example of "Application/post incubation plate design" below).
- Transfer 2 SkinEthic™ HCE epithelia units into the 2 first wells filled with 1 mL maintenance medium (at least 2 replicate tissues per test chemical).
- Verify the absence of air bubbles below the tissues.
- Incubate the plates at 37±2°C, 5±1% CO₂, ≥ 90% humidity at least 30 min.

Application/post incubation plate design

Two SkinEthic™ HCE tissues are used per chemical (Negative Control (NgC), Positive Control (PC), or test chemical).
- Only 1 chemical per plate
- The plate will be used for tissue conditioning, application and post incubation steps of the process.
Remark. In order to avoid cross contaminations between test chemicals leading to possible misclassifications in final results, only 1 chemical (NgC, PC, or test chemical) must be run per plate. The same plate will be used from tissue conditioning, application and post-incubation steps. Start with NgC, PC and then the test chemicals.

**Water-killed epithelium preparation**

Killed HCE tissues (also indicated as killed tissues) are used for MTT-interacting chemicals (p. 10) or MTT pre-check inconclusive chemicals due to color (p.12).

To prepare killed HCE tissues:

- Place the living epithelium in a 24-well plate pre-filled with 300 µL of distilled water.
- Incubate at 37±2°C, 5±1% CO₂, ≥ 90% humidity atmosphere for 24±1 hr.
- At the end of the incubation, discard the water.
- Keep killed epithelium frozen (dry) in freezer (-18 to -22°C) (*killed epithelium can be stored and used up to 6 months*).
- Tissues should be de-frozen before use at room temperature (at least 10 minutes) in 1 mL maintenance medium in 6-well plates.
- Further use of thawed killed HCE tissues is similar to living HCE tissues.
- Apply negative control and test chemical on killed tissues from the same batch.
- Proceed similarly to living tissues for application, rinsing, post-soak, etc.
- Perform this assay only once (on at least duplicate tissues) per test chemical when necessary.

**Test Material Exposure Procedures**

**Tissue treatment**

*Negative control (PBS-), positive control (Methyl Acetate) and test chemicals applications*

- For NgC, dispense 30±2 µL of PBS- directly topically onto the tissue, always using a positive displacement pipette. Gently spread if necessary on the epithelium surface.
- For PC, dispense 30±2 µL of PC directly topically onto the tissue, always using a positive displacement pipette. Gently spread if necessary on the epithelium surface.
- For test chemical, pre-moisten the tissues with 30±2 µL of PBS-.
- Remove the tissue from the 6-well plate and place onto a dish of weighting to avoid any contamination of the maintenance medium by the test chemical during its disposal.
- Dispense 30 mg ± 2 mg directly topically onto the tissue.
- Spread it on the epithelium surface without touching it and shake insert gently from side to side to ensure that the tissue is completely covered by the test chemical. Alternatively, pipette tip can be used to homogeneously cover the surface of the tissue with test chemicals.
- Treat tissues at adapted time intervals according to the necessary rinsing-off intervals, i.e. 60 seconds, to be adapted depending on the operator experience.
- Place the tissue, in the second column (see an example of Plate design on p.15), containing 1 mL maintenance medium.
- Verify the absence of air bubbles below tissues and incubate treated tissues for 4±0.1 hours at 37±2°C, 5±1% CO₂, ≥ 90% humidity with lids on.

Record time and details in the documentation sheet (*Annex 3*, p.29), as well as the weighting step.
End of treatment and rinsing procedure

- At the end of the exposure (4±0.1 hrs), the test chemical (and controls) will be removed by rinsing tissues with PBS - . Tissues are rinsed by using a 25 mL adapted multi-pipette. Adjust the distribution to 2 mL per push.
- Maintain the insert over a glass funnel with forceps (to collect the wash fluids in the wash bottle).
- Rinse with 25 mL of PBS - (at 5 to 8 cm distance from the tissue, directing to the wall of the insert in order to have a gentle action on the surface of the tissues) in order to remove the residual test chemical from the tissue surface. Use ever more the vortex movement to wash the tissue.
- Remove remaining PBS - onto the tissue by energised reversals. Do not use cotton swab to dry the tissues at this step.
- For tissues on which the test chemical has not been removed by standard washing procedure with PBS - , an alternative option is proposed. Fulfil insert with PBS - onto the tissue and use cotton swabs to gently remove any residual test chemical avoiding contact with tissue.

Post-Soak Period

- After rinsing, transfer immediately the rinsed tissue to a 12-well plate containing 4 mL of fresh pre-warmed maintenance medium.
- Immerge the tissue.
- Verify the absence of air bubbles below tissues and incubate the tissues for 30±2 minutes at RT. Note: This post-soak tissue immersion is intended to remove any test chemical inside the tissue.
- At the end of the post-soak immersion, each tissue will be removed from the maintenance medium. The medium will be decanted off the tissue by returning the insert.
- Carefully dry the bottom of the insert by gently taping on a dry absorbent paper (Figure A) and the surface with a cotton swab (Figure B).

Document this step using documentation sheet in Annex 3, p.29.

Post-Incubation Period

- Place the tissue, in the third column of a 6-well plate (see example of Plate design p.15), containing 1 mL maintenance medium.
- Verify the absence of air bubbles below tissues.
- incubate the tissues for 18 hours ± 30 min at 37±2°C, 5±1% CO₂, ≥ 90% humidity.

MTT conversion test

The MTT conversion test is carried out at the end of the 18 hours ± 30 min post-incubation period. The results of this test are documented using the documentation sheet in Annex 3, p.29.

Note: Additional specific tissue controls for coloring test chemicals (%NSC living ± %NSC killed) will be incubated with the maintenance medium (see Figure 1 on p.13, conditions 3 and 4).

- Prepare MTT medium according to "Preparation section" on p.8.
- In a pre-labelled 24-well plate, dispense 300 µL of MTT solution (1 mg/mL freshly prepared in maintenance medium) in 2 wells per test chemical (Conditions 1 and 2).
- For the specific coloring controls, dispense 300 µL of maintenance medium instead of MTT medium (Conditions 3 and 4).
- Remove remaining maintenance medium (from post-incubation step) below the tissue from the...
Transfer the tissues in a 24-well pre-labelled plate containing the MTT-containing wells (or maintenance medium for coloring chemical).

- Verify the absence of air bubbles under the tissues.
- Incubate the tissues for **3 hours ± 15 minutes** 37±2°C, 5±1% CO₂, ≥ 90% humidity.
- Record starting time of MTT incubation (Annex 3, p.29).
- After the 3 hrs ± 15 min incubation, perform a quick contact of the tissue with dry absorbent paper.
- Place the inserts in **300 µL PBS** (in 24-well plates) to remove the excess of MTT solution (for **conditions 1 and 2**) or maintenance medium (for **conditions 3 and 4**).
- Record ending time of MTT/Maintenance medium incubation (Annex 3, p.29).

**Formazan extraction.**

- Perform a quick contact of the tissue with dry absorbent paper.
- Pre-label 12-well plate containing **1.5 mL isopropanol**. To minimize any potential contamination of the isopropanol extraction solution with test chemical that may have remained on the tissue or with strongly colored test chemical, tissues should be extracted from the bottom only.
- During extraction, plates should be covered with “parafilm” to prevent evaporation. At least a layer of parafilm under the lid should be used (usually 3 layers: 2 layers on top of the wells under the lid. Put the lid on and cover with the third sheet of parafilm around the lid and plate; see Figure C and D below).
- Extract the MTT formazan crystals protected from light with gentle shaking on plate shaker (~ 120 rpm) for **2-3 hours at RT**.
- Remove the inserts from the 12-well plate.
- Homogenize the extraction solution vigorously up and down until a homogeneous solution is reached.

**Example of Formazan extraction - plate design.**

- Two SkinEthic™ HCE tissues are used per chemical (NgC, PC, or test chemical).
- Up to 6 chemicals per plate.

![Figure C](image1.png)

![Figure D](image2.png)
Endpoint Measurement

Optical Density (OD) endpoint measurement

For each tissue, transfer 200 µL / well of the formazan solution extract (i.e. 1.5 mL extraction solution) into two wells (2 x 200 µL) of a 96-well flat bottom microtiter plate (see examples below of plates’ design).

For Conditions 1 and 3:

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NgC= negative control; PC= positive control; TT1...TT13= Test treatment 1...13; BL= blank (isopropanol 100%)

For Conditions 2 and 4:

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</table>

KU= negative control killed tissue; TT1...TT13= Test treatment 1...13 on killed tissues; BL= blank (isopropanol 100%)

Note. Be careful of Isopropanol evaporation in 96-well plates: It is recommended to pool several test chemicals per plate but with a maximum of 60 wells filled per plate and to make the readings without delay in the same run (see example above). Moreover the filling time should not exceed 20 min.

- Read Optical Density (OD) by using a spectrophotometer microtiter plate reader equipped with a 570±30 nm, without using a reference filter.
- Use isopropanol as blank (200 µL / well).
- Link OD values with the appropriate treatment conditions and replicate on the raw data documents (or files).
- Perform the Quality Control of the raw data and adapt archiving upon needs.
**HPLC/UPLC-spectrophotometry endpoint measurement**

- **For negative control only**: transfer 200 µL/well of the formazan solution extract (i.e. 1.5 mL extraction solution) into two wells (2 x 200 µL) of a 96-well flat bottom microtiter plate and read Optical Density (OD) at 570±30 nm wavelength.

- Use isopropanol as blank (200 µL/well).

- **For all conditions included negative control**: transfer at least 100 µL into an HPLC/UPLC vial (samples can also be frozen 2 months maximum).

- Use a validated analytical method on a qualified HPLC/UPLC-UV/Visible system see documentation on validation of analytical method available in the Downloads section of this protocol on DB-ALM website.

- Measure peak area at the retention time of the Formazan at the wavelength defined in the validated analytical method.

- Link Area values with the appropriate treatment conditions and replicate on the raw data documents (or files).

- Perform the Quality Control of the raw data and adapt archiving upon needs.

**Acceptance Criteria**

**Common acceptance criteria**

- The mean Optical Density (OD$_{\text{NgC}}$) at 570±30 nm of the two replicate tissues treated with negative control should be $> 1.0$ with an upper acceptance limit of $\leq 2.5$.

- The acceptance criteria of the negative control by absorbance (optical density) should be met for both endpoints even if HPLC/UPLC-spectrophotometry is chosen as the endpoint measurement.

- The difference of viability between the two replicate tissues of a single test chemical should be $\leq 20\%$ in the same run whatever the test item (for NgC, PC, test chemical and all adapted controls).

- The PC data meet the acceptance criteria if the mean viability, expressed as % of the NC, is $\leq 20\%$ and the difference value is $\leq 20\%$.

- The acceptance criteria of the NgC and PC should be met for interpreting the test chemical data.

- For a given test chemical, a single testing run composed of 2 tissue replicates should be sufficient when the classification is unequivocal and if the difference value is $\leq 20\%$. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50 ± 5%, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

**Specific HPLC acceptance criteria**

Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (US FDA, 2001; Alépée et al., 2015).

These key parameters and their acceptance criteria are shown in the document "Validation of an analytical method on a HPLC/UPLC-spectrophotometry system" available in the Downloads section of this protocol on DB-ALM website.

Once the acceptance criteria defined in the document (see Downloads) have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this protocol.

A run is qualified if both the NgC and PC data fulfil the acceptance criteria requirements. Otherwise, the run will be considered as non-qualified. Non-qualified runs have to be documented and reported.

A single testing run composed of at least two tissue replicates should be sufficient for a test chemical when the resulting classification is unequivocal (independently of the endpoint: OD or HPLC/UPLC spectrophotometry).

However, in cases of borderline results, such as non-concordant replicate measurements, a second run may be considered, as well as a third one in case of discordant results between the first two runs.
Data Analysis

Main data calculation steps:

(a) Blanks: calculate the mean OD of isopropanol 100% from the 8 wells for each 96-well plate.

(b) Negative PBS-treated controls (NgC): subtract blank mean value from individual tissues ODs (2 values from each of two tissues). Calculate the mean OD for each individual tissue. Corrected mean OD for the 2 tissues corresponds to 100% viability.

(c) Positive control (PC): subtract blank mean value from individual tissues ODs (2 values from each of two tissues). Calculate the mean OD for each individual tissue.

(d) Test chemical: subtract blank mean value from individual tissues ODs (2 values from each of two tissues). Calculate the mean OD for each individual tissue.

(e) Viability %: calculate for each treated epithelium the percentage of viability relative to the mean OD of negative control. Calculate viability mean values for each test chemical.

(f) Variability for each test: calculate the viability difference between the two tissues replicates (for NgC, PC and test chemical).

The calculation procedures to follow under different conditions and according the analytical method chosen (see Figure 1, p.13 and Figure 2, p.14 for summary) are described in details in the following paragraphs.

For viability Tests only – Normal calculation procedure (Condition 1)

A. Calculation for OD reading

● MEAN OD CALCULATION

*Negative Control (NgC)

Individual OD Negative Control (NgC)

Mean OD Negative Control

\[\text{OD}_{\text{NgC}} = \frac{\text{OD}_{\text{NgC1}} + \text{OD}_{\text{NgC2}}}{2}\]

The mean OD of the two Negative Control replicates (PBS- treated) corresponds to 100% reference viability.

*Positive Control (PC)

OD Positive Control (PC)

Mean OD Positive Control

\[\text{OD}_{\text{PC}} = \frac{\text{OD}_{\text{PC1}} + \text{OD}_{\text{PC2}}}{2}\]

*Test Treatment

OD Test Treatment (TT)

Mean OD Test Treatment

\[\text{OD}_{\text{TT}} = \frac{\text{OD}_{\text{TT1}} + \text{OD}_{\text{TT2}}}{2}\]

● VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control1
% Negative Control2
% mean Negative Control

\[\%\text{NgC1} = \frac{\text{OD}_{\text{NgC1}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{NgC2} = \frac{\text{OD}_{\text{NgC2}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{NgC} = \frac{\%\text{NgC1} + \%\text{NgC2}}{2}\]

% Positive Control1
% Positive Control2
% mean Positive Control

\[\%\text{PC1} = \frac{\text{OD}_{\text{PC1}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{PC2} = \frac{\text{OD}_{\text{PC2}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{PC} = \frac{\%\text{PC1} + \%\text{PC2}}{2}\]

% Test Treatment 1
% Test Treatment 2
% Mean Test Treatment

\[\%\text{TT1} = \frac{\text{OD}_{\text{TT1}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{TT2} = \frac{\text{OD}_{\text{TT2}}}{\text{mean OD}_{\text{NgC}}} \times 100\]
\[\%\text{TT} = \frac{\%\text{TT1} + \%\text{TT2}}{2}\]
B. Calculation for HPLC/UPLC - spectrophotometry endpoint

● MEAN OD CALCULATION

*Negative Control (NgC)
Mean Area Negative Control
Area\textsubscript{NgC} = \frac{[\text{Area}\textsubscript{NgC1} + \text{Area}\textsubscript{NgC2}]}{2}

The mean Area of the two Negative Control replicates (PBS - treated) corresponds to 100% reference viability.

*Positive Control (PC)
Mean Area Positive Control
Area\textsubscript{PC} = \frac{[\text{Area}\textsubscript{PC1} + \text{Area}\textsubscript{PC2}]}{2}

*Test Treatment
Mean Area Test Treatment
Area\textsubscript{TT} = \frac{[\text{Area}\textsubscript{TT1} + \text{Area}\textsubscript{TT2}]}{2}

● VIABILITY CALCULATION: Individual means viabilities (%)

\% Negative Control\textsubscript{1}
\% Negative Control\textsubscript{2}
\% mean Negative Control
\%NgC\textsubscript{1} = \frac{[\text{Area}\textsubscript{NgC1}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%NgC\textsubscript{2} = \frac{[\text{Area}\textsubscript{NgC2}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%NgC = \frac{(%\%NgC\textsubscript{1} + %\%NgC\textsubscript{2})}{2}

\% Positive Control\textsubscript{1}
\% Positive Control\textsubscript{2}
\% mean Positive Control
\%PC\textsubscript{1} = \frac{[\text{Area}\textsubscript{PC1}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%PC\textsubscript{2} = \frac{[\text{Area}\textsubscript{PC2}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%PC = \frac{(%\%PC\textsubscript{1} + %\%PC\textsubscript{2})}{2}

\% Test Treatment \textsubscript{1}
\% Test Treatment \textsubscript{2}
\% Mean Test Treatment
\%TT\textsubscript{1} = \frac{[\text{Area}\textsubscript{TT1}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%TT\textsubscript{2} = \frac{[\text{Area}\textsubscript{TT2}}{\text{mean Area}\textsubscript{NgC}} \times 100
\%TT = \frac{(%\%TT\textsubscript{1} + %\%TT\textsubscript{2})}{2}

The mean relative viability is used for classification according to the Prediction Model on page 26.

MTT interacting test chemical calculation procedure (Condition 2)

Test chemicals that interfere with MTT can produce non-specific reduction of the MTT.
It is necessary to evaluate the OD or area due to the non-specific reduction (%NSMTT) and to subtract it before calculations of final viability.

OD or Area\textsubscript{ku}: OD or area of untreated killed tissues + MTT incubation
OD or Area\textsubscript{kt}: OD or area of test chemical treated killed tissues + MTT incubation
OD or Area\textsubscript{NgC}: mean OD or Area-negative control living tissues + MTT incubation

A. Calculation for OD reading

● Non-specific MTT reduction calculation (%NSMTT)

\% Killed Test Treatment \textsubscript{1}
\% Killed Test Treatment \textsubscript{2}
\% Mean Non Specific MTT reduction
\%NSMTT\textsubscript{1} = \frac{[(\text{OD}\textsubscript{kt1} - \text{OD}\textsubscript{ku})]}{\text{OD}\textsubscript{NgC}} \times 100
\%NSMTT\textsubscript{2} = \frac{[(\text{OD}\textsubscript{kt2} - \text{OD}\textsubscript{ku})]}{\text{OD}\textsubscript{NgC}} \times 100
\%NSMTT = \frac{(%\%NSMTT\textsubscript{1} + %\%NSMTT\textsubscript{2})}{2}

● CORRECTED FINAL VIABILITY (FVC)

\%Final viability \textsubscript{Test Treatment\textsubscript{1}}
\%Final viability \textsubscript{Test Treatment\textsubscript{2}}
Mean Final Viability
\%FV\textsubscript{C NSMTT\textsubscript{1}} = %\%TT\textsubscript{1} - %\%NSMTT
\%FV\textsubscript{C NSMTT\textsubscript{2}} = %\%TT\textsubscript{2} - %\%NSMTT
\% FV\textsubscript{C NSMTT} = \frac{(%FV\textsubscript{C NSMTT\textsubscript{1}} + %FV\textsubscript{C NSMTT\textsubscript{2}})}{2}
**B. Calculation for HPLC/UPLC - spectrophotometry endpoint**

- **Non-specific MTT reduction calculation (%NSMTT)**

  \[
  \% \text{NSMTT} = \left( \frac{\text{Area}_{k_1} - \text{Area}_{k_2}}{\text{Area}_{N_{GC}}} \right) \times 100
  \]

  \[
  \% \text{NSMTT} = \left( \frac{\% \text{NSMTT}_1 + \% \text{NSMTT}_2}{2} \right)
  \]

- **CORRECTED FINAL VIABILITY (FVC)**

  \[
  \% \text{FV}_{C \text{NSMTT}} = \% \text{TT} - \% \text{NSMTT}
  \]

  \[
  \% \text{FV}_{C \text{NSMTT}} = \left( \frac{\% \text{FV}_{C \text{NSMTT}_1} + \% \text{FV}_{C \text{NSMTT}_2}}{2} \right)
  \]

The mean final viability is used for classification according to the **Prediction Model** on p.26.

**Coloring test chemicals calculation procedure (Condition 3)**

For test chemicals detected as able to Color the tissues, it is necessary to evaluate the non-specific OD due to the residual chemical Color (unrelated to mitochondrial activity) and to subtract it before calculations of the "true" viability %.

**Note.** This calculation procedure is not applicable to HPLC/UPLC-spectrophotometry.

- **OD\text{TT-MTT}**: OD treated tissue without MTT incubation
- **OD\text{NgC}**: Mean OD Negative Control (living tissues + MTT incubation)

- **Non-specific Color CALCULATION (%NSC\text{living})**

  \[
  \% \text{NSC\text{living}} = \left( \frac{\% \text{MTT}_{TT-MTT}}{\% \text{MTT}_{NgC}} \right) \times 100
  \]

  \[
  \% \text{NSC\text{living}} = \left( \frac{\% \text{NSC\text{living}}_1 + \% \text{NSC\text{living}}_2}{2} \right)
  \]

- **CORRECTED FINAL VIABILITY (FVC)**

  \[
  \% \text{FV}_{C \text{NSC\text{living}}} = \% \text{TT} - \% \text{NSC\text{living}}
  \]

  \[
  \% \text{FV}_{C \text{NSC\text{living}}} = \left( \frac{\% \text{FV}_{C \text{NSC\text{living}}_1} + \% \text{FV}_{C \text{NSC\text{living}}_2}}{2} \right)
  \]

The mean final viability is used for classification according to the **Prediction Model** on p.26.
### Coloring +/- MTT interacting test chemical calculation procedure (Condition 4)

Colored interfering test chemicals are usually identified in pre-checks as being also potential direct MTT reducers due to their intrinsic Color, thus leading to the use of %NSMTT controls together with %NSC living controls.

However, the Non-Specific MTT Reduction (%NSMTT) control also includes the binding of the test chemical to the killed tissues and thus binding is corrected twice leading to an overestimation of the toxic effect. This can be corrected with the use of a third set of control (%NSC killed).

**Note.** The %NSC killed control is not applicable to HPLC/UPLC-spectrophotometry.

\[
\begin{align*}
\text{OD}_{\text{kt-MTT}} & : \text{OD Killed test tissue treated tissues without MTT incubation} \\
\text{OD}_{\text{NgC}} & : \text{mean OD negative control living tissues}
\end{align*}
\]

- **NON SPECIFIC Color WITHOUT MTT % CALCULATION (%NSC killed)**
  \[
  \%\text{NSC killed}_1 = \left( \frac{\text{OD}_{\text{kt-MTT}1}}{\text{OD}_{\text{NgC}}} \right) \times 100
  \\
  \%\text{NSC killed}_2 = \left( \frac{\text{OD}_{\text{kt-MTT}2}}{\text{OD}_{\text{NgC}}} \right) \times 100
  \\
  \text{Mean % Non-Specific Color without MTT} = \frac{\%\text{NSC killed}_1 + \%\text{NSC killed}_2}{2}
  \]

- **CORRECTED FINAL VIABILITY (FV_C)**
  \[
  \%\text{Final viability Test Treatment 1} = \%TT1 - \%\text{NSMTT} - \%\text{NSC living} + \%\text{NSC killed}
  \\
  \%\text{Final viability Test Treatment 2} = \%TT2 - \%\text{NSMTT} - \%\text{NSC living} + \%\text{NSC killed}
  \\
  \%\text{Mean Final Viability} = \frac{\%\text{FV_{C TT1}} + \%\text{FV_{C TT2}}}{2}
  \]

The mean final viability is used for classification according to the Prediction Model on p.26.

**Remarks on data analysis**

If the variability of the interfering test chemical is not significantly higher than normal, correction using adapted controls should be allowed as long as the interference is not extreme.

If variability is significantly higher than normal (above 140% of the negative control), it is assumed that the amount of test chemical retained by the tissue after exposure and post-treatment incubation varies significantly between different tests.

In this situation, the following rules are applied when **OD endpoint is chosen:**

- **IF** the mean of % Non-Specific Color on living tissues (%NSC living) or % Non-Specific MTT reduction (%NSMTT) of the qualified run is less than or equal to (≤) 50%,
  **THEN** the test chemical is considered to be compatible with the test method.

- **IF** the mean of %NSC living or %NSMTT or [%NSC living + %NSMTT - %NSC killed] of the qualified run is greater than (> ) 50% AND the classification remains the same upon correction,
  **THEN** the test chemical is considered to be compatible with the test method.

- **IF** the mean of %NSC living or %NSMTT or [%NSC living + %NSMTT - %NSC killed] of the qualified run is greater than (> ) 50% AND the classification of the qualified run changes upon correction,
  **THEN** this test chemical is considered to be incompatible with the test method. In this case, use of another method or of a default classification as classified (category 1) should be considered.

Results for test chemical producing %NSMTT and/or %NSC living and/or %NSC killed ≥ 50% of the negative control should be taken with caution.
In the table below examples of calculations to be performed depending on the conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Viab %TT</th>
<th>Mean Viab %NSMTT</th>
<th>Mean Viab %NSC living</th>
<th>Mean viab %NSC killed</th>
<th>Final Corrected Viability</th>
<th>Final Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Living+MTT</td>
<td>Killed+MTT</td>
<td>Living-MTT</td>
<td>Killed-MTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>81.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>%TT</td>
<td>81.2</td>
</tr>
<tr>
<td>2</td>
<td>101.2</td>
<td>11.2</td>
<td>-</td>
<td>-</td>
<td>%TT - %NSMTT</td>
<td>90.0</td>
</tr>
<tr>
<td>3</td>
<td>81.2</td>
<td>-</td>
<td>41.2</td>
<td>-</td>
<td>%TT - %NSC living</td>
<td>40.0</td>
</tr>
<tr>
<td>4</td>
<td>101.2</td>
<td>11.2</td>
<td>20</td>
<td>11</td>
<td>%TT - %NSMTT - %NSC living + %NSC killed</td>
<td>81.0</td>
</tr>
</tbody>
</table>

See specific section on calculation for OD reading

For Colored test chemicals interfering too strongly with the MTT-reduction assay an alternative endpoint may be required (e.g. **HPLC-UPLC-spectrophotometry**). In this case, one single test should be sufficient independently of how strong the Color interference is, unless the test chemical is also a strong MTT reducer (i.e., killed control values > 50% of the negative control) and correction from control tissues is required. In the table below examples of calculations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Viab %TT</th>
<th>Mean Viab %NSMTT</th>
<th>Final Corrected Viability</th>
<th>Final Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Living+MTT</td>
<td>Killed+MTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H)1</td>
<td>81.2</td>
<td>-</td>
<td>%TT</td>
<td>81.2</td>
</tr>
<tr>
<td>(H)2</td>
<td>101.2</td>
<td>11.2</td>
<td>%TT - %NSMTT</td>
<td>90.0</td>
</tr>
</tbody>
</table>

See specific section on calculation for HPLC/UPLC-Spectrophotometry endpoint
Prediction Model

The present test method is recommended to identify solid chemicals that do not require classification for eye irritation or serious eye damage according to UN GHS (UN GHS No Category) without further testing. However, the SkinEthic™ HCE EITS is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation).

The prediction model of the SkinEthic™ HCE EITS classifies chemicals into 2 groups:

- UN GHS No Category (NC), and
- "Category 1 / Category 2 (Cat. 1 / Cat. 2)" without further conclusion whether the test chemical belongs to the GHS Cat. 1 or Cat. 2.

Due to the high over-prediction rate shown in the SkinEthic™ HCE EITS validation (26.4%), part of the chemicals predicted as "Cat.1/ Cat.2" can actually be falsely positive. Therefore the OECD TG 492 (2017a) interprets the positive result of the SkinEthic™ HCE EITS as "no prediction can be made".

The prediction model (PM) is described below:

<table>
<thead>
<tr>
<th>Criteria for in vitro interpretation</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean tissue viability &gt; 50 %</td>
<td>No Category (NC)</td>
</tr>
<tr>
<td>Mean tissue viability ≤ 50 %</td>
<td>No prediction can be made</td>
</tr>
</tbody>
</table>

A single testing run should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run might be considered, as well as a third one in case of discordant results between the first two runs.

For a full evaluation of eye severe damage / eye irritation effects, the distinction will need to be addressed by another tier of a test strategy in the context of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2017c).
Annexes

Annex 1: Evaluation of Test chemicals - MTT direct interaction (3hrs±15min)

<table>
<thead>
<tr>
<th>Test chemical Name or code number</th>
<th>Start of Incubation Time:</th>
<th>End of incubation Time:</th>
<th>Interaction Blue Color Yes / No</th>
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</thead>
<tbody>
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</tbody>
</table>

Date: .................... ID and signature:..........................
Annex 2: Evaluation of test chemicals - Color interaction (30min±2min)

Laboratory: ............ Study N°..........

<table>
<thead>
<tr>
<th>Test chemical Name or code number</th>
<th>Start of Incubation Time:</th>
<th>End of incubation Time:</th>
<th>Ability to Color Yes / No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Date: ..................... ID and signature:..........................
### Annex 3: Incubation timings

Laboratory: ............. Study N°:.......... Batch N°:................ Series N°:.............

<table>
<thead>
<tr>
<th>Test chemical Name Or code</th>
<th>Treatment 4±0.1 hrs</th>
<th>Post-Soak Treatment 3±2 min</th>
<th>Post incubation 18 hrs ± 30 min</th>
<th>MTT incubation 3 hrs ± 15min</th>
<th>Formazan extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start time (hh:mm)</td>
<td>End time/Start time (hh:mm)</td>
<td>End time/Start time (hh:mm)</td>
<td>End time/Start time (hh:mm)</td>
<td>End time/Start time (hh:mm)</td>
<td>Date and End time (hh:mm)</td>
</tr>
</tbody>
</table>

Date: ..................... ID and signature:..........................
Annex 4: Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals

PRE-CHECK FOR COLOUR INTERFERENCE
Incubate 10 μL or 10 mg of test chemical in 90 μL of water for 30 minutes at room temperature

Does the mixture turn coloured?

No

Yes

Is the colour of the chemical too strong to allow a conclusive pre-check for direct MTT reduction?

No

Yes

PRE-CHECK FOR DIRECT MTT REDUCTION
Incubate 30 μL or 30 mg of test chemical in 300 μL of 1 mg/mL MTT solution for 3 hours at standard culture conditions

Does the mixture turn bluish-purple?

No

Yes

Consider one of the two following options

Use OD or HPLC/UPLC-spectrophotometry
Use OD or HPLC/UPLC-spectrophotometry
Use HPLC/UPLC-spectrophotometry
Use OD

Perform living tissue control concurrently with every test performed, following full testing procedure but incubating with medium instead of MTT (= %NSC\(_{\text{mm}}\))

Perform killed tissue control following full testing procedure (= %NSMTT) (one is sufficient to correct multiple tests)

AND

Perform living tissue control concurrently with every test performed, following full testing procedure but incubating with medium instead of MTT (= %NSC\(_{\text{mm}}\))

Perform killed tissue control following full testing procedure but incubating with medium instead of MTT (= %NSMTT + %NSC\(_{\text{mm}}\)) (one is sufficient to correct multiple tests)

AND

Perform killed tissue control following full testing procedure but incubating with medium instead of MTT (= %NSC\(_{\text{mm}}\)) (one is sufficient to correct multiple tests)

AND

No controls are required

Final %viability = %uncorrected test viability - %NSMTT

Final %viability = %uncorrected test viability - %NSC\(_{\text{mm}}\) - %NSMTT + %NSC\(_{\text{mm}}\)

Final %viability = %uncorrected test viability - %NSC\(_{\text{mm}}\)

No controls are required
Bibliography


OECD (2017a) OECD Test Guideline No 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. Link to document (last access 16.10.2017) OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects

OECD (2017b) OECD Series on Testing and Assessment No. 216: Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Reconstructed Human Cornea-Like Epithelium (RhCE) Test Methods for Identifying Chemicals not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage, Based on the Validated Reference Methods EpiOcular™ EIT and SkinEthic™ HCE EIT described in TG 492. Link to document (last access 16.10.2017) OECD Environmental Health and Safety Publications


• UN (2015)
  Globally Harmonised System of Classification and Labelling of Chemicals (GHS); New York and Geneva: United Nations. Sixth revised edition. Link to document (last access 15.06.2017)

• US FDA (2001)