DB-ALM Protocol

IN VITRO EYE HAZARD IDENTIFICATION TIME-TO-TOXICITY TEST METHOD ON SOLIDS (TTS) USING SKINETHIC™ HUMAN CORNEAL EPITHELIUM MODEL (HCE)

Last Update: June 2020

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Part A. Protocol Introduction

Protocol Name: SkinEthic™ HCE TTS
SkinEthic™ HUMAN CORNEAL EPITHELIUM TIME-TO-TOXICITY ON SOLIDS

Abstract: *IN VITRO* PREDICTION METHOD FOR EYE HAZARD IDENTIFICATION *INTO GHS CATEGORIES (NO CATEGORY / CATEGORY 2 / CATEGORY 1)* FOR SOLID CHEMICALS

Summary

The SkinEthic™ Human Corneal Epithelium (HCE) Time-to-Toxicity *in vitro* test method on Solids (SkinEthic™ HCE TTS) has been developed and established within L'Oréal for eye hazard identification (Alépée et al, 2021). This test method is intended to differentiate between UN GHS No Category, UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation), supported by *in vivo* Draize eye irritation data for comparative evaluation of results.
On the basis of the data currently available, the SkinEthic™ HCE TTS method was shown to be applicable to a wide range of solid chemicals, covering a variety of chemical types, chemical classes, functional groups. The test method allows the hazard identification of mono and multi-component test solid chemicals. Liquid chemicals cannot be evaluated with the current protocol; the assessment being performed using SkinEthic™ HCE Time-to-Toxicity test method on Liquids (TTL). Gasses and aerosols cannot be also evaluated with the current protocol.

Experimental Description

Biological Endpoint and Endpoint Measurement:
Cell viability determination, used as the endpoint, 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).

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 reduction % in viability is used to predict the eye hazard potential of the test chemical.

Experimental System:

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 mucosa 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 multi-layered 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: Three training days were necessary to establish the test method in a naïve laboratory. It included a practical training in which (i) the main steps of the protocol were emphasized (ii) a demonstration of the method was observed and (iii) then performed by the trainers. It also included 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 tissues conditioning, on Day 2 with cell treatment with chemicals and the MTT viability assay and on Day 3 with data acquisition. A trained experimenter can perform up to 13 test chemicals in a run.

Costs: Contract Research Organization (CRO) testing costs are available upon request to the CRO.

Status

The SkinEthic™ Human Corneal Epithelium (HCE) Time-to-Toxicity *in vitro* test method on Solids (SkinEthic™ HCE TTS) has been developed and established within L'Oréal by evaluating 69 chemicals (Alépée et al, 2021). A multicentric study was conducted to assess the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of the test method SkinEthic™ HCE TTS by testing 20 coded solid chemicals. From the method development / validation study and its independent peer review (Barroso el al, 2021) it was concluded that the SkinEthic™ HCE TTT is able to correctly identify solid chemicals by discriminating the three UN GHS categories for serious eye damage/eye irritation, i.e. UN GHS Category 1 (serious eye damage), Category 2 (eye irritation) and No Cat chemicals (UN, 2019). This test method is not intended to differentiate between UN GHS Category 2A (reversible effect on day 14) and Category 2B (reversible effect on day 7).
The SkinEthic™ HCE Time-to-Toxicity *in vitro* test method (for liquids: TTL and solids: TTS) was submitted to support OECD acceptance and currently an OECD draft test guideline (draft TG 492B) is under review to support regulatory acceptance.

Proprietary and/or Confidentiality Issues

The Reconstructed Human Tissue SkinEthic™ HCE technology, associated to production of model and media are proprietary to EPISKIN SA, France.
No intellectual property rights are associated with the present test method.

Abbreviations and Definitions

°C:	Degree Celsius
µL:	Microliter
%:	Percentage
EC:	European Commission
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
NSC_{killed}:	Non Specific Color in killed tissues–killed tissue without MTT incubation
NSC_{living}:	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 Ca^{2+} & Mg^{2+}
PC:	Positive Control
RhT:	Reconstructed human Tissue
RT:	Room Temperature
TT:	Test Treatment
TTL	Time-to-Toxicity on Liquids
TTS	Time-to-Toxicity on Solids
ULOQ:	Upper Limit Of Quantification
UN GHS:	United Nations Globally Harmonized System
UPLC:	Ultra-high Performance Liquid Chromatography
V:	Volume

- Run: a set of test chemicals plus Negative Control (NgC) and Positive Control (PC) all concurrently tested on at least 2 tissues replicates, conducted with the same tissue batch within the same day.
- Qualified Run: A run is qualified if it meets the acceptance criteria for the NgC and PC for each exposure time. Otherwise, a run is considered as Non-Qualified (invalid) for the time exposure for which the acceptance criteria are not met. Therefore, the test chemicals should be retested at this treatment time.
- 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.
- 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).

Version Number	Type of change	Author	Date
1	-	N.ALEPEE	June 25th 2019
2	Peer review Panel comments: Deletion of the positive control (lactic acid neat), simplification of the prediction model for Cat.2 and references update	N.ALEPEE	June 16th 2020

Part B. Procedure Details

Protocol Name: SkinEthic™ HCE *IN VITRO* TIME-TO-TOXICITY ON SOLIDS (TTS)

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Health and Safety Issues

General precautions:
The SkinEthic™ HCE tissues**,** ref: HCE/S model (www.episkin.com) are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm²) with the necessary culture media (maintenance medium). The human-derived HCE cells are free of contamination by bacteria, viruses, mycoplasma, and fungi. The sterility of the reconstructed tissue construct 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 sent by e-mail.

Nevertheless, 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.

MSDS Information:

Safety precautions:

MTT (H315, H319, H335, H341)
Isopropanol (H225, H319, H336)
Lactic Acid (H315, H318)

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

Quick flow chart: *HCE Time-to-Toxicity on Solids*

Time 1= Short Exposure 30'

Receipt: Transfer epithelium from agarose to maintenance medium in 6-well plate (1mL Tissue maintenance medium/well, 1 plate/ test chemical, 2 tissues / plate)

↓

Culture inserts equilibration period: Incubate at least overnight (37°C, 5% CO_2, ≥ 95% humidity)

↓

Fill fresh maintenance medium (1mL/ well) in the same 6-well plates and pre-warm (37°C, 5% CO_2, ≥ 95% humidity) at least 30 min before application. Transfer the tissue before application

↓

Treatment: 2 tissues each with:
80 µL **PBS** without Ca^{2+} & Mg^{2+} (Negative Control, NgC)
or 80 µL **Lactic acid 1% in water (w/v)** + 80 µL distilled water (Positive Control, PC)
or 80 mg **test chemical** (test treatment) + 80 µL distilled water + mesh for mixed

↓

Treatment Period: Incubate for 30 ± 2 min (37°C, 5% CO_2, ≥ 95% humidity)

↓

Rinse with PBS^- without Ca^{2+} & Mg^{2+} (25 mL: 2mL/jet)

↓

Post-Soak Immersion: Immerse tissues in 4mL fresh maintenance medium in 12-well plate

↓

Post-Soak Period: Incubate for 30 ± 2 min at RT

↓

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

↓

Incubate tissues for 3 hrs ± 15 min (37°C, 5% CO_2, ≥ 95% humidity)

↓

Rinse the MTT solution under the tissue with 300 µL PBS^- without Ca^{2+} & Mg^{2+} in 24-well plate

↓

Remove the insert, blotted on an absorbent paper and transferred into a well plate (dry) during overnight, 4°C or Perform the extraction directly

↓

Extraction: place the inserts on 1.5mL isopropanol in 12-well plate

↓

Extract formazan (at least 2 hours, RT, shaking ~120 rpm)

↓

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

Time 2= Long Exposure 120'

Receipt: Transfer epithelium from agarose to maintenance medium in 6-well plate (1mL Tissue maintenance medium/well, 1 plate/ test chemical, 2 tissues / plate)

↓

Culture inserts equilibration period: Incubate at least overnight (37°C, 5% CO_2, ≥ 95% humidity)

↓

Fill fresh maintenance medium (1 mL/ well) in the same 6-well plates and pre-warm (37°C, 5% CO_2, ≥ 95% humidity) at least 30 min before application. Transfer the tissue before application

↓

Treatment: 2 tissues each with:
80 µL **PBS** without Ca^{2+} & Mg^{2+} (Negative Control, NgC)
or 80 µL **Lactic acid 1% in water (w/v)** + 80 µL distilled water (Positive Control, PC)
or 80 mg **test chemical** (test treatment) + 80 µL distilled water + mesh for mixed

↓

Treatment Period: Incubate for 120 ± 5 min (37°C, 5% CO_2, ≥ 95% humidity)

↓

Rinse with PBS^- without Ca^{2+} & Mg^{2+} (25 mL: 2mL/jet)

↓

Post-Soak Immersion: Immerse tissues in 4 mL fresh maintenance medium in 12-well plate

↓

Post-Soak Period: Incubate for 30 ± 2 min at RT

↓

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

↓

Incubate tissues for 3 hrs ± 15 min (37°C, 5% CO_2, ≥ 95% humidity)

↓

Rinse the MTT solution under the tissue with 300µL PBS^- without Ca^{2+} & Mg^{2+} in 24-well plate

↓

Remove the insert, blotted on an absorbent paper and transferred into a well plate (dry) during overnight, 4°C or Perform the extraction directly

↓

Extraction: place the inserts on 1.5mL isopropanol in 12-well plate

↓

Extract formazan (at least 2 hours, RT, shaking ~120 rpm)

↓

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

B.1 Materials and Preparations

B.1.1 CELL OR EXPERIMENTAL SYSTEM

Human Corneal Epithelium model (SkinEthic™ HCE): The SkinEthic™ HCE tissues are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm²) with the necessary culture media (maintenance medium). The SkinEthic™ HCE tissues are produced by EPISKIN (France) (www.episkin.com). The sterility of the reconstructed tissue construct 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 sent by e-mail.
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.

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. The maintenance medium should be pre-warmed only at room temperature (and not at 37°C).

B.1.2 EQUIPMENT

Fixed Equipment

● Microbiological safety cabinet (laminar flow hood)	→ *Safe work under sterile conditions*
● Non-sterile ventilated cabinet	→ *Safe work with test chemicals, applications, washes*
● Cell incubator 37±2°C , 5±1% CO_2, ≥ 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

✓ 1 bottle	*reconstituting MTT reagent stock solution*
✓ 1 bottle or 1 glass Erlen	*diluting MTT in assay medium*
✓ 1 glass funnel	*dropping wash fluids in the bottle*
✓ Wash (waste) bottle (500 mL)	*collecting wash fluids*
✓ Adjustable multi-step pipette, 25 mL	*for rinsing tissues with 25 mL PBS-*
✓ Adjustable multi-step pipette, 25 mL	*distributing 1 mL and 4 mL maintenance medium*
✓ Adjustable multi-step pipettes, 5 mL	*distributing maintenance medium, MTT, PBS - and isopropanol*
✓ Adjustable micro-pipette – 0 to 200 µL	*pipetting 200 µL formazan extracts*
✓ Adjustable positive displacement micro-pipette 100µL or 250 µL	*application of 80 µL*
✓ Circular nylon mesh Ø = 7.5 mm (Sefar Fyltis, # Sefar Nitex 03-150/38) or equivalent	*use to spreading aid for test chemical*
✓ Stop-watches/Timers	*controlling contact and step times*
✓ Small sterile blunt-edged forceps	*handling tissue inserts*
✓ Spatula	*weighting powder*
✓ Small glass weight boat	*weighting powder*
✓ Mortar and pestle	*grinding granular*
✓ 6-well plates	*transfer tissue inserts upon receipt and treatment steps*
✓ 12-well plates	*post Soak immersion and formazan extraction*
✓ 24-well plates	*MTT incubation, rinsing MTT solution*
✓ 96-well plates	*reading Optical Density*
✓ “Parafilm”	*covering plates during formazan extraction*
✓ Cotton tip swabs	*drying the tissue surface*
✓ HPLC/UPLC vial	*HPLC/UPLC measurement*

✓ HPLC/UPLC reverse phase column	*HPLC/UPLC measurement*

B.1.3 MEDIA, REAGENTS, SERA, OTHERS

✓ Isopropanol (CASRN 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,* v*iability reagent*
✓ Dulbecco's D-PBS without Ca^{2+} & Mg^{2+} GIBCO 14190-144	*rinsing tissues and MTT* or equivalent (PBS^-) *solution, NgC, MTT solubilisation*
✓ Lactic acid (CASRN 50-21-5, Sigma L6661 or equivalent)	*Positive Control (PC)*
✓ Sterile distilled water	*application and checking for color test chemical*
✓ Tissue maintenance medium (provided by EpiSkin SA)	*tissues culture, incubations, post soak and MTT solution*
✓ Solvents HPLC/UPLC grade	*HPLC/UPLC measurement*
✓ Formazan (CASRN 57360-69-7 purity > 97%, *Sigma 88417 or equivalent)*	*HPLC/UPLC validation system*

B.1.4 PREPARATIONS

Media and Endpoint Assay Solutions

MTT stock solution preparation

- Prepare a 5 mg/mL 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 (1v+4v) (final concentration: 1 mg/mL) with maintenance medium. Keep at RT, protect from light until use (do not exceed 3 hours storage).
  Note: MTT solution is light sensitive. Protect it from light.

The culture media is delivered with the SkinEthic™ HCE tissue; it is stored in the fridge.

All these solutions and media are prepared or open under a safety cupboard.

Test chemicals

Solid test chemical (± color, ± MTT reducer) is topically applied onto HCE tissues.

Positive Control

Lactic acid **at 1%** (W/V) in water is used as Positive Control (PC).

Negative Control

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

NB: The negative and positive controls must be performed for each run by the user. This data is not provided by the tissues supplier.

Water- killed epithelium preparation (for MTT-interacting chemicals or MTT pre-check inconclusive chemicals due to color)

1) Place the living epithelium in a 24-well plate pre-filled with 300 µL of distilled water.
2) Incubate at 37±2°C, 5±1% CO_2, ≥ 90% humidity for 24±1 hr.
3) At the end of the incubation, discard the water.
4) Keep killed epithelium frozen (dry) in freezer (-18 to -22°C) (killed epithelium can be stored and used up to 6 months).
5) Tissues should be de-frozen before use at room temperature (at least 10 minutes) in 1mL maintenance medium in 6 well plates).
6) Further use of thawed killed tissues is similar to living tissues.
7) Apply Negative Control and test treatments on killed tissues from the same batch.
8) Proceed similarly to living tissues for application, rinsing, post-soak, etc.
9) Perform this assay only once (on at least duplicate tissues) per test chemical when necessary.

B.2 Method

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

B.2.1 Checking for direct MTT reduction of test chemicals (Annex 5)

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 OD is chosen as endpoint for viability assessment:

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

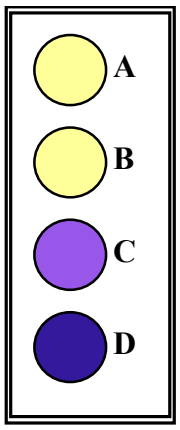
To identify this possible interference, each test chemical is checked for its ability to reduce MTT without tissue (step 1). In case of identified MTT interaction, proceed to step 2.

Step 1:

- Weigh 30 mg up to 80 mg of the test chemical to be evaluated in tube (type Eppendorf safe lock)

- Fill **tubes** with 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).



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

If the MTT solution color turns blue or purple, the test chemical interacts with the MTT (see illustration 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 value.

Step 2:

- Use killed tissues that possess no metabolic activity but can absorb and bind the test chemical like viable tissues.

- Each MTT interacting test chemical is applied onto at least two killed tissues using the TTS protocol. In addition to that, at least two killed tissues are treated with PBS^- as control (negative control killed tissue, KU).

- For details see section B.2.4 (condition 2).

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

- Document: Evaluation of test chemical – MTT direct interaction (Annex 1).
- 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 section B.3.2).

When HPLC/UPLC-spectrophotometry is chosen as endpoint for viability assessment:

Same procedure as for OD measurement.

B.2.2 Checking for color test chemicals only (Annex 5)

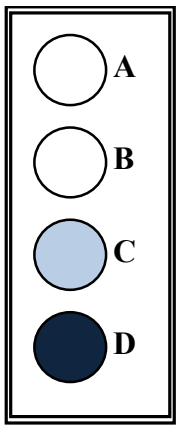
When OD is chosen as endpoint:

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 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. $\%NSC_{living}$ is determined after isopropanol extraction and OD reading in similar conditions (see specific calculation section B.3.3).



A: Control
B: Test chemical 1: no color
C: Slight coloration of an blue test chemical
D: Strong coloration of an dark blue test chemical

NB: Blue is an example. A coloring test chemical can have off course another color.

Step 1:

- Weigh 10±1 mg of the test chemical to be evaluated in tube (type Eppendorf safe lock)

- Fill Eppendorf tubes with 90±2 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 illustration example 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 observed, 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.

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.

Step 2:

- The Non-Specific Color ($\%NSC_{living}$) is quantified by using at least 2 living tissues per chemical. For details see section B.2.4 (Condition 3).
- 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).

- Document: Evaluation of Test chemicals – Color interaction (Annex 2).
- 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 section B.3.3).

<u>When HPLC/UPLC-spectrophotometry is chosen as endpoint:</u>

No pre-check or control are necessary.

B.2.3 Checking for color test chemicals with possible MTT direct interaction (Annex 5)

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 ($\%NSC_{killed}$) (see section B.2.4, condition 4).
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).

- Documents: (Annexes 1 & 2).
- 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 section B.3.4).

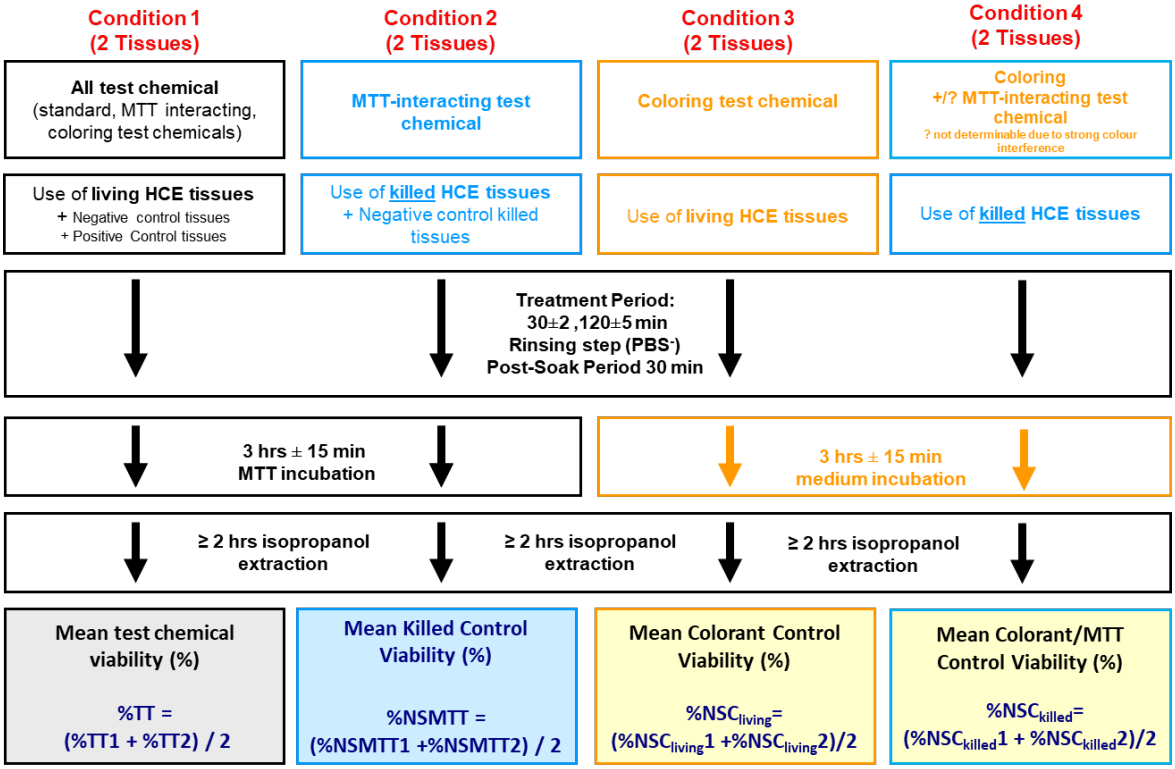
When HPLC/UPLC-spectrophotometry is chosen as endpoint:

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 section B.2.5.

Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or color interfering chemicals is described on Annex 5.

B.2.4 Summary of adapted controls depending of test chemical physical properties (when OD method is chosen) for each time of exposure.

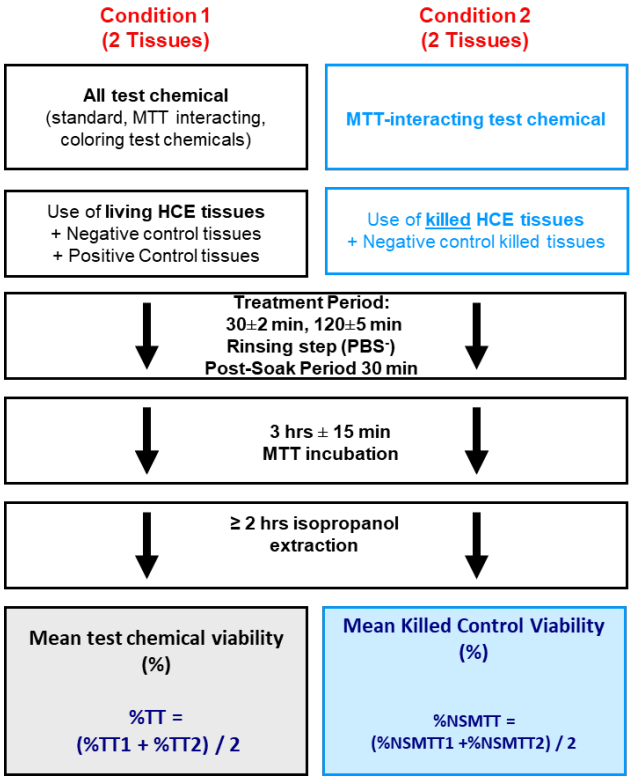


Case by case test conditions for OD reading

	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 ?	+ or ++	1 + 2 + 3+ 4	%TT - % NSMTT - $\%NSC_{living}$ + $\%NSC_{killed}$

Results for test chemicals producing %NSMTT and/or $\%NSC_{living}$ and/or $\%NSC_{killed}$ ≥ 60% of the negative control should be taken with caution.

B.2.5 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	-	-	1	%TT
Case 2	+	-	1 + 2	%TT - %NSMTT

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

B.2.6 Test chemical exposure procedures

Reception

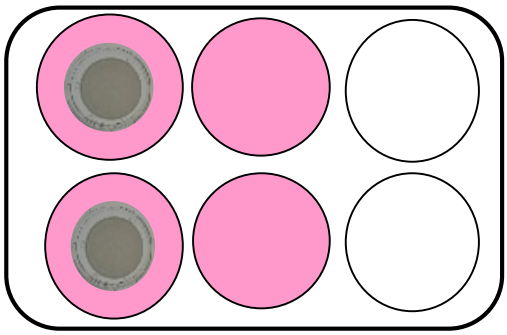
Label the plate lid with the appropriate test chemical information and the timing of treatment

- Upon receipt of the epithelium kits, **pre-warm maintenance medium at RT** (if necessary)
- Dispense the pre-warmed maintenance medium into the sterile 6-well plate: only 2 wells with 1 mL per plate
- Transfer each epithelium, from their transport packaging plate to 6-well plates containing 1 mL maintenance medium per well (2 replicate tissues per test chemical per time)
- 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

- Dispense the pre-warmed maintenance medium into the sterile 6-well plate: 2 wells with 1 mL for the treatment period (see example below) (at least 2 replicate tissues per test chemical per time).
- Incubate the plates at 37±2°C 37±2 °C, 5±1% CO_2, ≥ 90% humidity at least 30 min.
- Transfer the SkinEthic™ HCE epithelia units into the 2 following wells. Verify the absence of air bubbles below the tissues.

Reception and application plate design

 1st column: Reception – 1 mL of maintenance medium 2nd column: Application and incubation – 1 mL of maintenance medium	- Usually two SkinEthic™ HCE tissues are used per chemical: Negative Control (NgC), Positive Control (PC) or test chemical. - Only 1 chemical per plate and per time. - The plate will be used for topical application

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 and per time. Start with NgC, PC and then the test chemicals.

Tissue treatment

Safety instructions:

1. *Test chemicals should be handled following material safety datasheet. Store the test chemicals in ventilated safety cupboards. Respect special storage conditions if necessary (special temperature, protected from light, etc.) according to the material safety datasheet guidelines.*
2. *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.*

Negative control (PBS^-), positive control (lactic acid 1% in water) and test chemicals applications

- NgC, PC and test chemicals are tested at 2 application times: 30±2 min and 120±5 min
- For the 2 application times, **for NgC**, dispense **80±2 µL of PBS^-** directly topically onto the tissues, always using a positive displacement pipette.
- For the 2 application times, for **PC:** Dispense **80±2 µL of PC** and add **80±2 µL of distilled water** directly topically onto the tissues, always using a positive displacement pipette.
- For the 2 application times, for **test chemicals**: remove the tissue from the medium and place onto a dish of weighting. Dispense **80±2 mg of test chemical** and add **80±2 µL of distilled water** directly topically onto the tissues, always using a positive displacement pipette. *Always apply in the following order: Test chemical THEN distilled water. Ensure that chemicals cover the full tissues surface, therefore solids chemicals could be grounded to achieve that when needed.*

Carefully apply a nylon mesh Ø = 7.5 mm on the whole surface with tip or forceps and mixed.

- Treat tissues at adapted time intervals according to the necessary rinsing-off intervals, *i.e.*: 2 minutes, to be adapted depending on the operator experience.
- Verify the absence of air bubbles below tissues and incubate treated tissues for 2 application times: **30 min±2 min** and **120±5 minutes at 37±2 °C**, 5±1% CO_2, ≥ 90% humidity with lids on.

Record time and details in the documentation sheet (Annexes 3a and 3b).

End of treatment and rinsing procedure

- At the end of the exposure, 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 (not too close to 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).
- 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.
- 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).

Post-Soak Period

- After rinsing, immerse immediately the rinsed tissue to a new well (12 well plates) containing **4 mL** per well of **fresh maintenance medium**.

NgC alone in its plate (Figure A). Test chemicals: 2 to 3 maximum / plate at the opposite (Figure B)

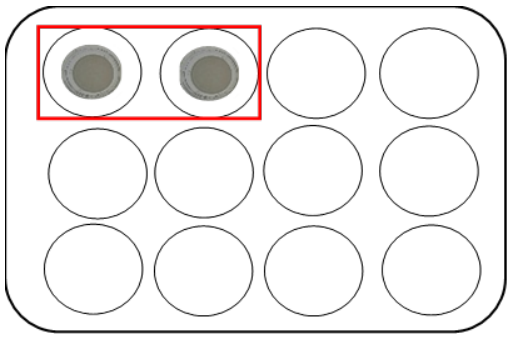


Figure A

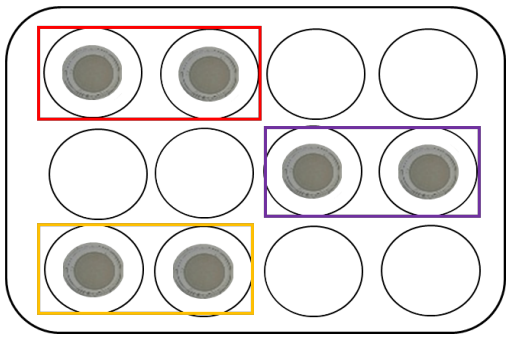


Figure B

- 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 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 C) and the surface with a cotton swab (Figure D).

- Document (Annexes 3a and 3b).

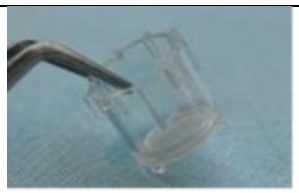


Figure C	Figure D

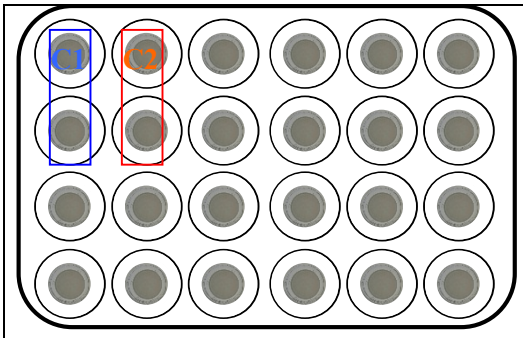
MTT conversion test

The MTT conversion test is carried out at the end of the 30±2 min immersion post soak period. (Document Annexes 3a and 3b).

*Note: Additional specific tissue controls for coloring test chemicals (%NSC_{living} ± %NSC_{killed}) will be incubated with the **maintenance medium** (see section B2.4, conditions 3 & 4).*

- Prepare MTT medium according to *section B.1.4*
- Dispense **300 µL** of MTT solution in wells of the 24-well plate (**1 mg/mL MTT** solution freshly prepared in maintenance medium) (Conditions 1 and 2).
- For the **specific coloring controls,** dispense **300 µL** of maintenance medium instead of MTT medium (Conditions 3 and 4).
  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.
- Record starting time of MTT/Maintenance medium incubation (Annexes 3a and 3b).
- To stop the MTT reaction, clean bottom inserts with **300 µL PBS^- in a 24-well plate** (for conditions 1, 2, 3 and 4).
- **Take a picture of plate**
- Each tissue will be removed from PBS^-, blotted on an absorbent paper and transferred into a well plate (dry)
- The formazan can be performed either immediately (see section Formazan extraction) or the day after (keep the plate at 4°C during overnight).

MTT incubation and rinsing - plate design (see example)

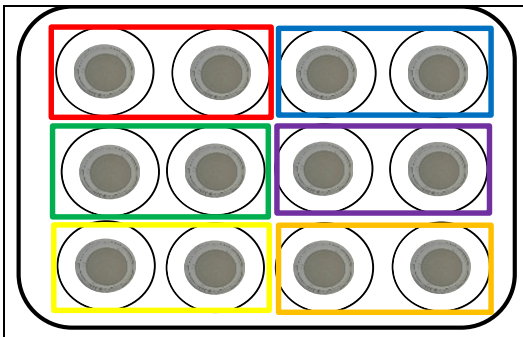
 MTT incubation: 300 µL of MTT medium (Conditions 1&2), or maintenance medium in the case of coloring controls (extemporaneously) (Conditions 3&4) Rinsing: 300 µL of PBS^- 1st column: Test chemical 1 (C1)	- Two SkinEthic™ HCE tissues are used per chemical (NgC, PC or test chemical).

2nd column: Test chemical 2 (C2)	

Formazan extraction.

- Transfer tissues to 12-wells plates containing 1.5mL isopropanol per well. Tissues are extracted from the **bottom only** 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.
- During extraction, plates should be covered with "parafilm" to prevent evaporation. At least a layer not stretched of parafilm under the lid should be used (usually 3 layers: 2 layers stretched on top of the wells under the lid. Put the lid on and cover with the third sheet of parafilm stretched around the lid and plate; see below Figures C & D).
- Extraction of formazan crystals is performed for at least 2 hours at RT protected from light with gentle shaking on plate shaker (~ 120 rpm).
- Remove the insert
- Homogenize the extraction solution vigorously up and down through the insert until a homogeneous solution is reached.

Formazan extraction - plate design (see example)



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

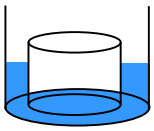


Figure C

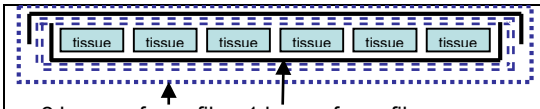


Figure D

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 a 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	empty	empty	empty	empty	empty	empty	empty	BL	BL	Tissue 1
F	TT11	TT12	TT13	empty	empty	empty	empty	empty	empty	empty	BL	BL	
G	TT11	TT12	TT13	empty	empty	empty	empty	empty	empty	empty	BL	BL	Tissue 2
H	TT11	TT12	TT13	empty	empty	empty	empty	empty	empty	empty	BL	BL	

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

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.

For conditions 2 and 4:

NSMTT:

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

KU = Negative control killed tissue; TT1 ... TT13 = Test treatment 1... 13; BL = blank (isoporanol 100%)

NSC_{killed}

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

TT1 ... TT13 = Test treatment 1... 13; BL = blank (isoporanol 100%)

- 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 (Annex 4) on a qualified HPLC/UPLC-UV/Visible system.
- 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.

B.2.7 Acceptance criteria

Common acceptance criteria

- The Optical Density (OD_{NgC}) at 570±30 nm of each 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 NgC, is > 40% at the time exposure of 30 minutes and 20%< PC ≤ 70% at the time exposure of 120 minutes. The difference value of the 2 replicates has to be ≤ 20%. The difference corresponds to the absolute difference between the 2 tissues viabilities and is as such expressed as a percentage viability.
- The acceptance criteria of the NgC and PC should be met for interpreting the test chemical data.
- In the real case scenario, for a given test chemical, 2 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 the cut off value ± 5%, a second run should be considered, as well as a third one in case of discordant results between the first two runs.
  For the multicentric study, three qualified runs should be performed per test chemical (within up to 5 runs) for each time exposure.

Specific HPLC/UPLC-spectrophotometry 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 Annex 4. Once the acceptance criteria defined in Annex 4 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 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.

B.3 Data Analysis

Main 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).

B.3.1 Viability – Standard calculation procedure (Condition 1)

B.3.1.1 Calculation for OD reading

Calculation should be performed for all treatment times

- MEAN OD CALCULATION:

*Negative Control (NgC)
§: a & b correspond to the OD of the tissue 1 and 2 replicates of the NgC, respectively

Individual OD Negative Control (NgC)

$$OD_{NgC\ a}{}^{\S} = OD_{NgC\ a\ raw} - OD_{blank\ mean}$$
$$OD_{NgC\ b}{}^{\S} = OD_{NgC\ b\ raw} - OD_{blank\ mean}$$

Mean OD Negative Control

$$\mathbf{OD_{NgC} = [OD_{NgC\ a} + OD_{NgC\ b}] / 2}$$

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

*Positive Control (PC)
§: a and b correspond to the OD of the tissue 1 and 2 replicates of the PC, respectively

OD Positive Control (PC)

$$OD_{PC\ a}{}^{\S} = OD_{PC\ a\ raw} - OD_{blank\ mean}$$
$$OD_{PC\ b}{}^{\S} = OD_{PC\ b\ raw} - OD_{blank\ mean}$$

Mean OD Positive Control

$$\mathbf{OD_{PC} = [OD_{PC\ a} + OD_{PC\ b}] / 2}$$

*Test Treatment
§: a and b correspond to the OD of the tissue 1 and 2 replicates of a TT, respectively

OD Test Treatment (TT)

$$OD_{TT\ a}{}^{\S} = OD_{\mathbf{TT\ a}\ raw} - OD_{blank\ mean}$$
$$OD_{TT\ b}{}^{\S} = OD_{\mathbf{TT\ b}\ raw} - OD_{blank\ mean}$$

Mean OD Test Treatment

$$\mathbf{OD_{TT} = [OD_{TT\ a} + OD_{TT\ b}] / 2}$$

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control $_a$	$\%NgC_a = [OD_{NgC\,a} / \text{mean } OD_{NgC}] \times 100$
% Negative Control $_b$	$\%NgC_b = [OD_{NgC\,b} / \text{mean } OD_{NgC}] \times 100$
% mean Negative Control	**$\%NgC = (\%NgC_a + \%NgC_b)/2$**
% Positive Control $_a$	$\%PC_a = [OD_{PC\,a} / \text{mean } OD_{NgC}] \times 100$
% Positive Control $_b$	$\%PC_b = [OD_{PC\,b} / \text{mean } OD_{NgC}] \times 100$
% mean Positive Control	**$\%PC = (\%PC_a + \%PC_b)/2$**
% Test Treatment $_a$	$\%TT_a = [OD_{TT\,a} / \text{mean } OD_{NgC}] \times 100$
% Test Treatment $_b$	$\%TT_b = [OD_{TT\,b} / \text{mean } OD_{NgC}] \times 100$
%Mean Test Treatment	**$\%TT = (\%TT_a + \%TT_b) / 2$**

B.3.1.2 Calculation for HPLC/UPLC - spectrophotometry endpoint

- MEAN AREA CALCULATION:

<u>*Negative Control (NgC)</u>

Mean Area Negative Control **$Area_{NgC} = [Area_{NgC\,a} + Area_{NgC\,b}] / 2$**

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

<u>*Positive Control (PC)</u>

Mean Area Positive Control **$Area_{PC} = [Area_{PC\,a} + Area_{PC\,b}] / 2$**

<u>*Test Treatment</u>

Mean Area Test Treatment **$Area_{TT} = [Area_{TT\,a} + Area_{TT\,b}] / 2$**

- VIABILITY CALCULATION: Individual means viabilities (%)

% Negative Control a	$\%NgC_a = [Area_{NgC\,a} / \text{mean } Area_{NgC}] \times 100$
% Negative Control b	$\%NgC_b = [Area_{NgC\,b} / \text{mean } Area_{NgC}] \times 100$
% mean Negative Control	**$\%NgC = (\%NgC_a + \%NgC_b)/2$**
% Positive Control a	$\%PC_a = [Area_{PC\,a} / \text{mean } Area_{NgC}] \times 100$
% Positive Control b	$\%PC_b = [Area_{PC\,b} / \text{mean } Area_{NgC}] \times 100$
% mean Positive Control	**$\%PC = (\%PC_a + \%PC_b)/2$**
% Test Treatment $_a$	$\%TT1 = [Area_{TT\,a} / \text{mean } Area_{NgC}] \times 100$
% Test Treatment $_b$	$\%TT2 = [Area_{TT\,b} / \text{mean } Area_{NgC}] \times 100$
%mean Test Treatment	**$\%TT = (\%TT_a + \%TT_b)/2$**

The mean relative viability is used for classification according to the prediction model (section B.4).

B.3.2 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
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

B.3.2.1 Calculation for OD reading

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

% Killed Test Treatment a	$\%NSMTT_a = [(OD_{kt\,a} - OD_{ku})/ OD_{NgC}]x100$
% Killed Test Treatment b	$\%NSMTT_b = [(OD_{kt\,b} - OD_{ku})/ OD_{NgC}]x100$
% Mean Non Specific MTT reduction	$\mathbf{\%NSMTT = (\%NSMTT_a + \%NSMTT_b) / 2}$

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment a	$\%FV_{C\,NSMTT\,a} = \%\,TT_a - \%NSMTT$
%Final viability Test Treatment b	$\%FV_{C\,NSMTT\,b} = \%\,TT_b - \%NSMTT$
Mean Final Viability	$\mathbf{\%FV_{C\,NSMTT} = (FV_{C\,NSMTT\,a} + FV_{C\,NSMTT\,b}) / 2}$

B 3.2.2 Calculation for HPLC/UPLC-spectrophotometry reading

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

% Killed Test Treatment a	$\%NSMTT_a = [(Area_{kt\,a} - Area_{ku})/ Area_{NgC}]x100$
% Killed Test Treatment b	$\%NSMTT_b = [(Area_{kt\,b} - Area_{ku})/ Area_{NgC}]x100$
% Mean Non Specific MTT reduction	$\mathbf{\%NSMTT = (\%NSMTT_a + \%NSMTT_b)/2}$

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment a	$\%FV_{C\,NSMTT\,a} = \%\,TT_a - \%NSMTT$
%Final viability Test Treatment b	$\%FV_{C\,NSMTT\,b} = \%\,TT_b - \%NSMTT$
Mean Final Viability	$\mathbf{\%FV_{C\,NSMTT} = (FV_{C\,NSMTT\,a} + FV_{C\,NSMTT\,b}) / 2}$

The mean final viability is used for classification according to the prediction model (section B.4).

B.3.3 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 that 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 (%NSCliving)

% Non-Specific Color tissue $_a$ $\%NSC_{living\ a} = [(OD_{TT\ a\text{-}MTT} / OD_{NgC}] \times 100$
% Non-Specific Color tissue $_b$ $\%NSC_{living\ b} = [(OD_{TT\ b\text{-}MTT} / OD_{NgC}] \times 100$

Mean % Non-Specific Color $\mathbf{\%NSC_{living} = (\%NSC_{living\ a} + \%NSC_{living\ b}) / 2}$

- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment $_a$ $\%FV_{C\ NSCliving\ a} = \%\ TT_a - \%NSC_{living}$
%Final viability Test Treatment $_b$ $\%FV_{C\ NSCliving\ b} = \%\ TT_b - \%NSC_{living}$

Mean Final Viability $\mathbf{\%FV_{C\ NSCliving} = (FV_{C\ NSCliving\ a} + FV_{C\ NSCliving\ b}) / 2}$

The mean final viability is used for classification according to the prediction model (section B.4).

B.3.4 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 that this $\%NSC_{killed}$ control is not applicable to HPLC/UPLC-spectrophotometry.*

$OD_{kt\text{-}MTT}$: OD killed treated tissues without MTT incubation
OD_{NgC}: mean OD negative control living tissues

1-NON SPECIFIC COLOR WITHOUT MTT % CALCULATION ($\%NSC_{killed}$):

% Non-Specific Color without MTT tissue $_a$ $\%NSC_{killed\ a} = (OD_{kt\text{-}MTT\ a}/OD_{NgC})x100$
% Non-Specific Color without MTT tissue $_b$ $\%NSC_{killed\ b} = (OD_{kt\text{-}MTT\ b}/OD_{NgC})x100$

Mean % Non-Specific Color without MTT $\mathbf{\%NSC_{killed} = (\%NSC_{killed\ a} + \%NSC_{killed\ b}) / 2}$

2- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment $_a$ $\%FV_{C\ TT\ a} = \%\ TT_a - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
%Final viability Test Treatment $_b$ $\%FV_{C\ TT\ b} = \%\ TT_b - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$

% Mean Final Viability $FV_{C\,TT} = (FV_{C\,TT\,a} + FV_{C\,TT\,b}) / 2$

The mean final viability is used for classification according to the prediction model (section B.4).

B.3.5 Remarks

If the variability of the interfering test chemical is not significantly higher than normal (≤140% of the negative control), correction using adapted controls should be allowed as long as the interference is not extreme. 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.

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
3	81.2	-	41.2	-	%TT - $\%NSC_{living}$	40
4	101.2	11.2	20	11	%TT-%NSMTT- $\%NSC_{living}$+$\%NSC_{killed}$	81
Section	B.3.1.1	B.3.2.1	B.3.3	B.3.4		

If variability is significantly higher than normal (>140% of the negative control), correction using adapted controls should be allowed as long as the interference is extreme. In this situation, the HPLC-UPLC-spectrophotometry endpoint is recommended.

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.

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
Section	B.3.1.2	B.3.2.2		

B.4. Prediction Model

The present SkinEthic™ HCE TTS test method is recommended to identify and classify solid chemicals for eye hazard identification according to UN GHS classification: No Category (not classified), Category 2 (eye irritation) and Category 1 (serious eye damage).

The prediction model (PM) is described below:

Criteria for *in vitro* interpretation		**Classification *In vitro* prediction**
Time treatment: 30±2 min	**Time treatment: 120±5 min**	
Mean tissue viability ≤ 40 %	≤ 60 %	Category 1 (Cat.1)
Any other combination of values		Category 2 (Cat.2)
> 40 %	> 60 %	No Category (No Cat.)

B.5 Quality assurance and archiving

To be adapted upon the need.

B.6 References

Alépée, N., Leblanc, V., Grandidier, MH., Teluob, S., Viricel, A., Adriaens, E., Michaut, V. SkinEthic HCE Time-to-Toxicity on Solids: A test method for distinguishing chemicals inducing serious eye damage, eye irritation and not requiring classification and labelling. (2021) *Toxicol. in Vitro* 75, 105203.

Alépée, N., Barroso, J., De Smedt, A., De Wever, B., Hibatallah, J., Klaric, M., Mewes, K.R., Millet, M., Pfannenbecker, U., Tailhardat, M., Templier, M., McNamee, P. (2015). Use of HPLC/UPLC-Spectrophotometry for Detection of Formazan in *In Vitro* Reconstructed Human Tissue (RhT)-Based Test Methods Employing the MTT-Reduction Assay to Expand their Applicability to Strongly Coloured Test Chemicals. *Toxicol. In Vitro* 29, 741-761.

Alépée N, Hibatallah J, Klaric M, Mewes KR, Pfannenbecker U, McNamee P (2016). Assessment of cosmetic ingredients in the in vitro reconstructed human epidermis test method EpiSkin™ using HPLC/UPLC-spectrophotometry in the MTT-reduction assay. *Toxicol In Vitro* 33,105-117.

Barroso, J., Eskes, C., Germolec, D., Kim, B-H., Kojima, H. (2021). Peer Review Panel Report on the scientific validity of the SkinEthic™ Human Corneal Epithelium (HCE) Time-To-Toxicity Test (TTT) on liquids (TTL) and on solids (TTS). January, 12, 2021.

Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* 65, 55-63.

Nguyen, D.H., Beuerman, R.W., De Wever, B., Rosdy, M. (2003). Three-dimensional construct of the human corneal epithelium for in vitro toxicology. In: Salem, H., Katz, S.A. (Eds), Alternative Toxicological Methods, CRC Press, pp. 147-159.

United Nations (UN) (2019), Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Eighth revised edition, New York and Geneva, United Nations Publications. Available at: [https://unece.org/ghs-rev8-2019].

US FDA (2001). Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration. May 2001. Available at: [http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf].

B.7 Annexes

Annex 1: Evaluation of test chemicals - MTT direct interaction (3 hrs)
Annex 2: Evaluation of test chemicals - Color interaction
Annexes 3a-b Incubation timings
Annex 4: HPLC/UPLC-spectrophotometry qualification system
Annex 5: Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals

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 signatures:……**

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

Laboratory: …… Study N°….....

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

Date: ……. **ID and signatures:……**

Annex 3a: Incubation timings

Time treatment: 30 min ± 2 min

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

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

Date: ……. **ID and signatures:……**

Annex 3b: Incubation timings

Time treatment: 120±5 min

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

Test substance Name Or code	Treatment 120 ± 5 min		Post-Soak Treatment 30 ± 2 min	MTT incubation 3 hrs ± 15 min	Formazan extraction	
	Start time (hh:mm)	End time /Start time (hh:mm)	End time /Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)

Date: ……. **ID and signatures:……**

Annex 4: Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint

A. Background

The approach used to validate an analytical method on a HPLC/UPLC-spectrophotometry system is based on The Federal Drug Administration (FDA) guidance for industry from May 2001 on Bio-analytical Method Validation (FDA, 2001). Within the FDA guidance, validation of a bio-analytical method encompasses all of the procedures that demonstrate a particular bio-analytical method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for intended use. The fundamental parameters for such a validation include: 1) selectivity; 2) precision and accuracy; 3) matrix effect; 4) carryover; 5) reproducibility and 6) stability. Validation involves documenting, through the use of specific laboratory investigations that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the system detection method.

B. Validation of an analytical method on a HPLC/UPLC-spectrophotometry system

Five different sample types were prepared to enable evaluation of key parameters for the approach to qualify the HPLC/UPLC endpoint. These were as follows:

- Solvent: isopropanol (IP)
- Living Blank: IP extract of a living tissue without MTT. This sample is an untreated SkinEthic™ HCE tissue on which the IP extraction step only is conducted. The same pool of blanks sample is used for the period of the qualification.
- Dead Blank: IP extract of killed tissues without MTT: The killed tissues are obtained following the step describes in section B.1.4.
- Standard sample: sample in IP with known concentration of formazan (CAS number 57360-69-7; purity > 97% or equivalent) is used to prepare the calibration curves. In this context, two limits are defined as follows:
    - Upper Limit Of Quantification (ULOQ) defined as being at least twice as high as untreated sample expressed as formazan concentration (i.e. for 200% cell viability).
    - Lower Limit Of Quantification (LLOQ) defined to enable the calibration curve to cover two orders of magnitude (i.e. 2% cell viability).

  Six concentrations are chosen from 0.823 to 200 µg/mL (1/3 dilutions) which cover the 2% cell viability at the lowest end of the concentration range and at least two times the highest Reconstructed human Tissue concentration for the upper part of the concentration range.

- QC samples: Sample in IP with known concentration of formazan at three different levels: low, medium and high. Concentrations of the QC samples are chosen to be with a constant factor between them and identified as:
    - QC low: 2 x LLOQ (i.e. 1.6 µg/mL)
    - QC medium: 16 µg/mL
    - QC high: 0.8 x ULOQ (i.e. 160 µg/mL)

C. Samples preparation

The different samples were prepared as follows:

- Stock solution :
    - Weigh 10 mg of formazan in an appropriate glass container and add 10 mL of IP (1000 µg/mL)
    - Stir overnight at room temperature with a magnetic bar (store up to 6 months at -20°C)

- ULOQ preparation:
  - Prepare a dilution of a factor 5 from the stock solution in IP using an appropriate container (ULOQ = 200 µg/mL) : 300 µL of stock solution + 1200 µL IP
- Calibration curve samples:
  - From ULOQ: 6 serial 1/3 dilutions in IP to obtain the following concentrations:
    i. 200 µg/mL
    ii. 66.6 µg/mL (1000 µL IP + 500 µL solution at 200 µg/mL)
    iii. 22.2 µg/mL (1000 µL IP + 500 µL solution at 66.6 µg/mL)
    iv. 7.41 µg/mL (1000 µL IP + 500 µL solution at 22.2 µg/mL)
    v. 2.47 µg/mL (1000µL IP + 500 µL solution at 7.41 µg/mL)
    vi. 0.823 µg/mL (1000 µL IP + 500 µL solution at 2.47 µg/mL)
- QC samples preparation:
  - QC stock solution (QC stock solution): 1600 µg/mL in IP: weigh 16 mg of formazan in an appropriate glass container and add 10 mL of IP
  - QC high (160 µg/mL): 1/10 dilution of the QC stock solution in IP or living blank
  - QC medium (16 µg/mL): 1/10 dilution of the QC high in IP or living blank
  - QC low (1.6 µg/mL): 1/10 dilution of the QC medium in IP or living blank

D. HPLC/UPLC-spectrophotometry analytical conditions

- Each laboratory established specific conditions of operation for their HPLC/UPLC-spectrophotometry system. For example, the L'Oréal system is an HPLC with UV-Visible detection. Use of a photodiode array detector was preferred rather than a single wavelength detector so that the quantified formazan could be confirmed by its spectrum identity. The chromatographic system used was an HPLC Waters Alliance 2695 with a Waters UV PAD 2996 detector, controlled with Empower Pro v.5.00 with analytical conditions as follows:

  - Column: Waters Xterra RP18 5µm 150*4.6mm
  - Column temperature: 50°C
  - Mobile phases A and B: HPLC grade Water and HPLC grade Acetonitrile
  - Flow rate: 1mL/min
  - Injection volume: 10 µL
  - Needle wash: IP
  - Seal wash in: water/methanol (90/10 v/v)
  - Stroke volume (µL): automatic
  - Syringe draw rate (µL/sec): slow
  - Needle wash time: extended
  - Wave length range: 250-700 nm
  - Sampling rate: 2.0 point / sec
  - Resolution: 1.2 nm
  - Gradient mode during 10 minutes was as detailed in the following table:

Time (Min)	Solvent A	Solvent B
0	100	0
1	100	0
3	0	100
6	0	100
6.1	100	0
10	100	0

Before the first injection the system was equilibrated during 20 minutes in the initial conditions (100% solvent A).

- HPLC reading internal control: during an analytical sequence, IP (blank matrix) is injected once and QC samples at 20 µg/mL and 200 µg/mL of formazan is injected approximately every 20 injections. After the QC samples another IP injection is done.

Data reading and storage

- Measure peak area of the formazan for each sample
- All data generated by the HPLC/UPLC-spectrophotometry should be printed (or saved as a '.pdf' files) and considered as raw data.
- Link Area values with the appropriate treatment conditions on the raw data documents (or files).
- Perform the Quality Control of the raw data.

<u>E. Acceptance criteria for the validation of an analytical method on a HPLC/UPLC-spectrophotometry system</u>

E.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte - here formazan - in the presence of other components in the sample.

For selectivity, analysis of blank samples of the appropriate biological matrix (here IP tissue extract) are obtained from at least 6 sources (i.e. at least 3 killed tissues - dead blank and 3 living tissues - living blank) from the same batch of tissues.

<u>Optional</u>: Dye (methylene blue CAS number 7220-79-3; maximum wavelength ~650 nm obtained from Sigma) is added to demonstrate that the system could quantify formazan in the presence of a color test chemical. In this way, IP containing methylene blue dye is evaluated. Nevertheless, it doesn't demonstrate absolute selectivity of the analytical method. It has to be controlled for each tested chemical.

Each blank sample is tested for interference with selectivity needing to be assured at the Lower Limit of Quantification (LLOQ).

E.2. Precision and Accuracy

Precision of an analytical method describes the closeness of individual measures of an analyte - here formazan - when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of matrix. The precision is expressed by the Coefficient of Variation (CV) calculated using the following relationship:

$$\text{CV \%} = \frac{\text{Standard Deviation}}{\text{Mean}} \text{ x } 100$$

Precision is further subdivided into 1) intra-batch precision and repeatability in a single analytical run, and 2) the inter-batch precision and repeatability between runs.
The accuracy of the analytical method will describe the closeness of mean test results obtained by the method to the true value (concentration) of the analyte - here formazan. Accuracy was determined by replicate analysis of samples containing known amounts of formazan using the following relationship:

$$\text{\% Accuracy} = \frac{(\text{Concentration}_{\text{Mean measured}} - \text{Concentration}_{\text{True}})}{\text{Concentration}_{\text{True}}} \text{ x } 100$$

Drawing from the FDA Guideline, precision and accuracy in IP were measured at 3 different QC formazan concentrations over 3 consecutive days on independent samples and stock solutions. Five replicates of QC samples are quantified on the first day over the three calibration curves performed. On the 2 following days, 3 replicates of the QC samples are quantified over the calibration curve performed the same day. The acceptance criterion was established that the precision determined at each QC level was not to exceed 15 % of CV for each independent determination. Furthermore, the mean determination values was expected to be within 15 % of the true value for each of the three QC levels over the calibration curves performed the same day.

E.3. Matrix Effect (here identified as RhT tissue insert effect)

RhT tissue insert effect is identified as the Matrix Effect (ME) that reflects the extraction efficiency of an analytical method within the limits of variability. The Matrix Effect (Rht tissue insert effect) is calculated using the following relationship:

$$\text{ME \%} = \frac{\text{Concentration}_{\text{living blank}}}{\text{Concentration}_{\text{IP solution}}} * 100$$

The matrix here is defined as the IP extract from the living blank. The Matrix Effect of the formazan in an assay is the detector response obtained from an amount of formazan added to living blank, compared to the detector response obtained for the true concentration of the pure authentic formazan standard (in IP).
Examination of the Matrix Effect is necessary to ensure that the difference between the standard sample (i.e. IP solution) and unknown samples (*i.e.* living blank) does not affect the true formazan concentration measured.

Matrix Effect (RhT tissue insert effect) for the determination of formazan was measured on one day using 5 independent samples of the same stock solution at the three QC concentrations (living blank samples compared to the calibration curve). The acceptance criterion for Matrix Effect (RhT tissue insert effect) is established that the mean value of the 5 samples at each QC concentration be between 85 % and 115 % according to the above relationship. Percentage of CV is measured for the three QC concentrations and meant not to exceed 15 %.

E.4. Carryover (Cross-contamination)

Carryover (cross-contamination) corresponds to the amount of residual formazan in the analytical system after analysis of the ULOQ sample. Carryover should be as low as possible to avoid over-estimation of unknown formazan samples.

To measure the carryover, a solvent sample is analyzed after the run of an ULOQ standard. The acceptance criterion is established that the area of the carryover is meant not to exceed 20 % of the LLOQ area.

E.5. Calibration/Standard Curve and Robustness/Reproducibility

A calibration (standard) curve demonstrates the relationship between instrument response area and known concentrations of the formazan. A sufficient number of standards need be used to adequately define the relationship between concentration and response. The calibration curve covers a given range of concentrations defined by the LLOQ and ULOQ.

The calibration curve for formazan is generated with at least 6 standard samples that included the LLOQ and ULOQ. A linear relation (forced through zero) between concentration and area is established. Such simple linear relation y = x axis, allows simplifying the further analytical process using a single point calibration. The concentrations of formazan used to generate the standard calibration curve are provided in the following table.

	Formazan concentrations (µg/mL) (CAS # 57360-68-7, Sigma or equivalent)
Calibration curve linear through zero (y = x axis)	0.823, 2.47, 7,41, 22.2, 66.6, 200

The following conditions are to be met in preparing the calibration curve:
Maximum 20 % deviation of the LLOQ from true concentration
Maximum 15 % deviation of standards other than LLOQ from true concentration

The deviation is calculated as follows:

$$\%\ \mathrm{Dev} = \frac{(\mathrm{Concentration}_{\mathrm{measured}} - \mathrm{Concentration}_{\mathrm{True}})}{\mathrm{Concentration}_{\mathrm{True}}}\ \mathrm{x}\ 100$$

Robustness corresponds to the ability of the analytical method to give similar results for the calibration curve within a day and from day to day. It is assessed with precision measurements.

Robustness which allows evaluation of the robustness and reproducibility of the quantification method intra- and inter-days is addressed as follows:

Intra-day: the calibration curve is repeated three times the same day with three different stock solutions. The QC samples series prepared with a fourth independent solution allowed measurement of within-run precision and accuracy with the three calibration curves.
Inter-day: A calibration curve is repeated on three consecutive days with three different stock solutions. Each day, a QC samples series prepared with an independent solution allowed measurement of precision and accuracy from day to day.

E.6. Stability

Formazan stability is a function of storage conditions. The stability of formazan in a particular matrix (i.e. IP, living blank) and containers (e.g. well plate, HPLC vial) is relevant only to the matrix and container used and is not extrapolated to other matrices and containers.

Stability procedures evaluated the stability of the formazan during sample collection and handling, short-term (bench top, room temperature) storage and after long-term (at the intended temperature) storage. Conditions used in stability experiments reflected situations likely to be encountered during actual sample handling and analysis.

Evaluation of stability also included an evaluation of formazan stability in stock solution. Such stability evaluation enabled use of the formazan stock solution over the period of the validation of the analytical method on HPLC/UPLC-spectrophotometry.

All stability determinations used a set of samples prepared from a freshly made stock solution of the formazan in the appropriate formazan-free IP solution and living blank. Stock solutions of the formazan for stability evaluation are prepared in an appropriate solvent (i.e. IP) at known concentration.

Formazan stability is evaluated at the three QC concentrations in three independent replicates in solvent as well as in living tissues blank. Stability is tested by comparing the area with those of freshly prepared samples. The accuracy of a stability sample is identified as meant to be within 15 % of the fresh sample.

Four different stability scenarios are evaluated. These are:

Stock solution stability: The stability of stock solutions of formazan is evaluated at room temperature for at least 6 hours.
Auto-sampler stability (doped solvent and doped living blank): The stability of the formazan is assessed over the anticipated run time for the batch size. Samples are kept on the auto-sampler until the next measurements. Evaluation of re-injections from the same vials is included.
Short term lab stability (doped living blank): This corresponded to the time frame that is to be expected in performance of the *in vitro* eye hazard identification test method i.e. completion of test method procedures including tissue treatment, spectrophotometry reading and data interpretation before the samples would be analyzed by HPLC/UPLC-spectrophotometry from 4 to 24 hours (based on the expected duration that samples would remain at room temperature in the intended study).
Long term storage stability (doped living blank): Evaluation of long-term stability enabled unknown samples to be analyzed up to at least a month at -20°C after generation. In this circumstance, clear definition of storage conditions is required (i.e. in the refrigerator or at room temperature according to the laboratory storage conditions).

Using the approach in the US FDA guidance document as a basis application of these key parameters, with associated acceptance criteria, to the validation of the analytical method on HPLC/UPLC-spectrophotometry for measurement of formazan to demonstrate acceptability of this analytical technique as an additional endpoint is summarized in the Table below.

Key parameters and acceptance criteria for qualification of an HPLC/UPLC-photometry system for measurement of MTT formazan extracted

Parameter	Protocol Derived from FDA Guidance	Acceptance Criteria
Selectivity	Analysis of isopropanol, living blank (isopropanol extract from living tissues without any treatment), dead blank (isopropanol extract from killed tissues without any treatment), and of a dye (e.g., methylene blue)	$Area_{interference}$ ≤ 20% of $Area_{LLOQ}$[1]
Precision	Quality Controls (MTT formazan) in isopropanol (n=5)	CV ≤ 15% or ≤ 20% for the LLOQ
Accuracy	Quality Controls in isopropanol (n=5)	%Dev ≤ 15% or ≤ 20% for LLOQ
Matrix Effect	Quality Controls in living blank (n=5)	85% ≤ ME% ≤ 115%
Carryover	Analysis of isopropanol after an ULOQ[2] standard	$Area_{interference}$ ≤ 20% of $Area_{LLOQ}$
Reproducibility (intra-day)	3 independent calibration curves (based on 6 consecutive 1/3 dilutions of MTT formazan in isopropanol starting at ULOQ); Quality Controls in isopropanol (n=5)	Calibration Curves: %Dev ≤ 15% or ≤ 20% for LLOQ Quality Controls: %Dev ≤ 15% and CV ≤ 15%
Reproducibility (inter-day)	Day 1: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 2: 1 calibration curve and Quality Controls in isopropanol (n=3) Day 3: 1 calibration curve and Quality Controls in isopropanol (n=3)	
Short Term Stability of MTT Formazan in Tissue Extract	Quality Controls in living blank (n=3) analysed the day of the preparation and after 24 hours of storage at room temperature	%Dev ≤ 15%
Long Term Stability of MTT Formazan in Tissue Extract, if required	Quality Controls in living blank (n=3) analysed the day of the preparation and after several days of storage at -20°C	%Dev ≤ 15%

[1]LLOQ: Lower Limit of Quantification, defined to cover 1-2% tissue viability
[2]ULOQ: Upper Limit of Quantification, defined to be at least two times higher than the highest expected MTT formazan concentration in isopropanol extracts from negative controls

Annex 5: Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or colour interfering chemicals