EYE IRRITATION TEST METHOD: LIQUID

IN VITRO PREDICTION ASSAY FOR ACUTE OCULAR IRRITATION OF LIQUID CHEMICALS

EYE IRRITATION TEST LIQUID:

SkinEthic™ Human Corneal Epithelial Model

(SkinEthic™ HCE)

S.O.P

CONTENTS

ABBREVIATIONS & DEFINITIONS

°C: Degree Celsius
µL: microliter
%: Percent
C: Classified
EC: European Commission Regulation
EITL: Eye Irritation Test Liquid
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 tissue
LLOQ: Lower Limit Of Quantification
mg: milligram
Min: minute
mL: milliliter
MTT: 3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NC: Not Classified
NgC: Negative Control
nm: nanometer
NSC_{killed}: No Specific Color in killed tissues – killed tissue without MTT incubation
NSC_{living}: Non Specific Color in living tissues – living tissue without MTT interaction
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: in vitro Reconstructed human Tissue
RT: Room Temperature
TT: Test Treatment
ULOQ: Upper Limit Of Quantification
UN GHS: United Nations Globally Harmonized System
UPLC: Ultra-high Performance Liquid Chromatography
V: volume

A. PROTOCOL INTRODUCTION

IN VITRO EYE IRRITATION TEST USING THE SkinEthic™ HCE TEST SYSTEM

The irritation potential of a test chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Corneal Epithelium (SkinEthic™ HCE) model (www.episkin.com). The tissue model is provided as a ready to use tool.

A.1 OBJECTIVES AND APPLICATION

Type of testing:
Replacement

Level of assessment:
Toxic potential, hazard identification

Purpose of testing:
Classification and labelling of test chemicals regarding their eye irritation potential.

Context of use:
The potential to induce eye irritation is an important consideration included in procedures for the safe handling, packing and transports of chemicals.
Current OECD guideline 405 (OECD, 2002) for acute eye irritation is based on the test method described by Draize (Draize et *al.* 1944). The test currently involves applying 0.1 mL or 0.1 g of test chemical to the eye of conscious rabbits for one to twenty-four hours. The animals are observed for up to 21 days, for signs of corneal opacity, area of injury, iritis, conjunctival redness and oedema, and discharge in the tested eye, allowing a subjective scoring of the different effects by the observer.
The European Union bans *in vivo* eye irritation testing on ingredients for cosmetic purpose in line with the 7th Amendment to the EU Cosmetics Directive deadlines, *Directive 76/768/ECC*. Prediction of eye irritation is also an international regulatory requirement for the REACH chemical legislation (Registration, Evaluation, Authorization and Restriction of Chemicals, June 2007). Therefore, a test strategy is needed to replace the regulatory Draize eye irritation test according to Method B.5 of EU Regulation 440/2008 (EU, 2008) or OECD TG 405 (OECD, 2002). It is necessary to set up and validate *in vitro* approaches in order to predict eye irritation without the need for animals.

Specific purpose of the test method:
The present test method is designed to predict and classify the ocular irritant potency of test chemicals according to safety regulations, using the Reconstructed Human Corneal Epithelium model (SkinEthic™ HCE). The validity (relevance and reliability) of the present test method has been demonstrated to discriminate test chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from testchemicals requiring classification and labelling

(Category 1 and Category 2) according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) and as implemented by the EU Classification, Labelling and Packaging regulation (EU CLP) (UN, 2011; EC, 2008) (Alépée *et al*., 2016a). The test method is not intended to differentiate between UN GHS/EU CLP Category 1 (serious eye damage) and UN GHS/EU CLP Category 2 (eye irritation). This differentiation would be left to another tier of a test strategy as described e.g., by Scott L. et *al.* (2010).

Applicability domain, known limitations:
The test method only classifies liquid test chemicals in 2 classes: as "No Category" or as eye classified (Category 1 / Category 2) according to UN GHS and EU CLP. The test method allows the hazard identification of mono and multi-component test chemicals. Gasses and aerosols as well as solid cannot be evaluated with the current protocol. Specific optical properties (color) of the test chemical or its particular interaction with the reagent (MTT reducers) or the tissue (tissue penetration, poor rinsing) may lead to some false estimates of the viability. In these cases additional controls must be used to detect and correct the test chemical interference with the measurement.

A.2 BASIS OF THE TEST METHOD

Acute irritation is a local inflammatory response of the eye to direct injury caused by the application and contact of an irritant liquid test chemical.

The test method consists of a topical exposure of the liquid test chemical onto the SkinEthic™ HCE, test system followed by cell viability assessment. Viability decrease in test chemical treated tissues is expressed comparatively to negative control. Percent (%) viability is used to predict and classify eye irritation potential following a defined prediction model.

A.3 EXPERIMENTAL DESCRIPTION

A.3.1 Endpoint

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]*, and conversion into a blue formazan salt that is quantitatively measured after extraction from tissues (Mossman, 1983). The reduction of cell viability in treated tissues is compared to negative controls and expressed as a % value. Measurements rely on optical density measurements at 570 nm (filter band pass ± 30 nm) by using a spectrophotometer microplate reader. As strongly colorant chemicals can interfere with this detection method, HPLC/UPLC-spectrophotometry is recommended to overcome this problem. Besides, this alternative endpoint detection can be used for all chemicals belonging to the applicability domain.

A.3.2 Endpoint value

The reduction of cell viability in test items treated tissues is compared to negative control (100% viable) and expressed as a %. The MTT reduction % in viability is used to predict the ocular damage potential.

A.3.3 Test 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, 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 about 4 to 7 cell layers, including columnar cells and wing cells.

A.3.4 Brief basic procedure

Upon receipt, the SkinEthic™ HCE tissue cultures are placed onto 1 mL fresh maintenance medium (6-well plate) and incubated overnight in standard culture conditions *(37°C, 5% CO_2, ≥ 95% humidity).* Following this equilibration period, the cultures are then transferred into a 24-well plate containing 300 µL fresh maintenance medium per well. 30 µL of test liquid chemical are applied topically onto the SkinEthic™ HCE tissues for 30 minutes ± 2min in standard culture conditions. Two tissue replicates are used per test chemical (Test Treatment: TT), concurrently to positive (PC) and negative (NgC) controls. After 30 minutes ± 2min treatment (Treatment Period), tissues are then rinsed to remove the test chemical and immersed into 1,5 mL fresh medium (750 µL underneath and 750 µL topically) for 30 minutes ± 2min in standard culture conditions (Post-Soak Period). The medium is then removed and the MTT assay is performed by transferring the tissues to wells containing 0.3 mL MTT medium (1 mg/mL). After 3 hrs MTT ± 15 min incubation at 37°C, 5% CO_2, the blue formazan salt formed is extracted with 1.5 mL isopropanol per tissue (24-well plates, extraction time : from a minimum of 4 hrs (at RT with shaking at 300 rpm) to overnight (until 96 hrs, 4°C, without shaking).
Two 200 µL aliquots per extract are transferred into a 96-well plate and the optical density is determined using a spectrophotometer at 570 nm (filter band pass ± 30 nm). Percentage viability of each treated tissue (test chemical) is individually calculated relative to the mean OD of Negative Control (100% viable).
An alternative endpoint could be used to determine the cell viability. The formazan extract samples could be analyzed by measuring its absorption spectrum at a single wavelength (555 nm) by HPLC/UPLC-spectrophotometry (Alépée *et al*., 2015; 2016b).
Based on the viability (expressed as %) endpoint, a cut-off was set at 60% reduction of viability relative to the Negative Control to define the eye severity potential of the test chemical (Alépée *et al*. 2016a).

Quick flow chart: *IN VITRO* HCE EYE IRRITATION TEST METHOD FOR LIQUIDS

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

↓

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

↓

Transfer tissues to fresh maintenance medium in 24-well plates

↓

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

↓

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

↓

Rinse with PBS^- without Ca^{2+} & Mg^{2+} (20 mL: 10mL/jet)

↓

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

↓

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

↓

Viability: Transfer tissues into MTT solution

↓

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

↓

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

↓

Extract formazan (minimum 4 hours, RT or overnight, 4°C)

↓

Perforate the insert and homogenize formazan extract

↓

Read OD with microplate spectrophotometer at 570 nm and/or analyse the extract samples by HPLC/UPLC-spectrophotometry

A.4 DATA ANALYSIS / PREDICTION MODEL

According to UN GHS and EU CLP classification, if the % viability is > 60%, the test chemical is predicted as Not Classified (No Category). If the % viability is ≤ 60%, the test chemical is predicted as Classified (Category 1 / Category 2).

***In vitro* Result**	**Classification (*In vivo* Prediction)**
Mean tissue viability > 60%	No category
Mean tissue viability ≤ 60%	Classified, Category 1 / Category 2

The prediction model does not discriminate UN GHS Cat. 1 from Cat. 2 classes.

A.5 TEST CHEMICALS & RESULTS SUMMARY

Not applicable at this stage.

A.6 DISCUSSION

Ethical issues: *in vitro* system

Special equipment: no specific equipment needed (classical laboratory devices)

Amount of training required: one training session is required before using the test method

A.7 STATUS

The *in vitro* Reconstructed human Tissue (RhT) test system, SkinEthic™ HCE for the eye irritation potential of test chemical, is currently in validation peer review process and OECD Test Guidelines program.

A.8 PROPRIETARY &/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.

B. TECHNICAL DESCRIPTION

Contact
Full Address: EPISKIN
4, rue Alexandre Fleming
69366 - LYON
France
Tel: +33 (0)4 37 28 22 00
E-mail: info@episkin.com

B.1 TEST SYSTEM

Reconstructed Human Corneal Epithelium SkinEthic™ HCE model

The SkinEthic™ HCE tissues**, ref: RHC/S/5 model** (www.episkin.com) are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm²) with the necessary culture media (maintenance medium). Each SkinEthic™ HCE tissue is controlled by the manufacturer.

Health and Safety issues

Results of the quality controls are supplied with the tissues. The epithelial cells are negative to anti-HIV and hepatitis C antibodies, and to hepatitis B antigens. The absence of mycoplasma has been verified on culture supernatant. 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.

The SkinEthic™ HCE tissues are produced by Episkin SA (France). 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 kits	Comment
Plate containing SkinEthic™ HCE units (area: 0.5 cm^2)	Tissues are shipped on semi solid agar's medium in order to maintain good shipment conditions for the tissues.
Bottle of Maintenance Medium	Basic culture medium for incubations

B.2 EQUIPMENT

B.2.1 Fixed equipment

● Microbiological safety cabinet (laminar flow hood)	→ *safe work under sterile conditions*
● Non-sterile ventilated cabinet	→ *safe work with chemicals, applications, washes*
● Cell incubator 37°C , 5% CO_2, ≥ 95% humidity	→ *tissues incubations*
● Plate reader (96 well) with a 570 nm (filter band pass ± 30 nm)	→ *Optical Density readings (MTT)*
● Laboratory balance (accuracy 0.1 mg)	→ *chemicals weighing*
● Shaker plates	→ *shaking before reading (formazan extraction sample)*
● HPLC/UPLC-*UV/Visible Analytical system*	→ *measurement of formazan extraction samples (UV/Visible detector with mono-wavelength in visible is enough, even if a photodiode array detector is preferred).*

B.2.2 Consumables

✓	1 sterile bottle	*reconstituting MTT reagent stock 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 combitips	*for rinsing tissues with 10 mL PBS-*
✓	Adjustable multi-step pipette, 25 mL combitips	*distributing 1 mL maintenance medium*
✓	Adjustable multi-step pipettes, 5 mL combitips	*distributing maintenance medium, MTT, PBS - and isopropanol*
✓	Adjustable micro-pipette – 0 to 200µL	*pipetting 200 µL formazan extracts*
✓	Adjustable positive displacement micro-pipette 0- 50 µL	*application of 30 µL*
✓	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 extraction*
✓	Cotton tip swabs	*drying the tissue surface*
✓	Nylon mesh Ø 0.5cm² (supplier SkinEthic MESH/0.5)	*for sticky or viscous liquid*
✓	*HPLC/UPLC vial (Waters ref: 186000385C as suggested)*	*HPLC/UPLC measurement*
✓	*HPLC/UPLC reverse phase column*	*HPLC/UPLC measurement*

B.3 MEDIA AND REAGENTS

✓ Isopropanol (CAS 67-63-0)	*formazan extraction*
✓ MTT reagent (3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide (CAS 298-93-1, Sigma M2128 as suggested)	*viability measurements, Viability reagent*
✓ Dulbecco's D-PBS without Ca^{2+} & Mg^{2+} GIBCO 14190-144 or equivalent without Ca^{2+} & Mg^{2+} (PBS^-)	*rinsing tissues , Negative Control, MTT solubilisation*
✓ Methyl acetate (CAS 79-20-9, Sigma 45999 as suggested)	*Positive Control*
✓ Sterile distilled water	*liquid test dilutions and checking for color test chemical*
✓ Tissue maintenance medium (SkinEthic™)	*tissues culture, incubations and MTT dilution*
✓ *SolvantsHPLC/UPLC grade*	*HPLC/UPLC measurement*
✓ *Formazan (CAS 37360-69-7 purity > 97%, Sigma 88417 as suggested)*	*HPLC/UPLC validation system*

B.4 PREPARATIONS

Safety precautions:
MTT (R26, R68, R22, 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.

B.4.1 Negative Control

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

B.4.2 Positive Control

Methyl acetate is used as Positive Control (PC).

NgC and PC should be tested concurrently to the test chemical.

B.4.3 Test chemical

Test chemical (+/- color, +/- MTT reducer) is topically applied onto HCE tissues.

B.4.4 Formazan Extraction solution

Isopropanol (100%) is used as Formazan Extraction solution.

B.4.5 MTT Solutions preparation

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

<u>MTT stock solution preparation</u>

- Prepare a 5 mg/mL solution in PBS^-
- Thoroughly mix this stock solution during 15 minutes at RT.
- Keep at 4°C protected from light up to 16 days.

<u>MTT ready to use solution preparation</u>

- 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).

B.4.6 Water- killed epithelium preparation (for MTT-interacting chemicals or MTT pre-check inconclusive 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°C, 5% CO_2, in a > 95% humidified for **24 hrs ± 1 hr**
3) At the end of the incubation, discard the water
4) Keep killed epithelium frozen (dry) in freezer at -18°C to -20°C (dead 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 300 µL maintenance medium)
6) Further use of thawed killed tissues is similar to living tissues.
7) Apply Negative Control and test treatments on dead tissues from the same batch.
8) Further use of thawed killed tissues is similar to living tissues.
9) Perform this assay only once (on duplicate tissue) per test chemical when necessary.

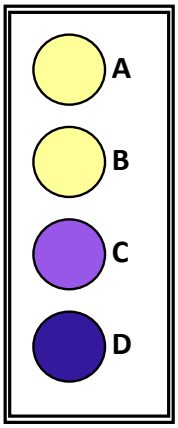
B.5 METHOD

B.5.1 Checking for direct MTT reduction of test chemicals

<u>When OD is chosen as endpoint for viability assessment:</u>

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

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

- Incubate the mixture for 3 hours ± 15 minutes at 37°C protected from light (test conditions).

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 epithelial 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 two killed tissues. In addition to that, two killed tissues are treated with PBS^- as control (negative control killed tissue = KU).

- For details see section B.5.4 Summary of adapted controls depending of test chemical physical properties.

The evaluation of direct MTT reduction of test chemical (steps 1 and 2) is performed only on one occasion (one single run).

- Document once: 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.7.3).

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

Same procedure as for OD measurement

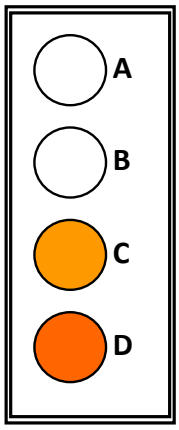
B.5.2 Checking for color test chemicals only

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 section B.7.4).



A: control
B: Test chemica 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 off course another color.

Step 1:

- Fill Eppendorf tubes with 90 µL of water

- Add 10 µL of the test chemical to be evaluated

- Vortex the solution for a few seconds

- Incubate the solution for 30 min ± 2 minutes 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.

When a colored solution is observed, it is then necessary to evaluate during the future studies the part of OD due to the non specific color (i.e. by using living epithelial 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 (**concurrently to every testing: i.e: for each series**).

Step 2 :

- Each intrinsically colored test chemical is applied on two living tissues. For details see section B.5.4 Summary of adapted controls depending of test chemical physical properties (Condition 3).

- Coloring test chemical controls are treated and handled like normal sample except that they **do not get into contact with MTT solution**: during the MTT assay, they are incubated in **Maintenance Medium** instead of MTT solution.

- Document once: 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 coloring test chemical), for a test chemical before calculating the final % viability (see specific calculation section B.7.4).

When HPLC/UPLC-spectrophotometry is chosen as endpoint:

No pre-check or control are necessary

B.5.3 Checking for color test chemicals with possible MTT direct interaction

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 adopted control is needed. Each coloring test chemical is applied onto 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.5.4 Summary of adapted controls depending of test chemical physical properties). **The evaluation of NSC_{killed} is performed only on one occasion (one single run).**

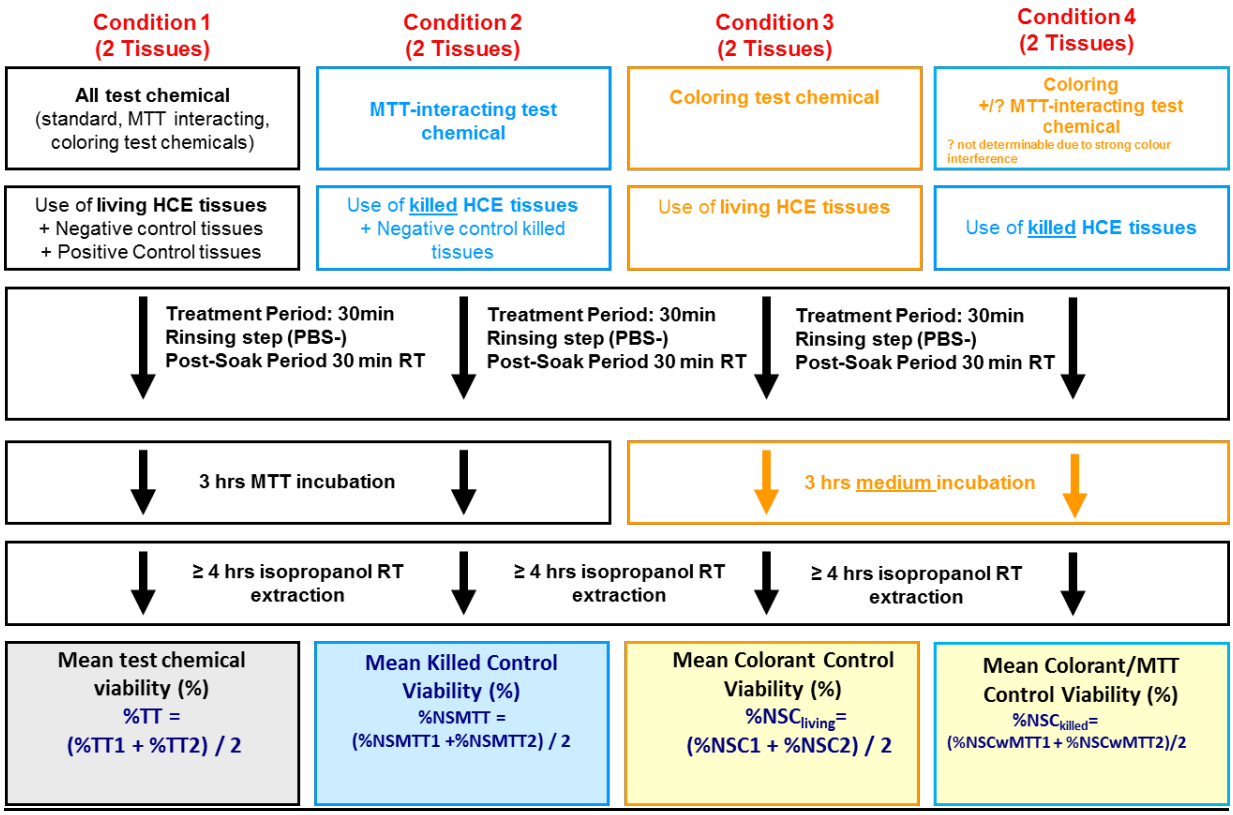
- Documents: Evaluation of Test chemicals – MTT direct interaction (Annex 1) and Evaluation of Test chemicals – Color interaction (Annex 2).
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration on killed tissue (to define the $\%NSC_{killed}$ value for a coloring test chemical), for a test chemical before calculating the final % viability (see specific calculation section B.7.5).

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, alternative separation method should be considered. Evaluation of direct MTT reduction will be performed to define the %NSMTT (Killed tissue with MTT incubation) (see section B.5.5 Summary of adapted controls depending of test chemical physical properties (When HPLC/UPLC-spectrophotometry method is chosen).

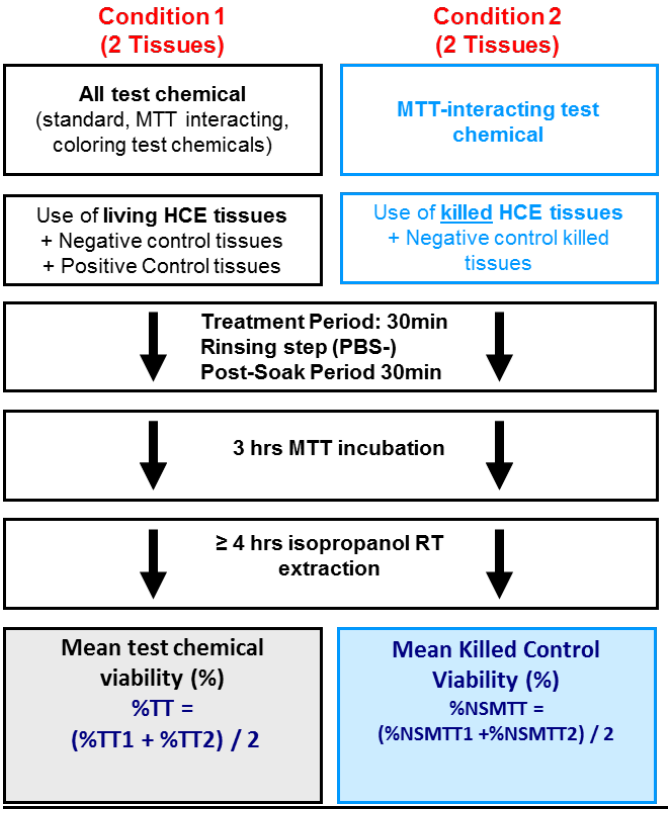
B.5.4 Summary of adapted controls depending of test chemical physical properties (when OD method is chosen)



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

B.5.5 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**

B.5.6 Test system procurement

Reconstructed Human Corneal Epithelium SkinEthic™ HCE model

The SkinEthic™ HCE tissues, **ref: RHC/S/5 model** (www.episkin.com) are reconstructed human corneal epithelium units (epithelium surface: 0.5 cm^2) shipped with the necessary culture media (maintenance medium). Each SkinEthic™ HCE tissue is controlled by the manufacturer.

B.5.7 Test chemical exposure procedure.

B.5.7.1 Tissue conditioning prior to testing

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.
- Verify the absence of air bubbles below tissues and incubate them at least overnight at 37°C, 5% CO_2, ≥ 95% humidity until treatment steps.

Tissue conditioning

- Pre-warm the maintenance medium at RT.
- Before test chemical treatment, label the plate lid with the appropriate test chemical information.

  Note: One plate is used for one treatment condition (NgC, PC and Tested chemical)

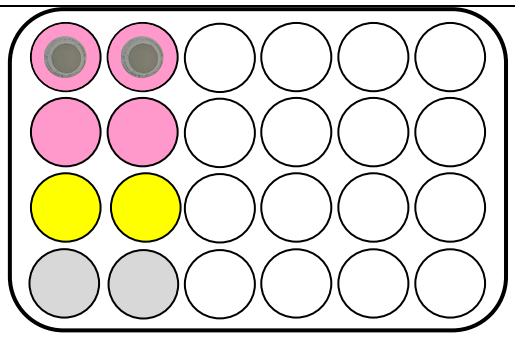
- Dispense the pre-warmed maintenance medium into a sterile 24-well plate : 2 wells with 300 µL for the 30 min treatment period, 2 wells with 750 µL for the 30 min post-soak period after the rinsing step.
- Pre-warm the maintenance medium dispensed on the plates at 37°C.
- Transfer 2 SkinEthic™ HCE epithelia units into the 2 first wells filled with 300 µL maintenance medium (1 Test chemical / 2 replicate tissues).
- Verify the absence of air bubbles below the tissues.

B.5.7.2 Tissue treatment

Safety instructions:

1. *Test chemicals should be handled following material safety datasheet. Store the test chemicals in ventilated safety cup boards. 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.*

Application/rinsing/MTT plate design (see example)



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

- 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.

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

- Dispense **30 µL ± 2µL** 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 minutes ± 2 minutes at 37°C, 5% CO_2, ≥ 95% humidity with lids on.

Record time and details in the documentation sheet (Annex 3: Incubation timings).

End of treatment and rinsing procedure

At the end of the exposure (30 min ± 2 min), the test chemical (and Controls) will be removed by rinsing tissues with **PBS⁻**. Tissues are rinsed by using a multi-pipette and adapted 50 mL combitips. Adjust the distribution to 10 mL per push.

- 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).
- 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). For a viscous test chemical, a cotton swab could be used to optimize the rinsing step (always in a full PBS⁻ well).
- Remove remaining PBS⁻ onto the tissue by energised reversals.

Note: *For tissues on which the test chemical has not been removed by standard washing procedure with PBS⁻, an alternative option is proposed (high viscosity). Fulfill insert with PBS⁻ onto the tissue and use cotton swabs to gently remove any residual test chemical avoiding contact with tissue.*

Note: *Do not use cotton swab to dry the tissues at this step.*

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 minutes ± 2 minutes** at 37°C, 5% CO_2, ≥ 95% 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 (Fig. A) and the surface with a cotton swab (Fig B).

- Document: (Annex 3: Incubation timings).

Figure A

Figure B

B.5.8 MTT conversion test

The MTT conversion test is carried out at the **end of the 30 min ± 2min immersion** period.

- Document (Annex 3: Incubation timings).

Note: *Additional specific tissue controls for coloring test chemicals will be incubated with the* ***maintenance medium*** *(not with the MTT solution – see section B.5.2).*

- Prepare MTT medium according to section B.4.5
- Dispense **300 µL** of MTT medium in 2 wells of the 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).
- 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) (3th line of plate design example). Verify the absence of air bubbles under the tissues.
- Incubate tissues for **3 hours ± 15 minutes** at 37°C, 5% CO_2, ≥ 95% 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). See plate example section B.5.7.2)
- Record starting time of MTT incubation (Annex 3: Incubation timings).
- 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 chemicals that may have remained on the tissue or with strongly colored test chemicals, tissues could be extracted from the bottom only (1.5 mL isopropanol into 12 well plate). During extraction, plates should be covered with "parafilm" to prevent evaporation. For extraction: at 4°C or RT 3 layers as

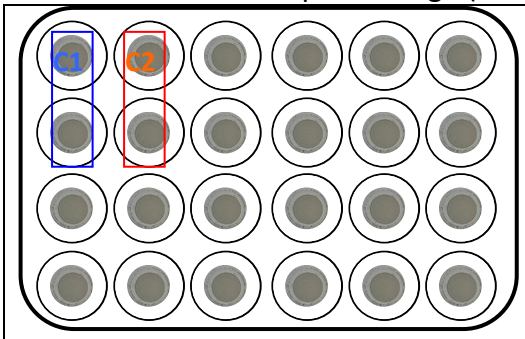
follows: 2 layers on top of the wells under the lid. Put the lid on and cover with a third sheet of parafilm around the lid and plate (Figures C & D).

- Extract the formazan crystals either

  - For 4 hours at RT (minimum) protected from light with gentle shaking on plate shaker (~ 300 rpm),
  - Or overnight at 4°C protected from light without shaking. The following day, shake at least 30 minutes at RT on plate shaker (~ 300 rpm).
  - Alternatively, extraction at 4°C protected from light without shaking can be conducted over the week end. Plates should be then carefully covered. After extended incubation period, shake at least 30 minutes at RT on plate shaker (~ 300 rpm). 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 (Figures E & F).
- Remove the empty insert.

Formazan extraction - plate design (see example)



1st column: Test chemical 1 (C1)

2nd 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.

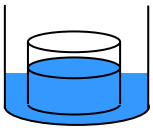


Figure C

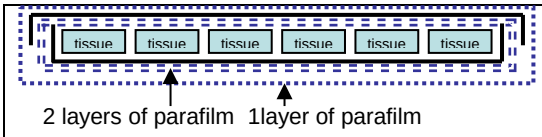


Figure D

Figure E

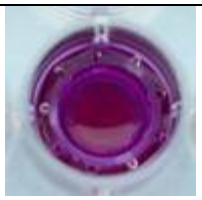


Figure F

- 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 according to the fixed plate design given in spread sheets, or similar laboratory specific design labelled appropriately, according to the example below. Do not use the "empty" wells for measurements (see example below).

For conditions 1 and 3:

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 1
B	NC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	
C	NC	PC	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	Tissue 2
D	NC	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	

NC = negative control; PC = positive control; TT … 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	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	Tissue 2
D	KU	TT1	TT2	TT3	TT4	TT5	TT6	TT7	TT8	TT9	TT10	empty	
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	

KU = Negative control killed tissue; TT1 … TT13 = Test treatment 1… 13; BL = blank (isoporanol 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.*

OD endpoint:

- Read Optical Density (OD) by using a spectrophotometer microtiter plate reader equipped with a 570 nm filter (pass-band max ± 30 nm), without using a reference filter.
- Use isopropanol as blank (200 µL / well).
- Link OD values with the appropriate treatment conditions and replicate on the raw data documents (or files).
- Perform the Quality Control of the raw data.

HPLC/UPLC-spectrophotometry endpoint:

- **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 nm), without using a reference filter.
- Use isopropanol as blank (200 µL / well).
- **For all conditions included negative control:** transfer at least 100 µl µl into an HPLC vial (samples can also be frozen at -20°C during 2 months maximum)
- Use a validated analytical method (Annex 4: HPLC/UPLC-spectrophotometry qualification system) 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.
- All data generated by the HPLC/UPLC-spectrophotometry system should be printed (or saved as a '.pdf' files) and considered as raw data.
- 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.

B.6 ACCEPTANCE CRITERIA

B.6.1 Definitions

- **Series:** a set of up to 13 test chemicals plus Negative Control (NgC) and Positive Control (PC) all concurrently tested on 2 tissues replicates.
- **Run**: a set of up to maximum 3 series can be conducted within the same day.

- **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).

B.6.2 Acceptance Criteria for Test and Run Results

Common acceptance criteria

1) The mean Optical Density (OD_{NgC}) at 570 nm (± 30 nm) of the two replicate tissues treated with NgC is ≥ 1.4 with an upper acceptance limit of ≤2.5.

The acceptance criteria of the NgC by absorbance (optical density) should be met

2) The Mean Viability of the two replicate tissues (2 values from each of the two tissues) treated with PC, expressed as % of the negative control, is ≤ 30%.

3) The difference of viability between the two replicate tissues of a single test chemical is ≤ 20 in the same run whatever the test item (for PC, NgC, TT and all adapted controls).

Specific HPLC acceptance criteria

An HPLC/UPLC run begin with the injection of 5 samples of 20 µg/mL of formazan and 5 samples of 200 µg/mL of formazan.

Measure peak area at the retention time of the formazan at the wavelength defined in the validated analytical method. Calculate Mean peak area at both level ($Mean_{20\ or\ 200}$) and standard deviation ($SD_{20\ or\ 200}$).

Retention time of formazan has to be consistent with those defined in the validation.

The %CV at both level ($\%CV_{20\ or\ 200}$= $SD_{20\ or\ 200}$*100/ $Mean_{20\ or\ 200}$) of these samples must be inferior at or equals to 15%.

During the run, every 20 samples, one QC sample of formazan at 200 µg/mL and another at 20 µg/mL (named $QC_{20\ or\ 200}$) are analyzed. Measure peak area at the retention time of the Formazan at the wavelength for each $QC_{20\ or\ 200}$.

The %Dev calculated with samples injected at the beginning of the run [$\%Dev_{20}$ =(QC_{20} – $Mean_{20}$)*100/ $Mean_{20}$ and $\%Dev_{200}$ =(QC_{200} –$Mean_{200}$)*100/ $Mean_{200}$] must be inferior at or equal to 15%.

If $QC_{20\ or\ 200}$ samples are not valid, the previous and the following set of 20 samples are invalided.

Notes:

i) A run is qualified (qualified run) if both the Negative and the Positive controls data fulfil the above criteria requirements. Otherwise, the run will be considered as non-qualified. Non-qualified runs have to be documented and reported.

ii) The absolute OD or Area values of the Negative Control (NgC) tissues and the % viability of the Positive Control are indicators of tissue response capacity in the testing laboratory after shipping and storage procedures and under use conditions.

iii) For a given test chemical, if the difference of viability between the two relating replicate tissues exceeds 20, the test chemical should be retested in one additional run. Non-qualified tests have to be documented and reported.

B.7 DATA ANALYSIS / CALCULATION STEPS AND RESULTS

B.7.1 Calculation steps

Main steps automatically followed by the prepared form:

(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: evaluated by calculating the viability difference between the two tissue replicates (for NgC, PC and test chemical).

B.7.2. For viability Tests only – Normal calculation procedure (Condition 1)

B.7.2.1- Calculation for OD reading

- MEAN OD CALCULATION:

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

Individual OD Negative Control (NgC)

$OD_{NgC1} = OD_{NgC1raw} - OD_{blank\ mean}$

$OD_{NgC2} = OD_{NgC2raw} - OD_{blank\ mean}$

Mean OD Negative Control **$OD_{NgC} = [OD_{NgC1} + OD_{NgC2}] / 2$**

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

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

OD Positive Control (PC)

$OD_{PC1} = OD_{PC1\ raw} - OD_{blank\ mean}$

$OD_{PC2} = OD_{PC2\ raw} - OD_{blank\ mean}$

Mean OD Positive Control **$OD_{PC} = [OD_{PC1} + OD_{PC2}] / 2$**

<u>*Test Treatment</u>

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 **$OD_{TT} = [OD_{TT1} + OD_{TT2}] / 2$**

- 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.7.2.2- Calculation for HPLC/UPLC - spectrophotometry endpoint

- MEAN AREA CALCULATION:

*Negative Control (NgC)

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

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

*Positive Control (PC)

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

*Test Treatment

Mean Area Test Treatment **$Area_{TT} = [Area_{TT1} + Area_{TT2}] / 2$**

- VIABILITY CALCULATION: Individual means viabilities (%)

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

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

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

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

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

%mean Test Treatment **$\%TT = (\%TT1 + \%TT2)/2$**

The mean relative viability is used for classification according to the prediction model (section B.8).

B.7.3. MTT interacting test chemical calculation procedure (Condition 2)

Data calculations for MTT interacting chemicals

Test chemical s 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.7.3.1- Calculation for OD reading

- NON SPECIFIC MTT REDUCTION CALCULATION (%NSMTT):

% Killed Test Treatment 1 $\%NSMTT1= [(OD_{kt1} - OD_{ku})/ OD_{NgC}]x100$
% Killed Test Treatment 2 $\%NSMTT2= [(OD_{kt2} - OD_{ku})/ OD_{NgC}]x100$

% 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 $\%FV_{C\ NSMTT} = (FV_{C\ NSMTT1} + FV_{C\ NSMTT2})/2$

B.7.3.2- Calculation for HPLC/UPLC reading

- 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 (section B.8).

B.7.4. Coloring test chemicals calculation procedure (Condition 3)

Data calculations for dyes and coloring test chemicals able to stain tissues.
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 %. 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)

1- NON SPECIFIC COLOR CALCULATION ($\%NSC_{LIVING}$)

% Non-Specific Color tissue 1	$\%NSC_{living}1 = [(OD_{TT1\text{-}MTT} / OD_{NgC}] \times 100$
% Non-Specific Color tissue 2	$\%NSC_{living}2 = [(OD_{TT2\text{-}MTT} / OD_{NgC}] \times 100$
Mean % Non-Specific Color	$\mathbf{\%NSC_{living} = (\%NSC_{living}1 + \%NSC_{living}2)/2}$

2- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1	$\%FV_{C\ NSC_{living1}} = \%\ TT1 - \%NSC_{living}$
%Final viability Test Treatment 2	$\%FV_{C\ NSC_{living2}} = \%\ TT2 - \%NSC_{living}$
Mean Final Viability	$\mathbf{\%FV_{C\ NSC_{living}} = (FV_{C\ NSC_{living1}} + FV_{C\ NSC_{living2}})/2}$

The mean final viability is used for classification according to the prediction model (section B.8).

B.7.5. 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 'Killed+MTT' controls together with 'Living-MTT' controls (NSC_{living}).

However, the "Non-Specific MTT Reduction" (NSMTT) obtained with 'Killed+MTT' controls 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 controls: 'Killed-MTT' (NSC_{killed}). This 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 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 **$\%NSC_{killed} = (\%NSC_{killed1} + \%NSC_{killed2})/2$**

2- CORRECTED FINAL VIABILITY (FV_C)

%Final viability Test Treatment 1 $\%FV_{C\ TT1} = \%\ TT1 - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
%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 (section B.8).

B.7.6. Remarks

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:

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

– IF the mean of $\%NSC_{living}$ or %NSMTT of qualified test is greater than (>) 50% AND their classification (C or NC) 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 of qualified test is greater than (>) 50% AND the classification of the qualified test 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, category 2) should be considered.

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

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
	Sect. B7.2	Sect. B7.3	Sect. B7.4	Sect. B7.5		

B.8 PREDICTION MODEL

A test chemical is predicted as ocular irritant, according to the United Nations Globally Harmonized System (UN GHS) of Classification and Labelling of Chemicals and as implemented in the European Commission Regulation (EC) No 1272/2008 on classification, labelling and packaging of chemicals and mixtures (EU CLP), if the mean relative tissue viability (%) of two tissues exposed to the test chemical is ≤ 60%.

Prediction Model according to UN GHS classification implemented in the European Union (EU CLP)

***In vitro* Result**	**Classification (*In vivo* Prediction)**
Mean tissue viability > 60 %	No Category
Mean tissue viability ≤ 60 %	Classified, Category 1 / Category 2

The prediction model does not discriminate the Cat. 1 and Cat. 2 classes.

References

Alépée N., Bessou-Touya S.,Cotovio J., de Smedt A., de Wever B., Faller C., Jones P., Le Varlet B., Marrec-Fairley M., Pfannenbecker U., Tailhardat M., van Goethem F., McNamee P., 2013. Cosmetics Europe multi-laboratory pre-validation of the SkinEthic™ reconstituted human corneal epithelium test method for the prediction of eye irritation. Toxicology in Vitro 27, 1476–1488

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, and McNamee, P. 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. 2015. Toxicol in Vitro 29, 741–761.

Alépée, N., Leblanc, V., Adriaens, E., Grandidier, M.H., Lelièvre, D., Meloni, M., Nardelli, L., Roper, C.S., Santirocco, E., Toner, F., Van Rompay, A.R., Vinall, C.J., Cotovio, J., 2016a. Inter-laboratory evaluation of SkinEthic HCE test method for testing serious eye damage/eye irritation on liquid chemicals. Toxicol in Vitro 31, 43–53.

Alépée, N., Adriaens, E., Grandidier, M.H., Meloni, M., Nardelli, L, Vinall, C. J., Toner, F., Roper, C.S., Van Rompay, A.R., Leblanc, V., Cotovio, J., 2016b. Multi-laboratory evaluation of SkinEthic HCE test method for testing serious eye damage/eye irritation using solid chemicals and overall performance of the test method with regard to solid and liquid chemicals testing. Toxicology in Vitro, in press.

Cotovio, J., Grandidier, M.H., Lelièvre, D., Bremond, C., Flamand, N., Loisel-Joubert, S., van der Lee, A., Capallère, C., Meunier, J.R., Leclaire, J., 2007. The use of the reconstructed human corneal model (HCE) to assess in vitro eye irritancy of chemicals. ALTEX 14 (special issue), 343–350.

Cotovio, J., Grandidier, M.H., Lelièvre, D., Bremond, C., Amsellem, C., Maloug, S., Ovigne, J.-M., Loisel-Joubert, S., van Der Lee, A., Minondo, A.-M., Capallère, C., Bertino, B., Alépée, N., Tinois-Tessonneaud, E., de Brugerolle de Fraissinette, A., Meunier, J.R., Leclaire, J., 2010. In vitro assessment of eye irritancy using the reconstructed human corneal epithelial SkinEthic™ HCE model: application to 435 substances from consumer products industry. Toxicology in Vitro 24, 523–537.

Draize, J., Woodard, G., Calvery, H., 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharm Exp Ther 82, 377–390.

European Commission (EC) (2008) REGULATION (EC) No 440/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). *Official Journal of the European Union* **L142**, 1-739.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. Journal of Immunological Methods 65, 55–63.

Nguyen, D.H., Beuerman, R.W., De Wever, B. and Rosdy, M. (2003) Three-dimensional construct of the human corneal epithelium for *in vitro* toxicology. In Alternatives Toxicological Methods, edited by Salem, H.and Katz S.A., CRC press, 47-159.

OECD (2002) Test Guideline 405. OECD Guideline for the Testing of Chemicals: Acute Eye Irritation/Corrosion. Paris, France: Organisation for Economic Cooperation and Development. Available at:

[http://www.oecd.org/env/testguidelines].

OECD (2005). OECD Series on Testing and Assessment No. 34. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Paris, France: Organisation for Economic Cooperation and Development. Available at:
[http://www.oecd.org/env/testguidelines].

Scott L., Eskes C., Hoffmann S., Adriaens E., Alepée N., Bufo M., Clothier R., Facchini D., Faller C., Guest R., Harbell J., Hartung T., Kamp H., Varlet BL., Meloni M., McNamee P., Osborne R., Pape W., Pfannenbecker U., Prinsen M., Seaman C., Spielmann H., Stokes W., Trouba K., Berghe CV., Goethem FV., Vassallo M., Vinardell P., Zuang V. (2010) A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. Toxicol In Vitro ; 24(1):1-9.

United Nations, 2011. Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fourth revised edition, UN New York and Geneva. Available at:
[http://www.unece.org/trans/danger/publi/ghs/ghs_rev04/04files_e.html].

van Goethem, F., Adriaens, E., Alepee, N., Straube, F., de Wever, B., Cappadoro, M., Catoire, S., Hansen, E., Wolf, A., Vanparys, P., 2006. Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. Toxicology in Vitro 20, 1–17.

B.9 ANNEXES

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

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

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

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

Annex 3: Incubation timings

Series N°:…………..

Test chemical Name Or code	Treatment 30 min ± 2 min		Post-Soak Treatment 30 min ± 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/Start time (hh:mm)	Date and End time (dd:hh:mm)

Annex 4: HPLC/UPLC-spectrophotometry qualification system

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 B4.6.
- Standard sample: sample in IP with known concentration of formazan (CAS number 57360-69-7; purity > 97%) 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 RhT 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.

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

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.

Optional: 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)
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 anlytical 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 is 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 irritation 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 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 endpoint is summarized in the table below.

Parameter	**Acceptance criteria**
Selectivity	$Area_{interference} \leq 20\%$ of $Area_{LLOQ}$
Precision	Coefficient of Variation ≤15% or ≤20% for LLOQ
Accuracy	% deviation ≤15% or ≤20% for LLOQ
Matrix effect	85% ≤ Matrix Effect% ≤ 115%
Carryover	$Area_{interference} \leq 20\%$ of $Area_{LLOQ}$
Reproductibility (intra-day)	Calibration Curves: % deviation ≤ 15% or ≤ 20% of $Area_{LLOQ}$ Quality Controls: % deviation ≤ 15% and CV ≤ 15%
Reproductibility (inter-day)	
Short Term Stability of MTT Formazan in SkinEthic™ HCE tissue Extract	% deviation ≤ 15%
Long Term Stability of MTT Formazan in SkinEthic™ HCE tissue Extract	% deviation ≤ 15%