DB-ALM Protocol n° 135 : SkinEthic™ Skin Irritation Test

Skin Irritation and Corrosivity

The SkinEthic™ Skin Irritation Test (42 minutes application + 42 hours post-incubation) is designed for the prediction of acute skin irritation potential of chemicals by measurement of their cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RHE) model. The protocol is compliant with the OECD Test Guideline No. 439 - *In Vitro* Skin Irritation : Reconstructed Human Epidermis Test Method.

Résumé

The purpose of SkinEthic™ Skin Irritation Test is to predict and classify the skin irritation potency of test chemicals by using the SkinEthic™ Reconstructed human Epidermis (RHE) model.

The relevance and reliability of the present test method has been demonstrated to discriminate skin irritant test chemicals and mixtures (Category 2 (Cat. 2)) from chemicals Not-Classified for skin irritation (No Category (NC)) according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (OECD, 2010; UN, 2015). This test method does not allow the classification of chemicals to the optional UN GHS Category 3 (mild irritants) (Method B.46, EU, 2009; OECD, 2015a).

The SkinEthic™ RHE test method underwent optimisation and validation studies (Alépée et al., 2010; Tornier et al., 2010) and it was reviewed by the the European Centre for the Validation of Alternative Methods (EURL-ECVAM) Scientific Advisory Committee (ESAC) (ESAC, 2008). This method was included in the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) Number 439 "*In vitro* Skin Irritation: Reconstructed Human *Epidermis* (RHE) Test Method" which was firstly adopted in 2010.

SkinEthic™ Skin Irritation Test is applicable to solids, liquids, semi-solids and waxes.
The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water.
Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. For this method, gases and aerosols have not been assessed yet.
While it is conceivable that these can be tested using SkinEthic™ RHE technology, the current protocol does not describe how to evaluate gases and aerosols.

Specific optical properties (colour) 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 optical density (OD) measurement. This limitation has been addressed in a project in which the use High/Ultra High Performance Liquid Chromatography Performance (HPLC-UPLC)-spectrophotometry for endpoint detection of formazan was established (Alépée et al., 2015b). Following this study, an updated version of the OECD TG439 was adopted in 2015 (OECD, 2015a).

Experimental Description

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], 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 control (NgC) and expressed as a % value. Measurements rely on **optical density (OD) 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.

Endpoint Value:

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

Experimental System(s):

The Skin Ethic™ reconstructed human epidermis model (RhE) commercialized by EPISKIN (Lyon, France) was first released by Martin Rosdy in 1989 (Rosdy and Clauss, 1990). Much of the work with this epidermal tissue engineering has followed the pioneering idea of Michel Prunerias (Prunerias et al., 1983). The SkinEthic™ RhE model, consists of normal human keratinocytes cultured until epidermis maturity (functional barrier function) on an inert 0.5 cm^2 polycarbonate filter at the air-liquid interface.

Discussion

- Ethical issues: *in vitro* system
- Special equipment: no specific equipment needed (classical laboratory devices)
- Amount of training required: a training session is recommended before using the test method

Status

Participation in Validation Studies:

The SkinEthic™ RHE test method underwent validation and optimisation studies (Alépée et al., 2010; Tornier et al., 2010) and a statement about this method was issued by ESAC in 2008 (ESAC, 2008).

Regulatory Acceptance:

The SkinEthic™ RhE test method was first adopted in an OECD TG 439 on 22 July 2010 .
In 2015, an updated version of OECD TG439 (OECD, 2015a) and Performance Standards (PS) for the validation of similar or modified RHE methods for skin irritation testing as described in TG 439 (OECD, 2015b) were published. In addition, OECD adopted an Integrated Approach on Testing and Assessment (IATA) for skin corrosion and skin irritation (OECD, 2014), which provides guidance on the integration of existing and new information in a modular approach for classification and labelling. The study demonstrated the use of the SkinEthic™ RHE model within the proposed OECD IATA have been also completed (Alépée et al., 2015a).

Proprietary and/or Confidentiality Issues

The Reconstructed Human Tissue SkinEthic™ RHE technology, associated to production of model and media are proprietary to EPISKIN SA, Lyon, France.

No intellectual property rights are associated with the present test method.

Health and Safety Issues

General Precautions

SkinEthic tissues are manufactured in compliance with ISO9001 certification. A quality control data sheet is provided with every batch of tissue including histology, viability and safety data. The epidermal cells are taken from healthy volunteer donors negative to anti-HIV-1 and 2, to hepatitis C antibodies, to hepatitis B antigens, and to syphilis. Nevertheless, normal handling procedures for biological materials should be followed:

(a) It is recommended to wear gloves during handling;

(b) After use, the epidermis, the material and all media in contact with it, should be decontaminated (for example, by using a 10% solution of bleach or appropriate containers), prior to disposal.

(c) Examine all kit components for integrity. If there is a question or a concern or something unusual call SkinEthic Laboratories, (Phone +33 4 37 28 22 00; Fax +33 4 37 28 22 01).

MSDS Information

- Store test chemicals in ventilated safety cup boards. Respect the special store conditions if necessary (e.g. special temperature, protected from light etc.)
- Non-coded test chemicals should be handled following chemical safety datasheet.
- Unknown and coded test chemicals with no or incomplete safety handling information should be considered as irritant and toxic and must be handled with maximum care. In accordance with chemical safety guidelines: use safety ventilated cabinet, wear gloves, eye and face protections.

MTT (R68, R36/37/38 - H315, H319, H335, H341)

Isopropanol (R11, R36, R67 - H225, H319, H336)

SDS (R11, R20/22, R37/38, R41 - H228, H302 / H332, H315, H318, H335, H412)

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

Abbreviations and Definitions

%: Percent

°C: Degree Celsius

µL: microliter

C: Classified

CAS RN: Chemical Abstract Service Registry Number

Cat.2: Category 2 (irritant)

ET50: Exposure time that induces 50% cell viability

EU CLP: European Classification, Labelling and Packaging Regulation

HPLC/UPLC: High-Performance/ Ultra-high Performance Liquid Chromatography

h: hour/hours

IATA: Integrated Approach on Testing and Assessment

KU: Negative control killed tissue

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}: Non-Specific Color in killed tissue - Killed tissue without MTT incubation

NSC_{living}: Non-Specific Color in living tissue - Living tissue without MTT incubation

NSMTT: Non-Specific MTT reduction in killed tissue – Killed tissue with MTT incubation

OD: Optical Density

OECD: Organisation for Economic Co-operation and Development

PBS^{-}: Phosphate Buffered Saline without Ca^{2+} & Mg^{2+}

PC: Positive Control

Qualified Run: A run is qualified if it meets the acceptance criteria for the NgC and PC. Otherwise, the run is considered as Non-Qualified (invalid).

Qualified Test: The test of a test chemical is qualified (qualified test) if it meets the acceptance criteria for the test within a qualified run. Otherwise, the test is considered as Non-Qualified (invalid).

Run: A set of test chemicals plus Negative Control (NgC) and Positive Control (PC), all concurrently tested on 3 tissues replicates.

RHE: Reconstructed Human Epidermis

RhT : Reconstructed Human Tissues

RT: Room Temperature

SD: Standard Deviation

SDS: Sodium Dodecyl Sulphate

TG: Test Guideline

Test chemical: The term "test chemical" is used in this protocol to refer to what is being tested and is not related to the applicability of the RHE test method to the testing of substances and/or mixtures.

Test: A test chemical concurrently tested on three tissuereplicates is called a "Test". A “Test” for a test chemical is defined 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.

UN GHS: United Nations Globally Harmonized System

V: Volume

Last update: 5 April 2018

PROCEDURE DETAILS, 22 May 2017

SkinEthic™ Skin Irritation Test
DB-ALM Protocol n° 135

The experimental procedure for **SkinEthic™ Skin Irritation Test** is briefly outlined here below. A detailed description of the different steps is available in teh following sections of this protocol.

Receipt: Transfer epidermis from agarose to Growth medium in:
6-well plate (1 mL /well) for overnight or in 24-well plate (300µL/well) at least 2 hours

↓

Culture inserts equilibration period:
(37±2°C, 5±1% CO_2, ≥ 90% humidity)

↓

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

↓

Treatment: At least on 3 tissues (usually 3 tissues) replicates per condition:

- Liquids and viscous: 16±2 µL (32 µL/cm^2) using nylon mesh
- Solids: 10 µL H2O + 16±2 mg (32 mg/cm2)
-Waxy/sticky: 16±2 mg (32 mg/cm2) using nylon mesh

↓

Treatment Period: Incubate for **42±1 min** at RT

↓

Rinse: Thoroughly 25 times with 1 mL PBS^-

↓

Post Treatment Period: Incubate for **42±1 h** (37±2°C, 5±1% CO_2, ≥ 90% humidity)
in Growth medium (2mL/well ; 6-well plate)

↓

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

↓

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

↓

Extraction: Immerse the inserts in 750 µL isopropanol, add 750 µL on the top of each tissue
(extraction from top and bottom of insert)

↓

Extract formazan at least 2 hours with gentle shaking at RT

↓

Perforate the insert and homogenize formazan extract

↓

Quantify the formazan extract by **OD -photometry** at 570±30 nm and/or by **HPLC/UPLC-spectrophotometry**

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Materials and Preparations

Cell or Test System

The SkinEthic™ RHE tissue model (EPISKIN–www.episkin.com) consists of normal human keratinocytes cultured until epidermis maturity (functional barrier function) on a 0.5 cm2 insert polycarbonate filter at the air-liquid interface.
The SkinEthic™ RHE model is cultured using a chemically defined growth medium (Rosdy and Clauss, 1990). On day of sending to users, a highly differentiated and stratified epidermis model is obtained comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum.
The SkinEthic™ RHE model presents a histological morphology comparable to the in vivo human tissue (Doucet et al., 1998). Its use for skin irritation testing involves topical application of test chemicals to the surface of the epidermis, and the subsequent assessment of their effects on cell viability (De Brugerolle et al., 1999; Tornier et al., 2006; Kandárová et al., 2006).

A specific protocol was developed for the testing of finished products applicable for cosmetics formulations (Rosdy, 1994; De Wever and Charbonnier, 2002). Optimisation and Validation of the current protocol have been established in 2010 (Alépée et al., 2010; Tornier et al., 2010). The quality system of the EPISKIN is ISO 9001 certified. Each production batch was provided with quality controls values and recommendations such as storage conditions, SkinEthic™ RHE instructions for use, lot number and origin, histology, cell viability, barrier function integrity (4.0h ≤ ET50 ≤ 10.0h).

Equipment

Fixed Equipment

Items	***Use***
Laminar flow hood	For safe work under sterile conditions
Non–sterile ventilated cabinet or laminar flow hood with chemical filter	For safe work with chemicals, applications, washes
Cell incubator (37±2°C, 5±1% CO_2, ≥ 90% humidity)	For incubating tissues prior and during assays
96-well plate photometer with a 570±30 nm filter	For Optical Density readings (MTT)
Laboratory balance (accuracy 0.1 mg)	For pipette verification, test chemical weighing
HPLC/UPLC-spectrophotometry	Performance Liquid Chromatography readings (MTT formazan)
Adjustable Pipette / multi-step Pipette	For rinse tissue
Adjustable Pipette / multi-step Pipette	For dispensing 300 µL MTT /medium
Multi-pipette + adapter for 25 mL tip	For washing
Adjustable Pipette	For dispensing 750 µL isopropanol
Adjustable Pipette	For dispensing 200 µL formazan extract from 24-well plate into 96-well plate for the plate photometer
Positive displacement pipette for 10 µL and 16 µL delivery	For application of liquid and viscous test chemicals
Plate shaker	For extraction of formazan
Stop-watches	To be used during application of test chemicals
Mortar and Pestle	For grinding granular
1 L beaker	For collecting PBS - washes

1 Funnel	For rinsing tissues with PBS -
1 gauged flask	For SDS 5% solution preparation
500 mL wash bottle	For rinsing tissue after test chemical exposure
Vortex mixer	For shaking MTT solution
Small curved flats spatula	For weighing and spreading solids, sticky chemicals

Consumables

Items	***Use***
Extra 6-well plates – sterile	To transfer tissue inserts to fresh media and post-incubation
Extra 24-well plates – sterile	For application + MTT incubation + formazan extraction steps
Extra 96-well plates – sterile	For OD measurements
Parafilm	Covering plates during formazan extraction
Sterile absorbent paper/sterile gauze	To remove agarose fragments or to dry inserts
Sterile, blunt-edged forceps	For handling tissue inserts
Sterile disposable pipettes, pipettes tips	For diluting, adding, and removing media and test chemicals. For topically applying test chemicals to tissues
Plastic wash bottles	For collecting PBS - rinses
Small glass weight boats	For weighing powders
Circular nylon mesh Ø = 7.5 mm (Sefar Fyltis, # Sefar Nitex 03-150/44) or equivalent	Use as a spreading aid for liquid test chemicals
Cotton tip swabs	For drying the tissue surface

Media, Reagents, Sera, others

SKINETHIC™ RHE set and media provided by EPISKIN:

Description
Epidermal tissues, small size (0.5 cm ^{2}), full maturity
SKINETHIC™ Maintenance Medium
SKINETHIC™ Growth medium

Upon reception of materials supplied by EPISKIN:

- Store the SkinEthic™ RHE tissues at room temperature until their transfer into SkinEthic™ growth Medium.
- Store the SkinEthic™ Maintenance and Growth Media in the fridge (2 to 8°C).

Reagents <u>not</u> provided with the SKINETHIC™ RHE kit:

Items	***Use***
Dulbeccos' PBS without Ca $^{2+}$ and Mg $^{2+}$	Use as negative control, for diluting MTT, and for rinsing tissues
MTT - Thiazolyl Blue Tetrazolium Bromide (CAS RN 298-93-1); Sigma, # M-5655 or M-2128)or equivalent	For the MTT assay
Sterile H_2O (distilled or aqua pure)	To be used for powder applications
5 % (aq) SDS (CAS RN151-21-3) (Sigma # L- 4509, purity min.>98.5%) or equivalent	To be used as positive control with each kit
2-propanol (isopropanol) (CAS RN 67-63-0) (Sigma-Aldrich # 190764) or equivalent	For formazan Crystal extraction
Solvents HPLC/UPLC grade	HPLC/UPLC measurement
Formazan (CAS RN 57360-69-7 purity > 97%, Sigma 88417 or equivalent)	HPLC/UPLC validation system

Preparations

Media and Endpoint Assay Solutions

MTT solutions

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

a) MTT stock solution preparation

Dissolve MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma M-2128 or M-5655) to a final concentration of 5 mg/mL in PBS$^-$ (or solution A, *see note below*). Thoroughly mix this stock solution during 15±2 minutes at RT.

Always protect the solution from light. Keep in the fridge up to 16 days or frozen between -18 to -22°C up to 1 year.

Note. *Composition of the A solution: Na2HPO4 0.142 g/L, glucose 1.802 g/L, HEPES 7.149 g/L, KCL 0.224 g/L, NaCl 7.597 g/L. Adjust pH to 7.4 with NaOH 4N. Filtrate at 0.22µm and store between 2 and 8°C.*

(b) MTT ready to use solution preparation

On day of testing, thaw the MTT stock solution (5 mg/mL) and dilute it with pre-warmed SkinEthic™ Maintenance Medium at room temperature up to 1 mg/mL.
Protect from light until use (do not exceed 3 hours stocking before use).

Formazan Extraction solution

Use 2-propanol (CAS N°67-63-0) from Sigma-Aldrich (ref 190764) or equivalent.

Test Compounds

Test chemical (± color, ± MTT reducer) is topically applied onto SkinEthic™ RHE tissues.

Proficiency chemicals

In OECD TG 439 (OECD, 2015a), it is recommended prior to routine use of the test to check technical proficiency of the laboratory using the Proficiency Chemicals listed in the **Table** below.

Chemical	CASRN	*In vivo* score OECD TG 404*	Physical state	UN GHS Category
Naphthalene acetic acid	86-87-3	0	Solid	No Cat.
Isopropanol	67-63-0	0.3	Liquid	
Methyl stearate	112-61-8	1	Solid	
Heptyl butyrate	5870-93-9	1.7	Liquid	No Cat. (Optional Cat. 3)
Hexyl salicylate	6259-76-3	2	Liquid	
Cyclamen aldehyde	103-95-7	2.3		Cat. 2
1-bromohexane	111-25-1	2.7		
Potassium hydroxide (5% aq.)	1310-58-3	3		
1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	Solid	
Heptanal	111-71-7	3.4	Liquid	

*OECD, 2015c

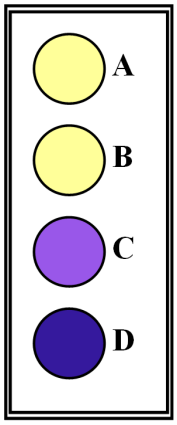
Checking for direct MTT reduction of test chemicals (Annex 1).

When OD is chosen as endpoint for viability assessment:

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

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.

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 tube with 16±2 µL or 16±2 mg of the test chemical to be evaluated or water for control.
- Add 300 µL of MTT ready to use solution (1 mg/mL) and mix.
- Incubate the mixture for 3 hours ± 5 minutes at 37°C protected from light (test conditions).
- If the MTT solution colour becomes 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 epidermis) to define the non-specific reduction of MTT (%NSMTT) value.

Step 2 :

- Use killed tissues that possess no metabolic activity but can absorb and bind the test chemical like viable tissues (*see page 13 for killed tissues preparation*).
- Each MTT interacting test chemical is applied on 3 killed tissues using the skin irritation protocol. In addition to that, three killed tissues remain untreated for control (negative killed control).

For details see page 11 (condition 2).

The **evaluation of direct MTT reduction of test chemical** (steps 1 and 2) is performed **only on one occasion** (a single run even if additional runs are required to classify the test chemical as **C:Classified** or **NC:Not Classified**).

- Evaluation of test chemical – MTT direct interaction is documented using **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 **Data analysis**, ***calculation procedure for Condition 2, page 25***) .

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

Same procedure as for OD measurement.

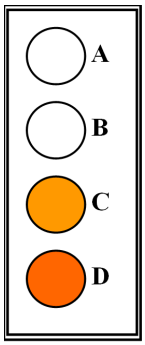
Checking for color test chemicals only (Annex 2)

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 test chemicals.
- For uncolored 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 Data analysis, ***calculation procedure for Condition 3, page 26***) .



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

Step 1:

- Fill tubes with **10 ±2 mg** (solids) or **10 ±1 µL** (liquids) of the test chemical to be evaluated.
- Add 90 µL of water.
- Vortex the solution for few seconds.
- Incubate at least for 30±2 minutes at RT.
- Perform a direct visual observation (*see illustration 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.

Then, it is necessary to evaluate during the future studies the part of OD due to the Non-Specific Color (i.e. by using living epidermis 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 skin irritation evaluation of a test chemical.

Step 2:

- The Non-Specific Color (%NSC $_{living}$) is first quantified by using three living tissues per chemical. For details see *page 11, 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).

- Evaluation of Test chemicals – Color interaction is documented using 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 Data analysis, ***calculation procedure for Condition 3***).

When HPLC/UPLC-spectrophotometry is chosen as endpoint:

No pre-check or control are necessary. For details see *page 12*.

Checking for color test chemicals with possible MTT direct interaction (Annex 1 and 2)

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 three killed tissues and incubated in Maintenance Medium instead of MTT solution to determine the Non-Specific Color on killed tissues (%NSC $_{killed}$) (see ***page 11***, **condition 4** and *page 13 for killed tissues preparation*).

The **evaluation of %NSC $_{killed}$ is performed only on one occasion** (one single valid run even if additional runs are required to classify the test chemical as **C** or **NC**).

- Evaluation of color test chemicals with possible MTT direct interaction is documented using **Annex 1** and **Annex 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 **Data analysis**, ***calculation procedure for Condition 4, page 26***).

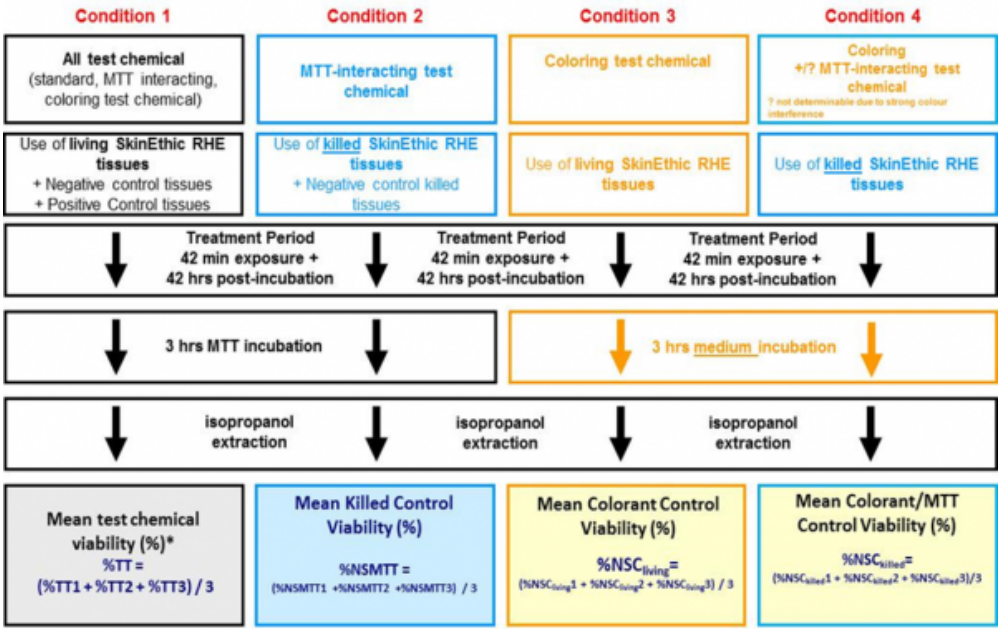
When HPLC/UPLC-spectrophotometry is chosen as endpoint:

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 $_{living}$ and %NSC $_{killed}$) are never required.

Based on this separation capacity of HPLC-UPLC-spectrophotometry 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 tissues with MTT incubation). For details see *page 12*.

Illustrative flowchart providing guidance on how to identify and handle direct MTT-reducers and/or color interfering chemicals is described on **Annex 4.**

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



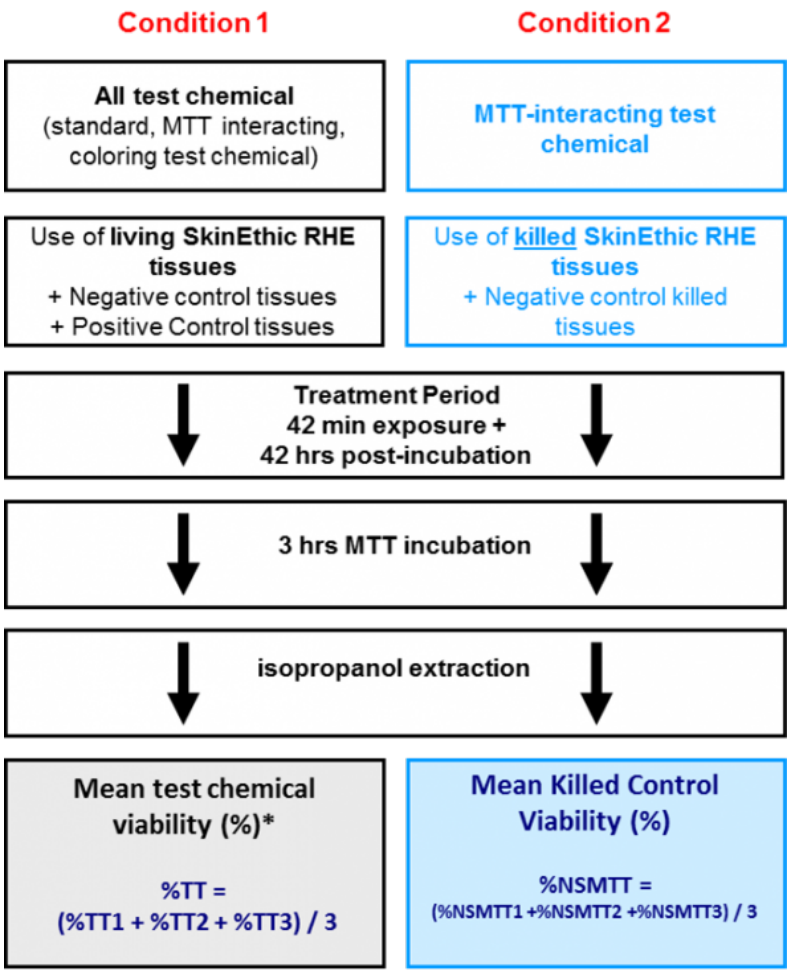
*Mean test substance viability (%) is calculated for 3 tissues

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 $_{killed}$ ≥ 50% of the negative control should be taken with caution.

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



*Mean test substance viability (%) is calculated for 3 tissues

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.

Positive Control(s)

Prepare SDS 5% solution (will be used as positive control (PC)).Preparation can be stored in the fridge for one month.

Note. The % SDS solution must be made in weight / volume (weighing of the SDS then add distilled water until the necessary volume to reach the final concentration of 5 % W/V) e.g. 1 g of pure SDS qs 20 mL water using a gauged flask.

Negative Control(s)

PBS - will be used as negative control (NgC).

Note. The negative control corresponds to the quality control named viability in OECD TG439 that must be performed for each run by the user. This data is not provided by the tissue supplier.

NgC and PC should be tested concurrently to the test chemical

Method

Test System Procurement

The SkinEthic™ RHE and the necessary culture media are received one or three day(s) following the shipment. Results of the quality controls are supplied by e-mail.

The quality system of the EPISKIN is ISO 9001 certified. A production batch is conform if quality controls criteria correspond to a normal histology (absence of significant histological abnormalities) with at least 4 viable cell layers, barrier function integrity (4.0 h ≤ ET50 ≤ 10.0 h, number of cell layers ≥ 4).

The sterility of the tissue construct should be checked by the supplier/end-user for absence of contamination by fungi and bacteria.
For the SkinEthic™ Media and SkinEthic™ RHE tissue models refer to the Technical Data and Certificate of Analysis sent by e-mail.

Reconstructed Human Epidermis SkinEthic™ RHE model

Tissues/Media Description	Storage Conditions	Shelf life
Epidermal tissues, small size (0.5 cm^2), full maturity	37°C	see technical data sheet
SKINETHIC™ Maintenance medium	2 to 8°C	
SKINETHIC™ Growth medium	2 to 8°C	

Note. The maintenance and growth culture media should be pre-warmed only at room temperature (and not at 37°C).

Reception of materials supplied by EPISKIN™ Laboratories:

Examine all kit components for integrity. If there is a question, a concern or something unusual, call +33 (0) 4 37 28 22 00 for support (sales@episkin.com).

1) Place the SkinEthic™ Maintenance medium in the fridge (2 to 8°C).

2) Keep the SkinEthic™ RHE tissues and the SkinEthic™ Growth medium at room temperature for the pre-incubation step.

Routine Culture Procedure

Water killed epidermis preparation

Water killed epidermidis (also indicated as killed tissues) are used to check for MTT-interacting chemicals (p.8) or MTT pre-check inconclusive due to colour (p.10) before starting the experiment.

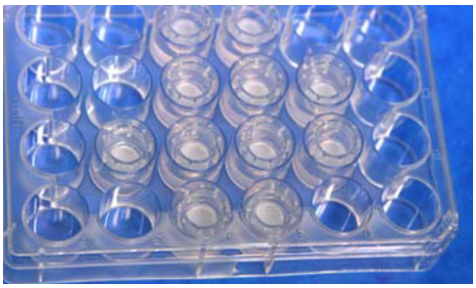
1. Place the living epidermis in a 24-well plate pre-filled with 300 µL of distilled water.
2. Incubate at 37±2°C, 5±1% CO_2, ≥ 90% humidity for 24±1 h.
3. At the end of the incubation, discard the water.
4. Keep dead epidermis frozen (dry) in freezer (killed epidermis can be stored and used up to 6 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 test treatment on killed tissues from the same batch than untreated killed tissues used as negative control.
8. Proceed similarly to living tissues for application, rinsing, post-incubation, etc.
9. Perform this assay only once (at least 3 tissues, usually 3 tissues) per test chemical when necessary.

Pre-incubation step: tissue condition prior testing

It is recommended to conduct this step under sterile conditions.

Pre-incubation step for tissues receipt (i.e. usually on Tuesday)

1. Fill an appropriate numbers of 6-well plates with 1 mL Growth culture medium.
2. Remove the adhesive tape from the agarose plate containing epidermal tissues. Open the 24-well plates.
3. Use sterile forceps to take off tissues from the agarose, clean the bottom of the insert on sterile absorbent paper or gauze to remove eventual remaining agarose pieces.
   See **Pictures 1** and **2**.



Picture 1

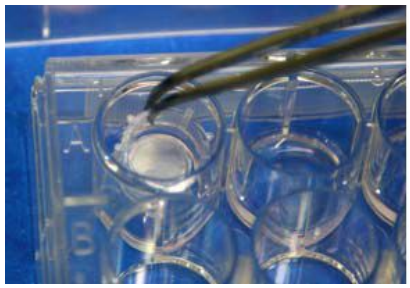
Picture 2

4. Check visually that no agarose is remaining and transfer the tissue on fresh medium by first slopping the insert before complete insert setting. See **Picture 3**.
5. Check the absence of air bubbles by watching underneath the 6-well plate. See **Picture 4**.
6. Place the SkinEthic™ RHE tissues at 37±2°C, 5±1% CO_2, ≥ 90% humidity until test chemical application.

Alternative pre-incubation step for tissues receipt (i.e.receipt on Wednesday)

Proceed to pre-incubation step for at least 2 hours.

1. Fill an appropriate numbers of 24-well plates with 300 µL Growth culture medium.
2. Remove the adhesive tape from the agarose plate containing epidermal tissues. Open the 24-well plates.
3. Use sterile forceps to take off tissues from the agarose, clean the bottom of the insert on sterile absorbent paper or gauze to remove eventual remaining agarose pieces. See **Pictures 1** and **2**.
4. Check visually that no agarose is remaining and transfer the tissue on fresh medium by first slopping the insert before complete insert setting at the air-liquid interface. See **Picture 3**.



Picture 3

5. Check the absence of air bubbles by watching underneath the 24-well plate. See **Picture 4.**
6. Place tissues at 37±2°C, 5±1% CO_2, ≥ 90% humidity until test chemical application.

Picture 4

Test Material Exposure Procedures

Tissue treatment

Use one plate per each test substance to prevent any adjacent effects of test substance fumes.
Pre-warm SDS 5% solution (Positive Control) at room temperature.

Plates preparation

Application plates :

1. Pre-warm the maintenance culture medium at room temperature.
2. Label a 24-well plate by condition: 3 wells per test chemical (code number, 3 replicates), positive control (PC) and negative control (NgC), respectively.
3. Fill the 3 wells with 300 µL pre-warmed Maintenance medium.
4. Use sterile forceps to transfer tissues by first slopping the insert before complete insert setting at the air-liquid interface. See **Picture 3**.
5. Check the absence of air bubbles by watching underneath the 24-well plate. See **Picture 4.**
6. Check the presence of all materials/equipment necessary for test chemical application, washing, drying and post-incubation steps.

Post-incubation plates :

1. Pre-warm the Growth culture medium at room temperature.
2. Label a 6-well plate by condition: 3 wells per test chemical (code number), positive control (PC) and negative control (NgC), respectively.
3. Fill the 3 wells with 2 mL pre-warmed Growth culture medium.

Treatment incubation: 42±1 minutes

It is strongly recommended to perform this step under sterile conditions.

Three tissues per test chemical are usually used (3 replicates). The application order is important since it will be the same for washing. Record Application timing using **Annex 3**.

Suggestion: Keep 1 minute interval between each tissue application. It is recommended keeping some minutes without test chemical application just before washing in order to be ready in time for this latter.

Note. Due to the application timing of 42±1 minutes, the application and rinsing phases should be performed in minimum two sets for testing the internal controls (NgC and PC) and test chemicals.
See examples below (**Tables SET1** and **SET2**).

Table SET1.

SET 1		**APPLICATION** Start : 13h30			**RINSING** Start : 14h13		
Plate 1	Control PBS	**42'**	**41'**	**40'**	**42'**	**41'**	**40'**
Plate 2	Control SDS	**38' 30**	**37'30**	**36'30**	**38'30**	**37'30**	**36'30**
Plate 3	TT1	**35'**	**34'**	**33'**	**35'**	**34'**	**33'**
Plate 4	TT2	**31'30**	**30'30**	**29'30**	**31'30**	**30'30**	**29'30**
Plate 5	TT3	**28'**	**27'**	**26'**	**28'**	**27'**	**26'**
Plate 6	TT4	**24'**	**23'**	**22'**	**24'**	**23'**	**22'**
Plate 7	TT5	**20'30**	**19'30**	**18'30**	**20'30**	**19'30**	**18'30**
Plate 8	TT6	**17'**	**16'**	**15'**	**17'**	**16'**	**15'**
Plate 9	TT7	**13'30**	**12'30**	**11'30**	**13'30**	**12'30**	**11'30**
Plate 10	TT8	**10'**	**9'**	**8'**	**10'**	**9'**	**8'**
Plate 11	TT9	**6'30**	**5'30**	**4'30**	**6'30**	**5'30**	**4'30**
		End : 14h12			End : 14h55		

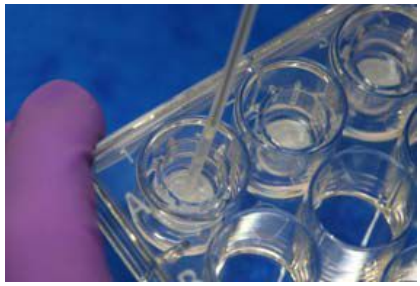
Table SET2.

SET 2		APPLICATION Start : 15h30			RINSING Start : 16h13		
Plate 12	Control PBS	42'	41'	40'	42'	41'	40'
Plate 13	Control SDS	38'30	37'30	36'30	38'30	37'30	36'30
Plate 14	TT10	35'	34'	33'	35'	34'	33'
Plate 15	TT11	31'30	30'30	29'30	31'30	30'30	29'30
Plate 16	TT12	28'	27'	26'	28'	27'	26'
Plate 17	TT13	24'	23'	22'	24'	23'	22'
Plate 18	TT14	20'30	19'30	18'30	20'30	19'30	18'30
Plate 19	TT15	17'	16'	15'	17'	16'	15'
Plate 20	TT16	13'30	12'30	11'30	13'30	12'30	11'30
Plate 21	TT17	10'	9'	8'	10'	9'	8'
Plate 22	TT18	6'30	5'30	4'30	6'30	5'30	4'30
		End : 16h12			End : 16h55		

Application volumes/quantities

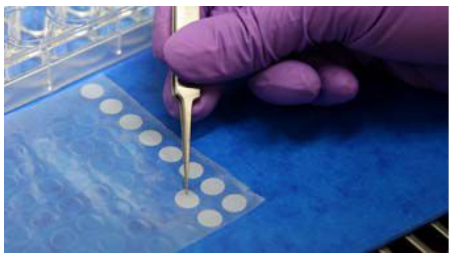
Liquid and viscous test chemicals:

1. Dispense 16±2 µL (i.e. 32 µL/cm²) of the undiluted test chemical on the top of each epidermis tissue (3 per test chemical: replicate 1, replicate 2, and replicate 3), using positive displacement pipette. Use the tip to spread the test chemical gently on the epidermis topical surface. See **Picture 5**.

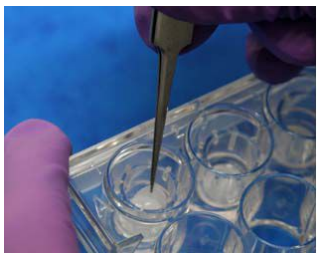


Picture 5

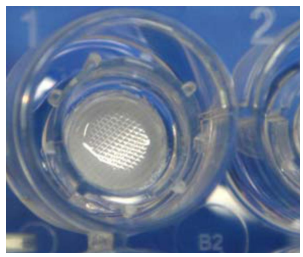
2. Carefully apply a nylon mesh (Ø = 7.5mm) on the whole surface with a tip or forceps. See **Pictures 6, 7** and **8**.



Picture 6



Picture 7

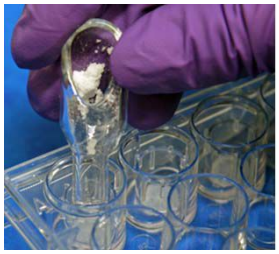


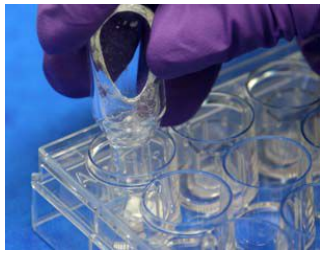
Picture 8

Solid tests chemicals:

1. If necessary, the test chemical should be crushed to a fine powder using a mortar and a pestle.
2. Gently spread 10 µL of distilled water using a positive displacement pipette to the epidermal surface in order to improve further contact between the powder and the epidermis.
3. Use special glass weigh boats (or similar tools avoiding electrostatic electricity and allowing a targeted application directly in the insert with no risk of test chemical scattering in the medium subnatant) to apply 16±2 mg (i.e. 32 mg/cm²) of the powder to the epidermis surface. If necessary, spread it on the epidermal surface. See **Pictures 9, 10** and **11**.


Picture 9


Picture 10


Picture 11

Waxy (sticky) test chemicals:

1. Allow for the tare with nylon mesh and directly weigh 16±2 mg (i.e. 32 mg/cm²) and spread sticky test chemical on this latter.
2. Apply the test chemical coated side of the nylon mesh on the epidermal surface and spread it gently on the whole surface.

Three tissues per test chemical are used. The application order is important since it will be the same for rinsing. Keep the plate (lids on) containing the treated SkinEthic™ RHE tissues for 42±1 minutes exposure in the ventilated cabinet at room temperature.

End of treatment and Post-treatment procedures

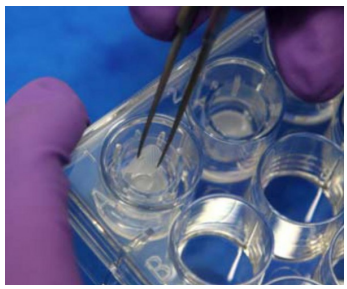
Rinsing and drying steps

End of the treatment and removal of the test chemical after 42± 1 min exposure at room temperature.
Strictly respect the application order (time based is recorded in **Annex 3**).

In order to prevent pollution the lids should be put on the plates continuously during the rinsing and drying steps. We also recommend putting a lid on the sterile PBS ⁻container.

Liquid, viscous and sticky test chemicals:

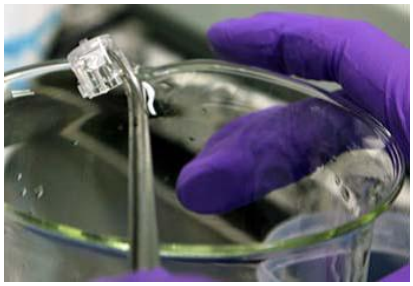
1. Fill a multi-pipette (adjusted for a 1 mL distribution) with 25 mL sterile PBS ⁻.
2. Open the 24-well plate.
3. Remove the nylon mesh with fine forceps from the epidermal surface of a treated tissue. See **Picture 12.**


Picture 12

4. Take the treated tissue with sterile forceps and close the 24-well plate (to protect the other tissues from washing solution projections).

Solid test chemicals:

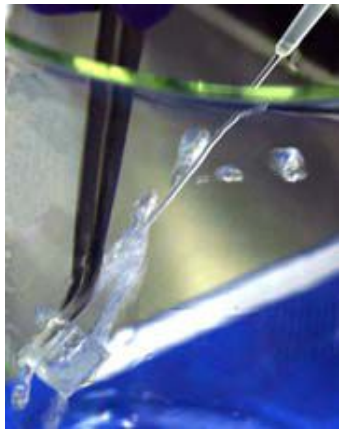
1. Fill a multi-pipette (adjusted for a 1 mL distribution) with 25 mL sterile PBS ⁻.
2. Open the 24-well plate. Take the solid test chemical treated tissue with sterile forceps and close the 24-well plate (to protect the other tissues from washing solution projections).
3. Remove the test chemical at the most, knock the forceps on the beaker, insert turned upside down. See **Picture 13**.



Picture 13

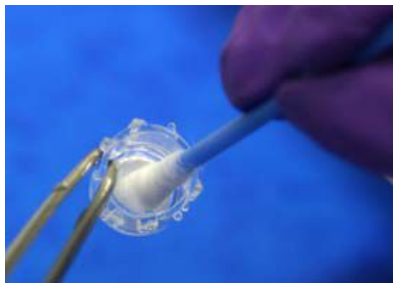
Rinsing step for treated tissues

1. Place a funnel in a large beaker or a bottle (to avoid underneath projections/contaminations of the SkinEthic™ RHE tissues).
2. Maintain the insert over the large funnel and rinse thoroughly 25 times with 1 mL PBS - at a 5-8 cm distance from the insert to remove all residual test chemical from the epidermal surface. See **Picture 14**.



Picture 14

3. After the last rinsing, empty the insert at the most (for example, knock the forceps on the beaker, insert turned upside down).
4. Dry the insert bottom on a sterile absorbent paper or gauze for 1-2 seconds.
5. Sweep gently the surface of the stratum corneum with both ends of a cotton tip (5-6 turns per end). See **Picture 15**.



Picture 15

6. Transfer the washed tissue on 2 mL growth culture medium by first sloping the insert before complete insert setting.
7. Check the absence of air bubbles.

Post treatment incubation: 42±1 hours

Incubate the treated, rinsed and dried epidermis tissues at 37±2°C, 5±1% CO_2, ≥ 90% humidified atmosphere for 42 hours (± 60 minutes).

Incubation start time corresponds to last tissue rinsing time of each set. Incubation timings are recorded using **Annex 3**.

MTT conversion test

Note. *Additional specific tissue controls for coloring test chemicals will be incubated with the* ***maintenance medium*** *(not with the MTT solution – see section "* ***Checking for color test chemicals****", p.9).*

- Prepare MTT medium according to the section *"Preparations", p.7.*
- Dispense **300 µL** of MTT medium in appropriate number of 24 wells plates (**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 adapted coloring chemical tissues controls). Verify the absence of air bubbles under the tissues.
- Incubate tissues for **3 hours ± 15 minutes** at 37±2°C, 5±1% CO_2, ≥ 90% humidity.
- After this MTT incubation period, rinse the inserts in **300 µL PBS÷** to remove the excess of MTT solution (for **Conditions 1** and **2**) or maintenance medium (for **Conditions 3** and **4**).

Record starting time of MTT incubation using **Annex 3**.

Formazan extraction

1. Label an appropriate numbers of 24-well plates. Fill the plate with 750 µL isopropanol.
2. After the MTT incubation period and remove the excess of MTT solution or maintenance medium.
3. Use forceps to transfer treated tissues.
4. Dry the insert bottom of the treated tissue on absorbent paper or gauze.
5. Transfer the tissue in isopropanol solution.
6. Add 750 µL isopropanol solution on the top of each tissue.
7. Ensure that tissue is completely covered by the isopropanol solution.
8. Consciously protect plate(s) from evaporation with at least a parafilm layer (usually 3 layers) over the plate and adding the lid on the plate.
9. Extract either
   a) 2 hours at room temperature protected from light with gentle agitation (about 120 rpm), or
   b) alternatively up to 72 hours in the fridge protected from light without shaking. The following day, shake at least 30 minutes at RT on plate shaker (~ 120 rpm).

Incubation timings are recorded using **Annex 3**.

Endpoint Measurement

At the end of formazan extraction the following two endpoint measurements can be performed.

Optical density (OD) endpoint

1. At the end of the formazan extraction incubation time, open the plate.
2. Remove the parafilm layer(s).
3. Maintain the insert with forceps.
4. Pierce tissue and polycarbonate filter with a tip in order to get the whole extraction solution in the corresponding well.
5. Homogenize the extraction solution by aspiring and dispensing 3 times to complete formazan crystals solubilisation.
6. Transfer 2 x 200 µL extraction solution per well (= 2 wells per tissue i.e. 2 replicates per tissue) into a 96-well plate (see ***Example of plate layout*** below). Be careful to isopropanol evaporation 96 well plates it is recommended-not to fill more than 42 wells/plate before readings.
7. Read the Optical Densities (OD) using a 96-well plate spectrophotometer ideally at 570 nm wavelengths (eventually between 540 to 600 nm). Isopropanol solution is used as blank. No reference filter should be used.

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

Example of plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
	Blank	NgC or KU or TT	PC or TT	TT	TT	TT	TT					
A	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
B	Blank	Tissue 1	Tissue 1	Tissue 1	Tissue 1	Tissue 1	Tissue 1	empty	empty	empty	empty	empty
C	Blank	Tissue 1	Tissue 1	Tissue 1	Tissue 1	Tissue 1	Tissue 1	empty	empty	empty	empty	empty
D	Blank	Tissue 2	Tissue 2	Tissue 2	Tissue 2	Tissue 2	Tissue 2	empty	empty	empty	empty	empty
E	Blank	Tissue 2	Tissue 2	Tissue 2	Tissue 2	Tissue 2	Tissue 2	empty	empty	empty	empty	empty
F	Blank	Tissue 3	Tissue 3	Tissue 3	Tissue 3	Tissue 3	Tissue 3	empty	empty	empty	empty	empty
G	Blank	Tissue 3	Tissue 3	Tissue 3	Tissue 3	Tissue 3	Tissue 3	empty	empty	empty	empty	empty
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty

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 microtiter plate and read Optical Density (OD) at 570 nm filter (pass-band max ± 30 nm), without using a reference filter.
- Use isopropanol as blank (200 µL / well).
- **For all conditions included negative control:** transfer at least 100 µL into an HPLC/UPLC-spectrophotometry vial (samples can also be frozen during 2 months maximum)
- Use a validated analytical method (see "***Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint*** " in **Downloads** section of this protocol) 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.

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

Acceptance Criteria

The SkinEthic™ RHE model kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the epidermis and the kit culture medium have been tested for the absence of viruses, bacteria and mycoplasma.
The quality of the final product is assessed by an MTT cytotoxicity test with sodium dodecyl sulphate (SDS) and by histological examination.

For reasons connected with the nature of the product, it is shipped before all of the necessary checks have been completed. A release form certifying the conformity (or otherwise) of the batch is sent to the user, via e-mail by the supplier, on the day of delivery of the kit.

Negative control (NgC)

A run meet the acceptance criteria if the mean Optical Density (OD NgC) of the three replicates tissues treated (usually 3 tissues) with NgC is ≥ 0.8 at 570nm (± 30nm) with an upper acceptance limit ≤ 3.0.

The absolute OD of the negative control (NgC) tissues (PBS^- treated) in the testing run is an indicator of tissue viability in the testing laboratory after shipping and storage procedures and under use conditions.

The Standard Deviation value is considered as valid if it is ≤ 18%, according to the Performance Standards (OECD, 2015b).

Positive control (PC)

The PC data meet the acceptance criteria if the mean viability, expressed as % of the NC, is ≤ 40% and the Standard Deviation value is ≤ 18 %.

Users should ensure that each batch of the tissue construct used meets defined criteria for the negative and positive controls. The run is qualified if it meets the acceptance criteria for the NgC and PC.

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

Assay acceptability criteria

For a given test chemical, a single testing run composed of 3 tissue replicates should be sufficient when the classification is unequivocal and if the Standard Deviation value is ≤ 18 %.

However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50 ± 5%, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

Specific HPLC/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 (FDA, 2001; Alépée et al., 2015b).
These key parameters and their acceptance criteria are shown in **Annex 4**.
Once the acceptance criteria have been met (as defined in "***Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint***" in **Downloads** section of this protocol), the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this procedure.

A **single testing run composed of at least three tissue replicates** should be **sufficient for a test chemical when the resulting classification is unequivocal** (independently of the endpoint: OD or HPLC/UPLC spectrophotometry). However, **in cases of borderline results**, such as non-concordant replicate measurements, a **second run** may be considered, **as well as a third one in case of discordant results** between the first two runs.

Data Analysis

Data calculation steps

Test chemical viability (%) calculation is presented for 3 tissues (see **Data analysis** and all the paragraphs dedicated to ***Calculation procedures***, pag es 23-26) . To be updated upon the need.

Main steps:

(a) Blanks

- Calculate the mean OD of isopropanol 100%.

(b) Negative PBS -treated controls (NgC)

- Subtract blank mean value from individual tissues ODs (2 values from each of three tissues).
- Calculate the mean OD for each individual tissue.
- Corrected mean OD for the 3 tissues corresponds to 100% viability.

(c) Positive control (PC)

- Subtract blank mean value from individual tissues ODs (2 values from each of three 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 three tissues).
- Calculate the mean OD for each individual tissue.

(e) Viability %

- Calculate for each treated epidermis 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 standard deviation between the three tissue replicates (for NgC, PC and test chemical) to evaluate the variability for each test.

Calculation procedure – For viability tests only (Condition 1)

Calculation for OD reading

- **MEAN OD CALCULATION:**

*Negative Control (NgC)
Individual OD Negative Control (NgC)

$OD_{NgC1} = OD_{NgC1raw} - OD_{blank\ mean}$
$OD_{NgC2} = OD_{NgC2raw} - OD_{blank\ mean}$
$OD_{NgC3} = OD_{NgC3raw} - OD_{blank\ mean}$

Mean OD Negative Control $\mathbf{OD_{NgC} = [OD_{NgC1} + OD_{NgC2} + OD_{NgC3}]/3}$

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

*Positive Control (PC)
OD Positive Control (PC)

$OD_{PC1} = OD_{PC1\ raw} - OD_{blank\ mean}$
$OD_{PC2} = OD_{PC2\ raw} - OD_{blank\ mean}$
$OD_{PC3} = OD_{PC3\ raw} - OD_{blank\ mean}$

Mean OD Positive Control $\mathbf{OD_{PC} = [OD_{PC1} + OD_{PC2} + OD_{PC3}]/3}$

*Test chemical Treatment OD
Test chemical Treatment (TT)

$OD_{TT1} = OD_{\mathbf{TT1}\ raw} - OD_{blank\ mean}$
$OD_{TT2} = OD_{\mathbf{TT2}\ raw} - OD_{blank\ mean}$
$OD_{TT3} = OD_{\mathbf{TT3}\ raw} - OD_{blank\ mean}$

Mean OD Test Treatment $\mathbf{OD_{TT} = [OD_{TT1} + OD_{TT2} + OD_{TT3}]/3}$

- **VIABILITY CALCULATION: Individual mean viabilities (%)**

% Negative Control 1 $\%NgC1 = [OD_{NgC1} / \text{mean } OD_{NgC}] \times 100$
% Negative Control 2 $\%NgC2 = [OD_{NgC2} / \text{mean } OD_{NgC}] \times 100$
% Negative Control 3 $\%NgC3 = [OD_{NgC3} / \text{mean } OD_{NgC}] \times 100$
% mean Negative Control $\mathbf{\%NgC = (\%NgC1 + \%NgC2 + \%NgC3)/3}$

Standard Deviation Negative Control $SD_{NgC} = \sqrt{((\Sigma(\%\ NgC - meanNgC)^2)/2)}$

% Positive Control 1 $\%PC1 = [OD_{PC1} / \text{mean } OD_{NgC}] \times 100$
% Positive Control 2 $\%PC2 = [OD_{PC2} / \text{mean } OD_{NgC}] \times 100$
% Positive Control 3 $\%PC3 = [OD_{PC3} / \text{mean } OD_{NgC}] \times 100$
% mean Positive Control $\mathbf{\%PC = (\%PC1 + \%PC2 + \%PC3) / 3}$

Standard Deviation Positive Control $SD_{PC} = \sqrt{((\Sigma(\%\ PC - meanPC)^2)/2)}$

% Test Treatment 1 $\%TT1 = [OD_{TT1} / \text{mean } OD_{NgC}] \times 100$
% Test Treatment 2 $\%TT2 = [OD_{TT2} / \text{mean } OD_{NgC}] \times 100$
% Test Treatment 3 $\%TT3 = [OD_{TT3} / \text{mean } OD_{NgC}] \times 100$
% Mean Test Treatment $\mathbf{\%TT = (\%TT1 + \%TT2 + \%TT3) / 3}$

Standard Deviation Test Treatment $SD_{TT} = \sqrt{((\Sigma(\%TT - meanTT)^2)/2)}$

Calculation for HPLC/UPLC- spectrophotometry endpoint

- **MEAN AREA CALCULATION:**

*Negative Control (NgC)
Mean Area Negative Control $\mathbf{Area_{NgC} = [Area_{NgC1} + Area_{NgC2} + Area_{NgC3}]/3}$

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

*Positive Control (PC)
Mean Area Positive Control $\mathbf{Area_{PC} = [Area_{PC1} + Area_{PC2} + Area_{PC3}]/3}$

*Test Treatment (TT)
Mean Area Test Treatment $\mathbf{Area_{TT} = [Area_{TT1} + Area_{TT2} + Area_{TT3}]/3}$

- **VIABILITY CALCULATION: Individual means viabilities (%)**

% Negative Control 1 $\%NgC1 = [Area_{NgC1} / \text{mean } Area_{NgC}] \times 100$
% Negative Control 2 $\%NgC2 = [Area_{NgC2} / \text{mean } Area_{NgC}] \times 100$
% Negative Control 3 $\%NgC3 = [Area_{NgC3} / \text{mean } Area_{NgC}] \times 100$
% mean Negative Control **%NgC= (%NgC1 + %NgC2 + %NgC3)/3**

% Positive Control 1 $\%PC1 = [Area_{PC1} / \text{mean } Area_{NgC}] \times 100$
% Positive Control 2 $\%PC2 = [Area_{PC2} / \text{mean } Area_{NgC}] \times 100$
% Positive Control 3 $\%PC3 = [Area_{PC3} / \text{mean } Area_{NgC}] \times 100$
% mean Positive Control **%PC= (%PC1 + %PC2 + %PC3)/3**

% Test Treatment 1 $\%TT1 = [Area_{TT1} / \text{mean } Area_{NgC}] \times 100$
% Test Treatment 2 $\%TT2 = [Area_{TT2} / \text{mean } Area_{NgC}] \times 100$
% Test Treatment 3 $\%TT3 = [Area_{TT3} / \text{mean } Area_{NgC}] \times 100$
% mean Test Treatment **%TT = (%TT1 + %TT2 + %TT3)/3**

*The mean relative viability is used for classification according to the **Prediction Model** (p.28).*

Calculation procedure – MTT interacting test chemical (Condition 2)

Data calculations for MTT interacting chemicals

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_{ku} or $Area_{KU}$: OD or Area untreated killed tissues + MTT incubation
OD_{kt} or $Area_{kt}$: OD or Area test chemical treated killed tissues + MTT incubation
OD_{NgC} or $Area_{NgC}$: mean OD or Area negative control living tissues + MTT incubation

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	$\%NSMTT2 = [(OD_{kt2} - OD_{ku})/ OD_{NgC}]x100$
% Killed Test Treatment 3	$\%NSMTT3 = [(OD_{kt3} - OD_{ku})/ OD_{NgC}]x100$
% Mean Non-Specific MTT reduction	**%NSMTT = (%NSMTT1 + %NSMTT2 + %NSMTT3)/3**

- **CORRECTED FINAL VIABILITY (FV_C)**

%Final viability Test Treatment 1	$\%FV_{C\ NSMTT1} = \%\ TT1 - \%NSMTT$
%Final viability Test Treatment 2	$\%FV_{C\ NSMTT2} = \%\ TT2 - \%NSMTT$
%Final viability Test Treatment 3	$\%FV_{C\ NSMTT3} = \%\ TT3 - \%NSMTT$
Mean Final Viability	$\%\ FV_{C\ NSMTT} = (\%FV_{CNSMTT1} + \%FV_{C\ NSMTT2} + \%FV_{CNSMTT3})/ 3$
Standard Deviation Final Viability	$SD_{FV} = \sqrt{((\sum (\%FV\text{-}meanFV)^2)/2)}$

Calculation for HPLC/UPLC-spectrophotometry reading

- **NON-SPECIFIC MTT REDUCTION CALCULATION (%NSMTT)**

% Killed Test Treatment 1	$\%NSMTT1 = [(Area_{kt1} - Area_{ku})/ Area_{NgC}]x100$
% Killed Test Treatment 2	$\%NSMTT2 = [(Area_{kt2} - Area_{ku})/ Area_{NgC}]x100$
% Killed Test Treatment 3	$\%NSMTT3 = [(Area_{kt3} - Area_{ku})/ Area_{NgC}]x100$
%Mean Non-Specific MTT reduction	**%NSMTT = (%NSMTT1 + %NSMTT2 + %NSMTT3)/3**

- **CORRECTED FINAL VIABILITY (FV_C)**

%Final viability Test Treatment 1	$\%FV_{C\ NSMTT1} = \%\ TT1 - \%NSMTT$
%Final viability Test Treatment 2	$\%FV_{C\ NSMTT2} = \%\ TT2 - \%NSMTT$
%Final viability Test Treatment 3	$\%FV_{C\ NSMTT3} = \%\ TT3 - \%NSMTT$
Mean Final Viability	$\%\ FV_{C\ NSMTT} = (\%FV_{CNSMTT1} + \%FV_{C\ NSMTT2} + \%FV_{CNSMTT3})/ 3$
Standard Deviation Final Viability	$SD_{FV} = \sqrt{((\sum (\%FV\text{-}meanFV)^2)/2)}$

The mean final viability is used for classification according to the ***Prediction Model*** *(p.28).*

Calculation procedure - Coloring test chemicals (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)

- **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	$\%NSC_{living\ 2} = [(OD_{TT2\text{-}MTT} / OD_{NgC}] \times 100$
% Non-Specific Color tissue 3	$\%NSC_{living\ 3} = [(OD_{TT3\text{-}MTT} / OD_{NgC}] \times 100$
Mean % Non-Specific Color	$\mathbf{\%NSC_{living} = (\%NSC_{living1} + \%NSC_{living2} + \%NSC_{living3})/3}$

- **CORRECTED FINAL VIABILITY (FV_C)**

%Final viability Test Treatment1	$\%FV_{C\ NSC\ living\ 1} = \%\ TT1 - \%NSC_{living}$
%Final viability Test Treatment 2	$\%FV_{C\ NSC\ living2} = \%\ TT2 - \%NSC_{living}$
%Final viability Test Treatment 3	$\%FV_{C\ NSC\ living3} = \%\ TT3 - \%NSC_{living}$
Mean Final Viability	$\mathbf{\%FV_{C\ NSCliving} = (\%FV_{C\ NSCliving1} + \%\ FV_{C\ NSCliving2} + \%\ FV_{C\ NSCliving3})/3}$

Standard Deviation Final Viability $\mathbf{SD_{FV} = \sqrt{((\sum (\%FV\text{-}meanFV)^2)/2)}}$

*The mean final viability is used for classification according to the **Prediction Model** (p.28).*

Calculation procedure - Coloring +/? MTT interacting test chemical (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 ($\%NSC_{killed}$) together with ‘Living-MTT' controls ($\%NSC_{living}$).
However, the "Non-Specific MTT Reduction" obtained with %NSMTT 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 overestimation 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

- **NON-SPECIFIC COLOR WITH KILLED TISSUES % CALCULATION ($\%NSC_{killed}$):**

% Non-Specific Color with killed tissue 1	$\%NSC_{killed1} = (OD_{kt\text{-}MTT1} / OD_{NgC}) \times 100$
% Non-Specific Color with killed tissue 2	$\%NSC_{killed2} = (OD_{kt\text{-}MTT2} / OD_{NgC}) \times 100$
% Non-Specific Color with killed tissue 3	$\%NSC_{killed3} = (OD_{kt\text{-}MTT3} / OD_{NgC}) \times 100$
Mean % Non-Specific Color without MTT	$\mathbf{\%NSC_{killed} = (\%NSC_{killed1} + \%NSC_{killed2} + \%NSC_{killed3})/3}$

- **CORRECTED FINAL VIABILITY ($FV_{C\ TT}$)**

%Final viability Test Treatment 1	$\%FV_{C\ TT1} = \%\ TT1 - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
%Final viability Test Treatment 2	$\%FV_{C\ TT2} = \%\ TT2 - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
%Final viability Test Treatment 3	$\%FV_{C\ TT3} = \%\ TT3 - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
% Mean Final Viability	$\mathbf{\%FV_{C\ TT} = (\%FV_{C\ TT1} + \%FV_{C\ TT2} + \%FV_{C\ TT3})/3}$

Standard Deviation Final Viability $\mathbf{SD_{FV} = \sqrt{((\sum (\%FV\text{-}meanFV)^2)/2)}}$

*The mean final viability is used for classification according to the **Prediction Model** (p.28).*

Remarks

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

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

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

- ***IF*** the mean of % Non-Specific Color on living tissues (%NSC $_{living}$) or % Non-Specific MTT reduction (NSMTT) or [%NSC $_{living}$ + %NSMTT - %NSC $_{killed}$] of the 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 or [%NSC $_{living}$ + %NSMTT - %NSC $_{killed}$] of the 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 or [%NSC $_{living}$ + %NSMTT - %NSC $_{killed}$] of the 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” should be considered. Results for test chemical producing %NSMTT and/or %NSC $_{living}$ and/or %NSC $_{killed}$ ≥ 50% of the negative control should be taken with caution.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Mean Viab %NSC $_{living}$	Mean viab %NSC $_{killed}$	Final Corrected Viability	Final Viability
	Living+MTT	***Killed+MTT***	***Living-MTT***	***Killed-MTT***		
1	81.2	-	-	-	%TT	81.2
2	101.2	11.2	-	-	%TT - %NSMTT	90.0
3	81.2	-	41.2	-	%TT - %NSC $_{living}$	40.0
4	101.2	11.2	20	11	%TT - % NSMTT - %NSC $_{living}$ + %NSC $_{killed}$	81.0
See page	*23*	*25*	*26 (condition 3)*	*26 (condition 4)*		

For colored test chemicals interfering too strongly with the MTT-reduction assay an alternative endpoint may be considered (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 %NSMTT> 50% of the negative control) and correction from control tissues is required.

In the following table conditions for HPLC/UPLC-spectrophotometry measurement (H in the table) are provided.

Condition	Mean Viab %TT	Mean Viab %NSMTT	Final Corrected Viability	Final Viability
	Living+MTT	***Killed+MTT***		
(H)1	81.2	-	%TT	81.2
(H)2	101.2	11.2	%TT - %NSMTT	90.0
See page	*23*	*25*		

Prediction Model

This **test method** provides an ***in vitro* procedure** that may be used **for the hazard identification of irritant chemicals and mixtures in accordance with UN GHS Category 2**.

According to UN GHS classification (UN, 2015), the irritancy potential of test chemicals is predicted for distinguishing between Category 2 (skin irritating) and non-classified (non-skin irritating) test chemicals.
The irritancy potential of test chemicals is predicted by mean tissue viability of tissues exposed to the test chemical. The test chemical is considered to be “Category 2” if the mean relative viability after 42±1 minutes exposure and 42±1 hours post-incubation is less or equal (≤) to 50% of the negative control.

The prediction model (PM) is described below:

Criteria for *in vitro* interpretation	**Classification**
Mean tissue cell viability ≤ 50%	Category 2
Mean tissue cell viability > 50%	No Category

It does not allow the classification of chemicals to the optional UN GHS Category 3 (mild irritants).

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

For a full evaluation of local skin effects after a single dermal exposure, it is recommended to follow some sequential testing strategy that includes the conduct of *in vitro* tests for skin corrosion and skin irritation (i.e. by using the SkinEthic™ test methods (OECD, 2014)).

Annexes

Annex 1: Evaluation of Test chemicals - MTT direct interaction

Laboratory: …….. Study:……...

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

Date: …….. Signature: ……..

Annex 2: Evaluation of test chemicals - Color interaction (at least 15 min)

Laboratory: …….. Study N°:……..

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

Date: …….. Signature: ……..

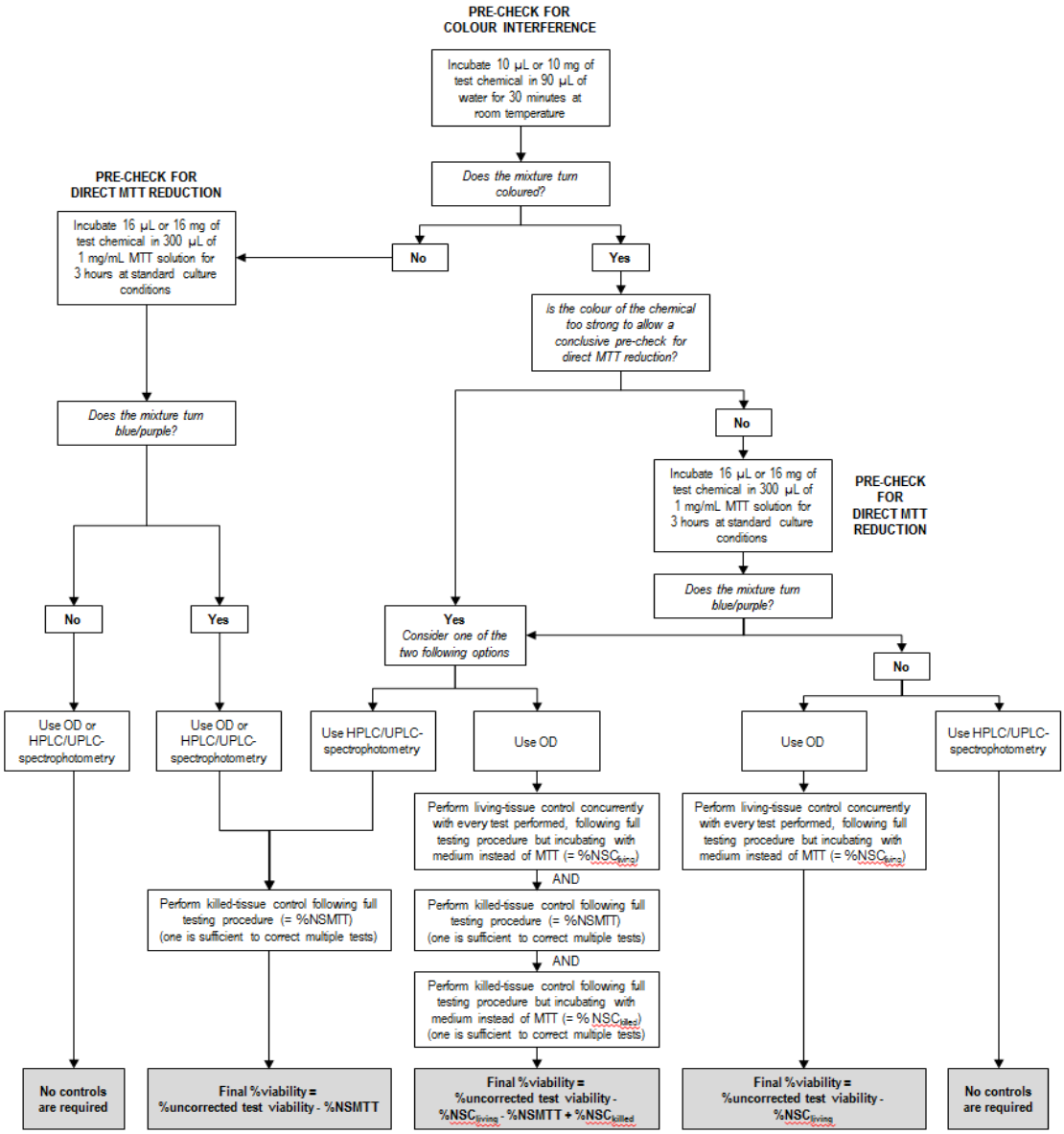
Annex 3: Incubation timings

Laboratory: …….. Study N°:…….. Assay N°: ……..

Test chemical Name Or code	Treatment			Post incubation		MTT incubation		Formazan extraction	
	42 ± 1 min			42 ± 1 h		3 h ± 15 min			
	Tissue	Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)
NgC	1								
	2								
	3								
PC	1								
	2								
	3								
	1								
	2								
	3								
	1								
	2								
	3								
	1								
	2								
	3								
	1								
	2								
	3								

Date: …….. ID and Signature: ……..

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



Source OECD, 2015a

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