DB-ALM Protocol n° 190 : SkinEthic™ HCE Eye Irritation Test Liquid (EITL)

Eye Irritation

The SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test Liquid (EITL) is an *in vitro* assay used to assess the acute ocular irritation potential of liquid/viscous 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 liquid/viscous chemicals (EITL), whereas the SkinEthic™ HCE EITS is for solid chemicals (**DB-ALM Protocol No. 191**).

The **SkinEthic™ HCE EITL**, hereafter described, has been validated in a study coordinated by L'Oreal for which its relevance and reliability have been demonstrated (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, is recommended to identify liquid/viscous 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 EITL 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 EITL protocol has been shown to be applicable to a wide range of liquid/viscous chemicals, covering a large variety of chemical types, chemical classes, functional groups, molecular weights, LogPs, chemical structures, coloured and/or direct MTT reducers, etc. (Alépée et *al*., 2016b). The test method allows the hazard identification of mono and multi-component test liquid/viscous chemicals. Gasses and aerosols cannot be also evaluated with the current protocol.

Solid chemicals cannot be evaluated with the current protocol but their assessment can be performed using SkinEthic™ HCE EITS assay (Alépée et *al*., 2016b) which is described in the **DB-ALM Protocol No. 191**.

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 liquid chemicals (**EITL protocol**) has been evaluated in a multicentre validation study coordinated by L'Oréal (Alépée et *al.*, 2016a).

The **SkinEthic™ HCE EITL 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 EITL from data on 60 chemicals is in the order of 92% within laboratories and 93% between laboratories, respectively (Alépée et *al.*, 2016a).
Considering the data obtained in the validation study (Alépée et *al.*, 2016a), the SkinEthic™ HCE EITL, has an overall accuracy of 85% (based on 105 liquid/viscous chemicals), sensitivity of 99% (based on 55 *in vivo* classified chemicals), false negative rate of 1% (based on 55 chemicals), specificity of 70% (based on 50 *in vivo* No category chemicals) and false positive rate of 30% (based on 50 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 solid (see **DB-ALM Protocol No. 191**) and liquid/viscous 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 **EITL protocol** is able to correctly identify **liqui/viscous 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 liquid/viscous (EITL protocol, e.g. the current DB-ALM

Protocol No.190) and solid (EITS protocol, DB-ALM Protocol No.191) 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

°C:	Degree Celsius
µL:	Microliter
%:	Percentage
No pred:	No prediction can be made
EC:	European Commission
EITL:	Eye Irritation Test Liquid
EITS:	Eye Irritation Test Solid
EU CLP:	European Classification Labelling and Packaging Regulation
HCE:	Human Corneal Epithelium
HPLC:	High Performance Liquid Chromatography
hr/hrs:	Hour/hours
IP :	Isopropanol
KU :	Negative control killed treated tissue
LLOQ:	Lower Limit Of Quantification
mg:	Milligram
Min:	Minute
mL:	Milliliter

MTT:	3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazoliumbromide
NC:	Not Classified
NgC:	Negative Control
nm:	Nanometer
NSCkilled:	Non Specific Color in killed tissues–killed tissue without MTT incubation
NSCliving:	Non Specific Color in living tissues–living tissue without MTT incubation
NSMTT:	Non Specific MTT reduction in killed tissue–killed tissue with MTT incubation
OD:	Optical Density
PBS- :	PBS without Ca2+ & Mg2+
PC:	Positive Control
Qualified run:	a run is qualified if it meets the acceptance criteria for the NgC and PC. Otherwise, the run is considered as Non-Qualified (invalid).
Qualified Test:	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).
Series:	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 per test item.
Run:	a set of up to 3 series conducted with the same tissue batch within the same day.
RhT:	Reconstructed human Tissue
RT:	Room Temperature
Test:	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.
TT:	Test Treatment
ULOQ:	Upper Limit Of Quantification
UN GHS:	United Nations Globally Harmonized System
UPLC:	Ultra-high Performance Liquid Chromatography
V:	Volume

Last update: 16 October 2017

PROCEDURE DETAILS, Latest version 7 June 2017

SkinEthic™ HCE Eye Irritation Test Liquid (EITL)
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QUICK Flowchart

The experimental procedures for the *in vitro* SkinEthic™ HCE EITL 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% CO_2, ≥ 90% humidity)

↓

Transfer tissues to fresh maintenance medium in 24-well plates (300µL/well)

↓

Treatment: 2 tissues each with 30±2 µL PBS without Ca^{2+} & Mg^{2+} (Negative Control)
or 10±2 µL PBS^- & 30±2 µL methyl acetate (Positive Control)
or 10±1 µL PBS^- & 30±2 µL test chemical (Test chemical)

↓

Treatment Period: Incubate for 30±2 min (37±2°C , 5±1% CO_2, ≥ 90% humidity)

↓

Rinse with PBS^- (20 mL: 10 mL/jet)

↓

Post-Soak Immersion: Immerse tissues in 1.5 mL fresh maintenance medium

↓

Post-Soak Period: Incubate for 30±2 min (37±2°C, 5±1% CO_2, ≥ 90% humidity)

↓

Viability: Transfer tissues into MTT solution

↓

Incubate tissues for 3 hrs ± 15 min (37±2°C, 5±1% CO_2, ≥ 90% humidity)

↓

Extraction: Immerse the inserts in 1.5 mL isopropanol (formazan extraction)

↓

Extract formazan (minimum 4 hours at RT or overnight in the fridge)

↓

Perforate 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**

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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 cm^2) 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**).

Description of the kit	Comment	Storage conditions	Shelf life
SkinEthic™ HCE units (0.5 cm^2)	Tissues are shipped on semi solid agar's medium in order to maintain good shipment conditions for the tissues	RT	see technical data sheet
SkinEthic™ Maintenance Medium	Culture medium for incubations	Fridge	see technical data sheet

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

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

Consumables

Item	Use
1 sterile bottle	*reconstituting MTT reagent solution*
1 sterile bottle	*diluting MTT in assay medium*
1 glass funnel	*dropping wash fluids in the bottle*
Wash bottle (500 mL)	*collecting wash fluids*
Adjustable multi-step pipette, 50 mL	*for rinsing tissues with 10 mL PBS-*
Adjustable multi-step pipette, 25 mL	*distributing 1 mL maintenance medium*
Adjustable multi-step pipette, 5 mL	*distributing maintenance medium, MTT, PBS - and isopropanol*
Adjustable micro-pipette – 0 to 200µL	*pipetting 200 L formazan extracts*
Adjustable positive displacement micro-pipette 0- 50 µL	*application of 30 L*
Stop-watches/Timers	*controlling contact and step times*
Small sterile blunt-edged forceps	*handling tissue inserts*
96-well plates	*reading Optical Density*
24-well plates	*treatment steps*
6-well sterile plates	*transfer tissue inserts upon receipt*
“Parafilm”	*covering plates during formazan*
Cotton tip swabs	*drying the tissue surface*
Nylon mesh	*for sticky or viscous liquid*
HPLC/UPLC vial	*HPLC/UPLC measurement*
HPLC/UPLC reverse phase column	*HPLC/UPLC measurement*

Media, Reagents, Sera, others

Item	Use
Isopropanol (CAS 67-63-0)	*formazan extraction*
MTT reagent (3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide (CASRN 298-93-1, Sigma M2128 or equivalent)	*viability measurements, viability reagent*
Dulbecco's D-PBS without Ca2+ & Mg2+ GIBCO 14190-144 or equivalent (PBS-)	*rinsing tissues , Negative Control (NgC), MTT solubilisation*
Methyl acetate (CASRN 79-20-9, Sigma 45997 or equivalent)	*Positive Control (PC)*
Sterile distilled water	*liquid test dilutions and checking for color test chemical*
Tissue maintenance medium (SkinEthic™)	*tissues culture, incubations and MTT dilution*
Solvents HPLC/UPLC grade	*HPLC/UPLC measurement*
Formazan (CAS 37360-69-7, purity > 97%,Sigma 88417 or equivalent)	*HPLC/UPLC validation system*

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

Application of test chemicals

Liquid/viscous 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 EITL protocol **for regulatory purposes** , as recommended in OECD Test Guidelines 492, laboratories should demonstrate **technical proficiency** by correctly predicting the **eight proficiency liquid/viscous chemicals** (see Table 1 in the following page).

Table 1. List of proficiency liquid/viscous chemical

Chemical Name	CAS RN	Organic Functional Group [1]	Physical State	Viability (%) [2]	Prediction	MTT Reducer	Colour Interfer.
***In Vivo* Category 1 [3]**							
Methylthio glycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	L	5.5±7.4	No prediction can be made	Y (strong)	N
Hydroxy ethyl acrylate	818-61-1	Acrylate; Alcohol	L	1.6±1.0	No prediction can be made	N	N
***In Vivo* Category 2A [3]**							
Tetraaza tetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous) [4]	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	L	1.3±0.6	No prediction can be made	N	Y (weak)
***In Vivo* Category 2B [3]**							
Diethyl toluamide	134-62-3	Benzamide	L	2.8±0.9	No prediction can be made	N	N
***In Vivo* No Category [3]**							
1-Ethyl-3-methyl imidazolium ethyl sulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	L	79.4±6.2	No Cat	N	N
Dicapryl ether	629-82-3	Alkoxy; Ether	L	95.2±3.0	No Cat	N	N
Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	L	96.5±3.5	No Cat	N	N
Poly ethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Viscous	89.1±2.9	No Cat	N	N

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.; L=liquid.

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

2 Based on results obtained with SkinEthic™ HCE EIT in the validation study (Alépée et al ., 2016b and 2016a) .

3 Based on results from the in vivo rabbit eye test (OECD TG 405) and using the UN GHS.

4 Classification as 2A or 2B depends on the interpretation of the UN GHS criterion for distinguishing between these two categories, i.e., 1 out of 3 vs 2 out of 3 animals with effects at day 7 necessary to generate a Category 2A classification. The in vivo study included 3 animals. All endpoints apart from corneal opacity in one animal recovered to a score of zero by day 7 or earlier. The one animal that did not fully recover by day 7 had a corneal opacity score of 1 (at day 7) that fully recovered at day 9.

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.

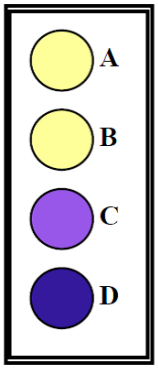
Checking for direct MTT reduction of test chemicals (Annex 4)

Relative conversion of MTT by the tissue being the parameter evaluated in this assay, it is therefore necessary to assess the non-specific reduction of MTT by the test chemical 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 before starting the experiment (ideally the week before the study/run).

  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**.



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

Step 1:

- Fill tubes or wells of a 24-well plate with 300 µL of MTT solution (1 mg / mL).

- Add 30±2 µL of the test chemical to be evaluated or water for control, 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.

Step 2 :

- Use killed tissues that possess no metabolic activity but can absorb and bind the test chemical like viable tissues (*see page 16 more details*).

- Each MTT interacting test chemical is applied onto at least two killed tissues using the EITL 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*) is followed to evaluate the direct MTT reduction of test chemicals

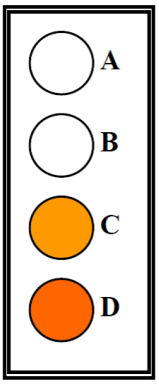
Checking for color test chemicals only (Annex 4)

- ***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 on living tissues ($\%NSC_{living}$). 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 liquid test chemicals.
- For uncolored liquid 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.
$\%NSC_{living}$ is determined after isopropanol extraction and OD reading in similar conditions (*see specific calculation under "Data Analysis" section, from p.21*)



A: *control*
B: Test chemical 1: *no color*
C: *slight coloration of an orange Test chemical*
D: *strong coloration of an orange Test chemical*
NB: Orange is an example.
A coloring chemical can have of course another color

Step 1:

- Fill Eppendorf tubes with 90±2 µL of water.
- Add 10±1 µL of the test chemical to be evaluated.
- 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 $\%NSC_{living}$ value (*see step 2 below*).

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 eye evaluation of a test chemical (concurrently to every testing: i.e.: for each series).

Step 2 :

-The Non-Specific Color ($\%NSC_{living}$) is quantified by using at least 2 living tissues per chemical. For details see **Figure 1** (Condition 3), p.13.

-Coloring test chemical controls are treated and handled like normal treated tissues samples except that they do not get into contact with the MTT solution as they are incubated in maintenance medium.

An independent %NSC_{living} control needs to be conducted with 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_{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 (%$\mathbf{NSC_{killed}}$) (see **Figure 1**, condition 4 on p.13).

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

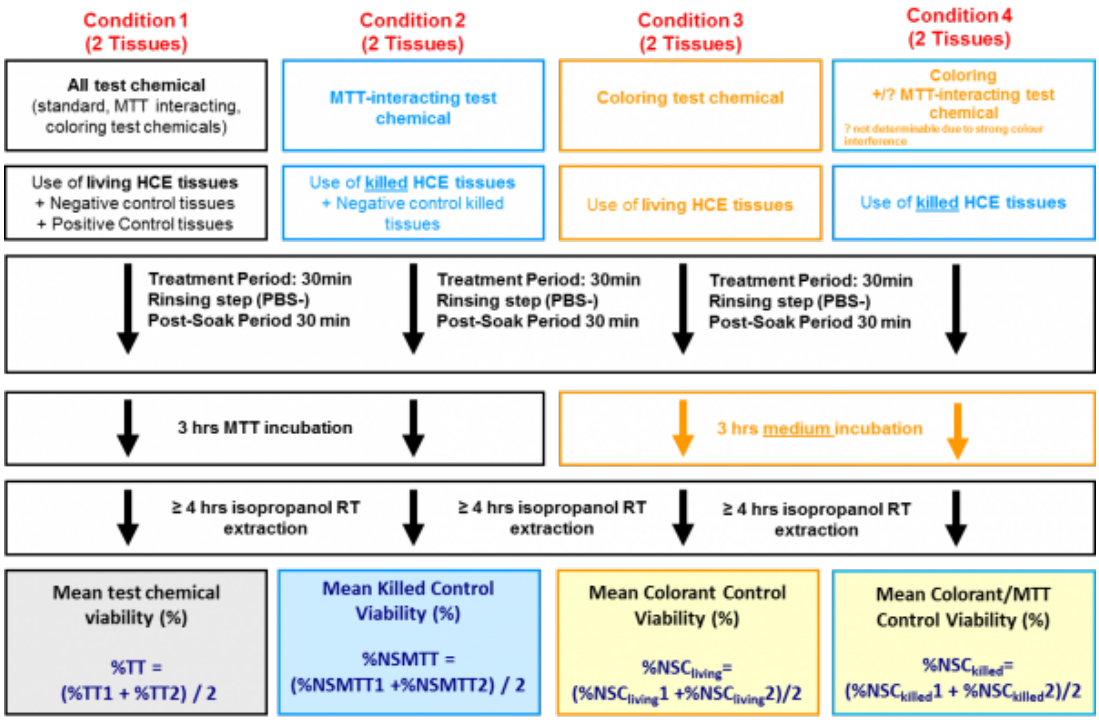
- Results are documented using the documentation sheets available as **Annex 1**, p.27 and **Annex 2**, p.28.
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration on killed tissues (to define the %NSC_{killed} value for a coloring MTT-reducer test 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_{living} or %NSC_{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 2 killed tissues with MTT incubation). See **Figure 2**, p.14.

Figure 1. Summary of adapted controls depending of test chemical physical properties (when OD method is chosen)

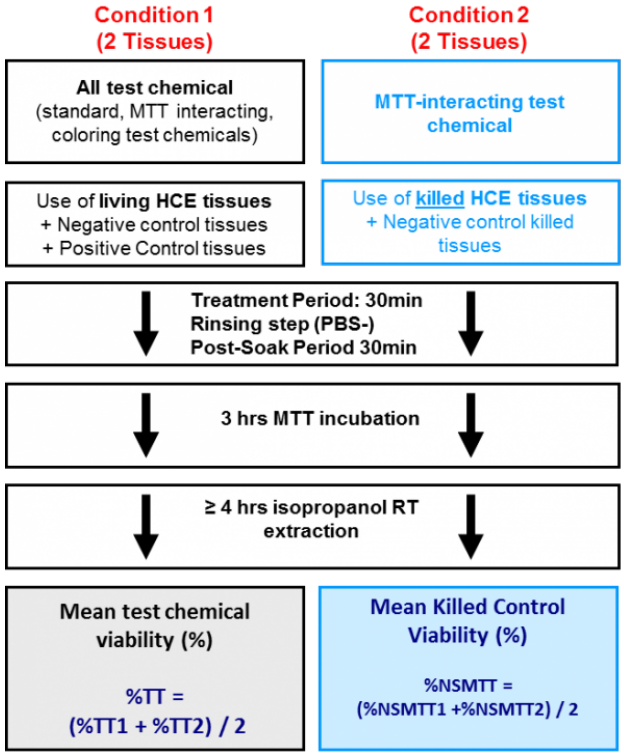


Case by case test conditions for OD reading

	MTT interaction	Coloration interference	Test conditions	Final Corrected Viability
Case 1	-	-	1	%TT
Case 2	+	-	1 + 2	%TT - %NSMTT
Case 3	-	+	1 + 3	%TT - $\%NSC_{living}$
Case 4	+ or ?	+	1 + 2 + 3+ 4	%TT - % NSMTT - $\%NSC_{living}$+ $\%NSC_{killed}$

Results for test chemicals producing %NSMTT and/or $\%NSC_{living}$ and/or $\%NSC_{killed}$ ≥ 50% of the negative control should be taken with caution.

Figure 2. Summary of adapted controls depending of test chemical physical properties (when HPLC/UPLC-spectrophotometry method is chosen)



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

	MTT interaction	Coloration interference	Test conditions	Final Corrected Viability
Case 1	-	- or +	1	%TT
Case 2	+	- or +	1 + 2	%TT - %NSMTT

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

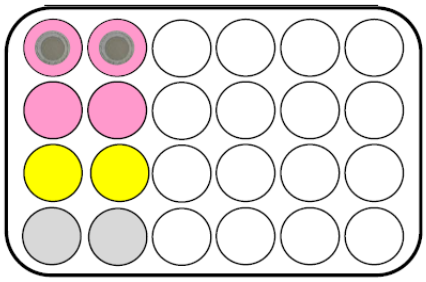
- Upon receipt of the epithelium kits, **pre-warm maintenance medium at RT** (if necessary) and **transfer each epithelium** from their transport packaging plate to 6-well plates containing 1 mL maintenance medium per well. ***Note:*** *The maintenance medium should be pre-warmed only at room temperature (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% CO_2, ≥ 90% humidity until treatment steps.

Tissue conditioning

Note: *One plate is used for each test chemical, 2 wells per plate.*

- Pre-warm the maintenance medium at RT.
- Before test chemical treatment, label the plate lid with the appropriate test chemical information.
- Dispense the pre-warmed maintenance medium into a sterile 24-well plate: 2 wells with 300 µL for the 30 min treatment period, 2 wells with 750 µL for the 30 min post-soak period after the rinsing step (see example of "*Application/rinsing/MTT plate design*" below).
- Transfer 2 SkinEthic™ HCE epithelia units into the 2 first wells filled with 300 µL 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% CO_2, ≥ 90% humidity at least 30 min.

Application/rinsing/MTT plate design



1st line: Application – 300 µL of maintenance medium
2nd line: Immersion 750 µL of maintenance medium
3rd line : 300 µL of MTT medium (**Conditions 1&2**), or maintenance medium in the case of coloring controls (extemporaneously) (**Conditions 3&4**)
4rd line: 300 µL of PBS^-

-Usually 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 topical application, rinsing/immersing and MTT conversion 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 application to MTT test. 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% CO_2, ≥ 90% humidity 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

Test chemicals, Negative Control (PBS-) and Positive Control (Methyl Acetate) applications

- For NgC, dispense **30±2 µL of PBS-** directly topically onto the tissue, always using a positive displacement pipette.
- For PC and test chemical, dispense **10±1 µL of PBS-** and add **30±2 µL of PC or test chemical** directly topically onto the tissue, always using a positive displacement pipette.
- Gently spread it on the epithelium surface without touching it.
- Ensure to cover all tissue surfaces. If necessary, gently move the plate or the insert by performing circular or elliptic movements.
- A nylon mesh can be used for spreading a sticky or viscous chemical.
- 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.
- Verify the absence of air bubbles below tissues and incubate treated tissues for **30±2 minutes** at 37±2°C, 5±1% CO_2, ≥ 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 (30±2 min), the test chemical (and controls) will be removed by rinsing tissues with **PBS-** . Tissues are rinsed by using a 50 mL adapted multi-pipette. Adjust the distribution to 10 mL per push.
- Rinse **2 times** with **10 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 tissues. You can proceed to one more rinsing step if it's necessary (color test chemical).
- Remove remaining PBS- onto the tissue by energised reversals. *Do not use cotton swab to dry the tissues at this step.*

- Process the tissues one at a time; maintain the insert over a glass funnel with forceps (to collect the wash fluids in the wash bottle).
- For tissues on which the test chemical has not been removed by standard washing procedure with PBS $^{-}$, an alternative option is proposed (high viscosity). 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 new well containing **750 µL** per well of **fresh pre-warmed maintenance medium** (wells of the same plate).
- Immerge the tissue by applying topically 750 µL of fresh maintenance medium pre-warmed at RT on each tissue.
- Verify the absence of air bubbles below tissues and incubate the tissues for **30±2 minutes** at 37±2°C, 5±1% CO_2, ≥ 90% humidity.

***Note** . This post-soak tissue immersion is intended to remove any test chemical inside the tissue.*

- At the end of the Post-Soak incubation, 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 .

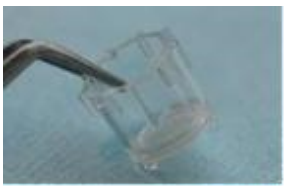


Fig.A

Fig.B

MTT conversion test

The MTT conversion test is carried out at the **end of the 30 min ± 2min immersion post-soak period.** The results of this test are documented using the documentation sheet in **Annex 3**, p.29.

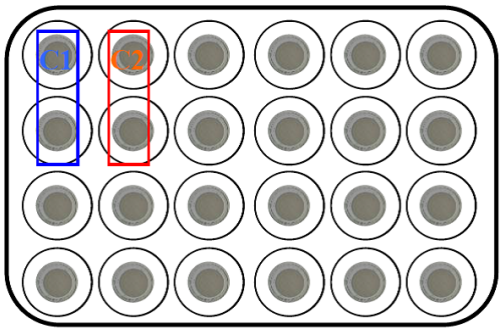
***Note** : Additional specific tissue controls for coloring test chemicals (%NSC $_{living}$ ± %NSC $_{killed}$) will be incubated with the **maintenance medium** (see **Figure 1** on p.13 , **conditions 3** and **4**).*

- Prepare MTT medium according to " *Preparation section* " on p.8.
- Dispense **300 µL** of MTT solution in 2 wells of the plate (**1 mg/mL MTT** solution freshly prepared in maintenance medium) (**Conditions 1** and **2, Figure 1**, p.13).
- For the **specific coloring controls,** dispense **300 µL** of maintenance medium instead of MTT medium (**Conditions 3** and **4, Figure 1**, p.13).
- Remove remaining maintenance medium below the tissue by gently tapping the inserts on dry absorbent paper and transfer tissues to the MTT-containing wells (or maintenance medium for coloring chemical) (see *3 rd line of plate design example* , p.15). Verify the absence of air bubbles under the tissues.
- Incubate tissues for **3 hours ± 15 minutes** at 37±2°C, 5±1% CO_2, ≥ 90% humidity.
- After this MTT incubation period, rinse the inserts in **300 µL PBS** to remove the excess of MTT solution (for **conditions 1** and **2**) or maintenance medium (for **conditions 3** and **4**).
- Record starting time of MTT/Maintenance medium incubation (**Annex 3,** p.29).

Formazan extraction

- Perform a quick contact of the tissue with dry absorbent paper.
- Transfer tissues to new 24-wells plates containing 750 µL isopropanol per well. Additionally, 750 µL isopropanol is added topically onto each tissue insert. 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 (1.5 mL isopropanol).
- 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 **Figures C** and **D** below).
- Extraction of formazan crystals can be performed alternatively as:
  - *For 4 hours at RT* (minimum) protected from light with gentle shaking on plate shaker (~ 120 rpm).
  - *Overnight in the fridge* protected from light without shaking. The following day, shake at least 30 minutes at RT on plate shaker (~ 120 rpm).
  - Other formazan extraction conditions than those described above may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results, e.g. over week end storage in the fridge (tissue treatment was performed on Friday). Ensure that plates should always be carefully covered, protected from light without shaking. After extended incubation period, shake at least 30 minutes at RT on plate shaker (~ 120 rpm) and ensure that plates recover a room temperature.
- Perforate the inserts (using a 200 µL tip on a micropipette)
- Homogenize the extraction solution vigorously up and down through the insert until a homogeneous solution is reached. See **Figures E** and **F** below.
- Remove the empty insert.

Example of Formazan extraction - plate design.



1 st column: Test chemical 1 (C1)
2 nd column: Test chemical 2 (C2)

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

Figure C

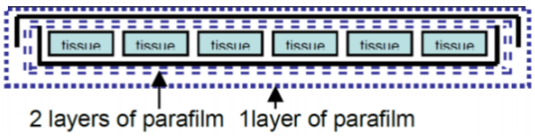


Figure D

Figure E

Figure F

Endpoint Measurement

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:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 1
B	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	
C	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 2
D	NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	
E	TT11	TT12	TT13								BL	BL	Tissue 1
F	TT11	TT12	TT13								BL	BL	
G	TT11	TT12	TT13								BL	BL	Tissue 2
H	TT11	TT12	TT13								BL	BL	

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

For conditions 2 and 4:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	Killed Tissue 1
B	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	
C	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	Killed Tissue 2
D	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	
E	TT11	TT12	TT13								BL	BL	Killed Tissue 1
F	TT11	TT12	TT13								BL	BL	
G	TT11	TT12	TT13								BL	BL	Killed Tissue 2
H	TT11	TT12	TT13								BL	BL	

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 filter.
- Use isopropanol as blank (200 µL / well).
- Link OD values with the appropriate treatment conditions and replicates 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 OD at 570±30 nm filter.
- 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_{NgC}) at 570±30 nm of the two replicate tissues treated with negative control should be > 1.0 with an upper acceptance limit of ≤ 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 ≤ 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 ≤ 30% and the difference value is ≤ 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 ≤ 20 %.
  However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 60 ± 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 as 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 procedure.

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.

Viability – Standard calculation procedure (Condition 1)

A. Calculation for OD reading

- MEAN OD CALCULATION

* Negative Control (NgC)	
Individual OD Negative Control (NgC)	$OD_{NgC1} = OD_{NgC1raw} - OD_{blank\ mean}$ $OD_{NgC2} = OD_{NgC2raw} - OD_{blank\ mean}$
Mean OD Negative Control	$\mathbf{OD_{NgC} = [OD_{NgC1} + OD_{NgC2}] / 2}$

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

* Positive Control (PC)	
OD Positive Control (PC)	$OD_{PC1} = OD_{PC1\ raw} - OD_{blank\ mean}$ $OD_{PC2} = OD_{PC2\ raw} - OD_{blank\ mean}$
Mean OD Positive Control	$\mathbf{OD_{PC} = [OD_{PC1} + OD_{PC2}] / 2}$

* Test Treatment	
OD Test Treatment (TT)	$OD_{TT1} = OD_{\mathbf{TT1}\ raw} - OD_{blank\ mean}$ $OD_{TT2} = OD_{\mathbf{TT2}\ raw} - OD_{blank\ mean}$
Mean OD Test Treatment	$\mathbf{OD_{TT} = [OD_{TT1} + OD_{TT2}] / 2}$

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control1	$\%NgC1 = [OD_{NgC1} / \text{mean } OD_{NgC}] \times 100$
% Negative Control2	$\%NgC2 = [OD_{NgC2} / \text{mean } OD_{NgC}] \times 100$
% mean Negative Control	**%NgC= (%NgC1 + %NgC2)/2**

% Positive Control1	$\%PC1 = [OD_{PC1} / \text{mean } OD_{NgC}] \times 100$
% Positive Control2	$\%PC2 = [OD_{PC2} / \text{mean } OD_{NgC}] \times 100$
% mean Positive Control	**%PC= (%PC1 + %PC2)/2**

% Test Treatment 1	$\%TT1 = [OD_{TT1} / \text{mean } OD_{NgC}] \times 100$
% Test Treatment 2	$\%TT2 = [OD_{TT2} / \text{mean } OD_{NgC}] \times 100$
% Mean Test Treatment	**%TT = (%TT1 + %TT2)/2**

B.Calculation for HPLC/UPLC - spectrophotometry endpoint

- MEAN AREA CALCULATION

*Negative Control (NgC)

Mean Area Negative Control $\quad$ **$Area_{NgC} = [Area_{NgC1} + Area_{NgC2}] / 2$**

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

*Positive Control (PC)

Mean Area Positive Control $\quad$ **$Area_{PC} = [Area_{PC1} + Area_{PC2}] / 2$**

* Test Treatment

Mean Area Test Treatment

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control1 $\quad$ $\%NgC1 = [Area_{NgC1} / \text{mean } Area_{NgC}] \times 100$
% Negative Control2 $\quad$ $\%NgC2 = [Area_{NgC2} / \text{mean } Area_{NgC}] \times 100$

% mean Negative Control $\quad$ **%NgC= (%NgC1 + %NgC2)/2**

% Positive Control1 $\quad$ $\%PC1 = [Area_{PC1} / \text{mean } Area_{NgC}] \times 100$
%Positive Control2 $\quad$ $\%PC2 = [Area_{PC2} / \text{mean } Area_{NgC}] \times 100$

% mean Positive Control $\quad$ **%PC= (%PC1 + %PC2)/2**

% Test Treatment 1 $\quad$ $\%TT1 = [Area_{TT1} / \text{mean } Area_{NgC}] \times 100$
% Test Treatment 2 $\quad$ $\%TT2 = [Area_{TT2} / \text{mean } Area_{NgC}] \times 100$

% Mean Test Treatment $\quad$ **%TT = (%TT1 + %TT2)/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* **$Area_{KU}$** : OD or Area untreated killed tissues + MTT incubation
OD *or* **$Area_{kt}$** : OD or Area test chemical treated killed tissues + MTT incubation
OD *or* **$Area_{NgC}$** : mean OD or Area negative control living tissues + MTT incubation

A.Calculation for OD reading

- Non-specific MTT reduction calculation (%NSMTT)

% Killed Test Treatment 1 $\quad$ $\%NSMTT1 = [(OD_{kt1} - OD_{ku}) / OD_{NgC}] \times 100$
% Killed Test Treatment 2 $\quad$ $\%NSMTT2 = [(OD_{kt2} - OD_{ku}) / OD_{NgC}] \times 100$

% Mean Non Specific MTT reduction $\quad$ **%NSMTT = (%NSMTT1 + %NSMTT2)/2**

- CORRECTED FINAL VIABILITY (FVC)

%Final viability Test Treatment1 $\quad$ $\%FV_{C\ NSMTT1} = \%\ TT1 - \%NSMTT$
%Final viability Test Treatment 2 $\quad$ $\%FV_{C\ NSMTT2} = \%\ TT2 - \%NSMTT$

Mean Final Viability $\quad$ **$\%\ FV_{C\ NSMTT} = (FV_{C\ NSMTT1} + FV_{C\ NSMTT2})/2$**

B.Calculation for HPLC/UPLC - spectrophotometry endpoint

- Non-specific MTT reduction calculation (%NSMTT)

% Killed Test Treatment 1	%NSMTT1= [(Area $_{kt1}$ -Area $_{ku}$)/ Area $_{NgC}$]x100
% Killed Test Treatment 2	%NSMTT2= [(Area $_{kt2}$ -Area $_{ku}$)/ Area $_{NgC}$]x100
% Mean Non Specific MTT reduction	**%NSMTT = (%NSMTT1 + %NSMTT2)/2**

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1	*%FV $_{C\ NSMTT1}$ = % TT1- %NSMTT*
%Final viability Test Treatment 2	*%FV $_{C\ NSMTT2}$ = % TT2- %NSMTT*
Mean Final Viability	**% *FV $_{C\ NSMTT}$* = (FV $_{C\ NSMTT1}$ +FV $_{C\ NSMTT2}$)/2**

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

- Non-specific Color CALCULATION (%NSC $_{living}$)

% Non-Specific Color tissue 1	%NSC $_{living}$ 1= [(OD $_{TT1\text{-}MTT}$ / OD $_{NgC}$] x 100
% Non-Specific Color tissue 2	%NSC $_{living}$ 2= [(OD $_{TT2\text{-}MTT}$ / OD $_{NgC}$] x 100
Mean % Non-Specific Color	**%NSC $_{living}$ = (%NSC $_{living}$ 1+%NSC $_{living}$ 2)/2**

- CORRECTED FINAL VIABILITY (FV $_C$)

%Final viability Test Treatment 1	*%FV $_{C\ NSCliving1}$ = % TT1-* %NSC $_{living}$
%Final viability Test Treatment 2	*%FV $_{C\ NSCliving2}$ = % TT2-* %NSC $_{living}$
Mean Final Viability	**% *FV $_{C\ NSCliving}$* = (FV $_{C\ NSCliving1}$ + FV $_{C\ NSCliving2}$)/2**

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 .*

OD $_{kt\text{-}MTT}$: OD Killed test tissue treated tissues without MTT incubation
OD $_{NgC}$: mean OD negative control living tissues

- NON SPECIFIC Color WITHOUT MTT % CALCULATION (%NSC $_{killed}$)

% Non-Specific Color without MTT tissue 1	%NSC $_{killed1}$ = (OD $_{kt\text{-}MTT1}$ /OD $_{NgC}$)x100
% Non-Specific Color without MTT tissue 2	%NSC $_{killed2}$ = (OD $_{kt\text{-}MTT2}$ /OD $_{NgC}$)x100
Mean % Non-Specific Color without MTT	***%NSC $_{killed}$ = (%NSC $_{killed1}$ + %NSC $_{killed2}$)/2***

- CORRECTED FINAL VIABILITY (FV $_{C}$)

%Final viability Test Treatment 2	*%FV $_{C\ TT1}$ = % TT1- %NSMTT-* %NSC $_{living}$ + %NSC $_{killed}$
%Final viability Test Treatment 2	*%FV $_{C\ TT2}$ = % TT2- %NSMTT-* %NSC $_{living}$ + %NSC $_{killed}$
% Mean Final Viability	***FV $_{C\ TT}$ = (FV $_{C\ TT1}$ + FV $_{C\ TT2}$)/2***

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

Remarks

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 (≤) 60%,
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 (>) 60% **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 (>) 60% **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 $_{killed}$ ≥ 60% of the negative control should be taken with caution.

In the table below examples of calculations to be performed depending on the conditions.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Mean Viab %NSC $_{living}$	Mean viab %NSC $_{killed}$	Final Corrected Viability	Final Viability
	Living+MTT	*Killed+MTT*	*Living-MTT*	*Killed-MTT*		
1	81.2	-	-	-	%TT	81.2
2	101.2	11.2	-	-	%TT - %NSMTT	90.0
3	81.2	-	41.2	-	%TT - %NSC $_{living}$	40.0
4	101.2	11.2	20	11	%TT - % NSMTT - %NSC $_{living}$ + %NSC $_{killed}$	81.0
See specific section on calculation for OD reading	***p.21 (A)***	***p.22 (A)***	***p.23 (condition 3)***	***p.24 (condition 4)***		

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 > 60% of the negative control) and correction from control tissues is required. In the table below examples of calculations.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Final Corrected Viability	Final Viability
	Living+MTT	*Killed+MTT*		
(H)1	81.2	-	%TT	81.2
(H)2	101.2	11.2	%TT - %NSMTT	90.0
See specific section on calculation for HPLC/UPLC-Spectrophotometry endpoint	***p.22 (B)***	***p.23 (B)***		

Prediction Model

The present test method is recommended to identify liquid/viscous 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 EITL 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 EITL 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 EITL validation (30%), 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 EITL as “no prediction can be made”.

The prediction model (PM) is described below:

Criteria for *in vitro* interpretation	**Classification**
Mean tissue viability > 60 %	No Category
Mean tissue viability ≤ 60 %	No prediction can be made

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)

Laboratory: …… Study : ……

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Interaction Blue Color Yes / No

Date: …….. ID and signature:……

Annex 2: Evaluation of test chemicals - Color interaction (30min±2min)

Laboratory: …… Study N°…....

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Interaction Blue Color Yes / No

Date: …….. ID and signature:……

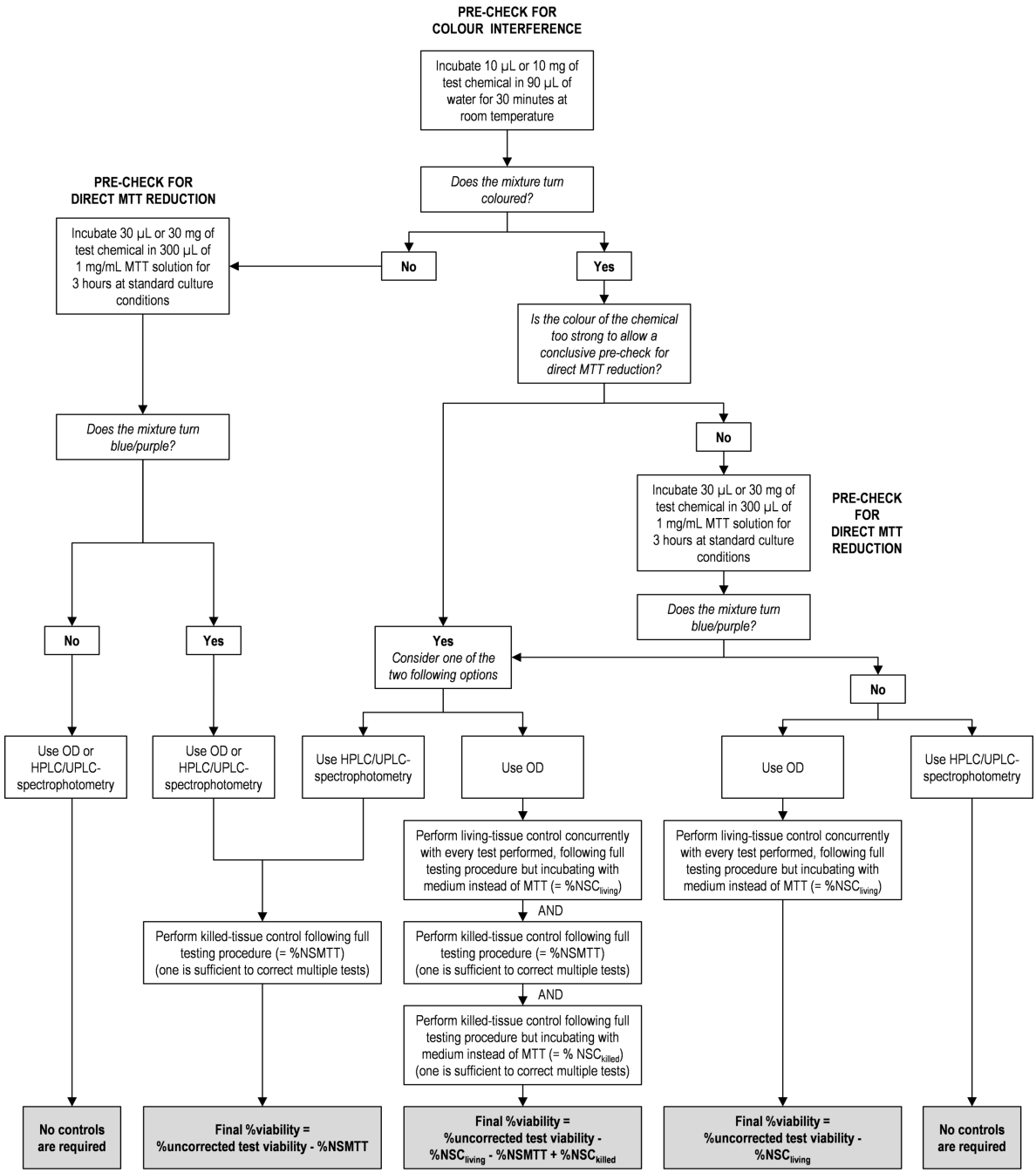
Annex 3: Incubation timings

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

Test chemical Name Or code	Treatment 30±2 min		Post-Soak Treatment 30±2 min	MTT incubation 3 hrs ± 15min	Formazan extraction
	Start time (hh:mm)	End time/Start time (hh:mm)	End time/Start time (hh:mm)	End time/Start time (hh:mm)	Date and End time (dd:hh:mm)

Date: …….. ID and signature:……

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



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