DB-ALM Protocol n° 188 : SkinEthic™ RHE Skin Corrosion Test

Skin Irritation and Corrosivity

The method is designed for the prediction of acute skin corrosion of chemicals by measurement of cytotoxic effect on the SkinEthic™ Reconstructed Human Epidermis (RHE) model.

Résumé

The SkinEthic RHE Skin Corrosivity Corrosion Test method is designed to predict and classify the skin corrosion potency of test chemicals according to safety regulations, using the reconstructed human epidermis model, SkinEthic™ RHE.
This test method allows the identification of non-corrosive and corrosive substances and mixtures (test chemicals) in accordance with the UN GHS (UN, 2015). This method further supports the sub-categorisation of corrosive substances and mixtures into optional Sub-category 1A, in accordance with the UN GHS, as well as a combination of sub-categories 1B and 1C (i.e. 1A versus 1B-and-1C versus NC) (Alépée et al., 2014) (Desprez et al., 2015). It does not allow discriminating between skin corrosive sub-category 1B and sub-category 1C due to the limited set of well-known in vivo corrosive sub-category 1C (OECD, 2016a).

This test method 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. 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 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) and adopted in Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TG) 431 whose last revision was adopted in 2016 (OECD, 2016).

The test method was granted regulatory approval as a replacement for the in vivo skin corrosivity test (OECD, 2015a) and it is used for hazard identification and classification of corrosive potential in order to fulfil the regulatory requirements concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (EU, 2008). The OECD Series on Testing & Assessment No. 219 describes the Performance Standards for the assessment of proposed similar or modified in vitro reconstructed human epidermis (RHE) test methods for skin corrosion testing as described in TG 431 (Intended for the developers of new or modified similar test methods) in accordance with the principles of Guidance Document No. 34 (OECD, 2005; OECD 2015b).

While this test method does not provide adequate information on skin irritation, it should be noted that OECD TG 439 specifically addresses the health effect skin irritation in vitro and is based on the same SkinEthic™ RHE test system, though using another protocol (OECD, 2015c).
For a full evaluation of local skin effects after a single dermal exposure, the Guidance Document No. 203 on Integrated Approaches for Testing Assessment (IATA) should be consulted (OECD, 2014). This IATA approach includes the conduct of in vitro tests for skin corrosion (such as described in this test method) and skin irritation. The study demonstrated the use of the SkinEthic™ RHE model within the proposed OECD IATA guidance document have been also completed (Alépée et al., 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 controls (NgC) 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 test 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 test chemicals belonging to the applicability domain.

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 skin corrosion potential.

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:

Optimisation and validation studies have been completed for the SkinEthic™ RHE test method (Kandárová *et al*., 2006b; Alépée *et al*., 2010; Tornier *et al*., 2010) leading to its endorsement by the EURL ECVAM Scientific Advisory Committee (ESAC) in 2006 (ESAC, 2006).

Regulatory Acceptance:

The SkinEthic™ RHE skin corrosion test method was first adopted in an OECD TG 431 on July 2010 for the identification of non-corrosive and corrosive test chemicals. The last OECD revision adopted on 29 July 2016 supports the subcategorization of corrosives (OECD, 2016). The SkinEthic™ RHE test method was adopted in the OECD GD 203 - Integrated Approach on Testing and Assessment (IATA) for skin corrosion and skin irritation in 2014 (OECD, 2014).

Proprietary and/or Confidentiality Issues

The Reconstructed Human Tissue SkinEthic™ RHE 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.

Health and Safety Issues

General Precautions

SkinEthic™ RHE tissues are manufactured in compliance with ISO 9001 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, a concern or something unusual, +33 (0) 4 37 28 22 00, for support (**sales@episkin.com**).

Safety instructions for working with chemicals:

1-Store test chemicals in ventilated safety cupboards. Respect special store conditions if necessary (special temperature, protected from light etc.).

2-Non-coded test chemicals should be handled following chemical safety datasheet.

3-Unknown and coded test chemicals with no or incomplete safety handling information should be considered as corrosive and toxic and must be handled with maximum care. In accordance with chemical safety guidelines: use safety ventilated cabinet, wear gloves, eye and face protection.

MTT and corrosive materials are dangerous. Work in a non-sterile, ventilated cabinet; wear protective gloves, and a mask and safety glasses, as necessary.

MSDS Information

Safety precautions:

MTT (R68, R36, R37, R38 / H315, H319, H335, H341)

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

8N KOH (H290, H302, H314)

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: Corrosive

Cat.: Category

Chemical: The term "test chemical" is used in this protocol to refer to what is being tested (substances and/or mixtures).

CASRN: CAS Registry Number

ET50: Exposure time that induce 50% cell viability

HPLC: High performance liquid chromatography

hr/hrs: Hour/hours

IATA: Integrated Approach on Testing and Assessment

KU: Negative control killed tissue

KOH: Potassium Hydroxide

mg: Milligram

Min: Minute(s)

mL: milliliter

MTT: 3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NC: Non corrosive

NgC: Negative Control

nm: Nanometer

NSC_{killed}: Non Specific Color in killed tissues - Killed tissues without MTT incubation

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

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

OD: Optical Density

OECD: Organisation for Economic Cooperation and Development

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

PC: Positive Control

RHE: Reconstructed Human Epidermis

RT: Room Temperature

s: Seconde(s)

TG: Test Guideline

UN GHS: United Nations Globally Harmonized System

UPLC: Ultra-high Performance Liquid Chromatography

V: Volume

Run: A set of test chemicals plus Negative Control (NgC) and Positive Control (PC) all concurrently tested on at least 2 tissues replicates at both exposure periods of 3 min ± 15 s and 1 h ± 1 min (except PC at 1 h ± 1 min only) conducted with the same tissues batch 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).

Test: A test chemical concurrently tested on at least two tissues (usually 2 replicates) per exposure period 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.

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).

Last update: 30th October 2017

PROCEDURE DETAILS, Latest version: 30th October 2017

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The experimental procedure for Skin Ethic™ skin Corrosion Test is briefly outlined here below. A detailed description of the different steps is available in the following sections of this protocol.

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

↓

Culture inserts equilibration period: Incubate between 3 hours and 24 hours (37±2°C, 5±1% CO_2, ≥90% humidity)

↓

Transfer tissues to fresh maintenance medium in 24-well plates

↓

Treatment: At least 2 tissues per exposure time:

Liquids and viscous: 40±3 µL (80 µL/cm^2) using nylon mesh
Solids: 20±2 µL H_2O + 20±3 mg (40 mg/cm^2)
Waxy/sticky: 20±2 µL H_2O + 20±3 mg (40 mg/cm^2) using nylon mesh

↓

Treatment Period: Incubate for **3 min ± 15 s** at RT and **1 hr ±1 min** at RT

↓

Rinse: Thoroughly 20 times with 1mL PBS-

↓

Viability: Transfer tissues into MTT solution

↓

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

↓

Extraction: Immerse the inserts in 1.5 mL isopropanol
(extraction from top and bottom of insert)

↓

Extract formazan at least 2 hours
with gentle shaking at RT or overnight in the fridge without shaking

↓

Perforate the insert and homogenize formazan extract

↓

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

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Materials and Preparations

Cell or Test System

The SkinEthic™ RHE tissue model (Episkin SA) consists of normal human keratinocytes cultured until epidermis maturity (functional barrier) on at 0.5 cm^2 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.

The SkinEthic™ RHE model is cultured using a chemically defined growth medium (Rosdy and Clauss, 1990). A highly differentiated and stratified epidermis model is obtained comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum (Fartasch and Ponec, 1994; Kandárová, 2006a; Kandárová *et al*., 2006b). The SkinEthic™ RHE model presents a histological morphology comparable to the *in vivo* human tissue (Doucet *et al*., 1998).

The quality system of the EPISKIN is ISO 9001 certified. Each batch production 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 (4.0 ≥ ET50 ≥ 10.0 hrs). 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, by e-mail, on the day of delivery of the kit.

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°C, 5% CO_2, ≥ 90% relative 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
Adjustable Pipette / multi-step Pipette	For 1 mL assay medium
Adjustable Pipette / multi-step Pipette	For dispensing 300 µL MTT /medium
Adjustable Pipette	For dispensing 750 µL (x2) propan-2-ol
Positive displacement pipette for 20 µL and 40 µL delivery	For application of liquid and viscous test materials
Plate shaker	For extraction of formazan

Adjustable Pipette	For dispensing 200 µL formazan extract from 24-well plate into 96-well plate for the plate photometer
Stop-watches	To be used during application of test materials
Mortar and Pestle	For grinding granular
1 L beaker	For collecting PBS - washes
1 Funnel	For rinsing tissues with PBS -
Adjustable Pipette or 500 mL wash bottle	For rinsing tissue after test material exposure
Vortex mixer	For shaking MTT solution
Small curved flats spatula	For weighing and spreading solids, sticky chemicals
HPLC/UPLC-spectrophotometry	Performance Liquid Chromatography readings (MTT formazan)

Consumables

Items	***Use***
Extra 6-well plates – sterile	To transfer tissue inserts to fresh media and checking of direct MTT interaction with test chemicals
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
Absorbent paper / 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
Circular nylon mesh Ø = 7.5mm (Sefar Fyltis, # Sefar Nitex 03-150/44 or equivalent)	Use as a spreading aid for liquid test materials,provided a pre-test shows the compatibility of test material and nylon mesh
Cotton tip swabs	For drying the tissue surface
Small glass weight boats	For weighing powders

Media, Reagents, Sera, others

SkinEthic™ RHE set and media provided by Episkin

- Epidermal tissues, small size (0.5 cm ^{2}), full maturity
- SkinEthic™ Maintenance Medium

Upon reception of materials supplied by EPISKIN: store the SkinEthic™ RHE tissues at room temperature until their transfer into SkinEthic™ Maintenance Medium. -Store SkinEthic™ Maintenance Medium in the fridge (2 to 8°C).

Reagents not provided with the SkinEthic™ RHE kit:

Items	***Use***
Dulbeccos' PBS without Ca^{2+} and Mg^{2+} (PBS^{-})	Use for diluting MTT, and for rinsing tissues
MTT - Thiazolyl Blue Tetrazolium Bromide (CASRN 298-93-1; Sigma # M-5655 or M-2128 or equivalent)	For the MTT assay
8N KOH (CASRN 1310-58-3)	To be used as positive control
Sterile H_2O (distilled or aqua pure)	To be used as negative control and for powder applications
2-propanol (isopropanol) (CASRN 67-63-0 ; Sigma-Aldrich # 190764 or equivalent)	For formazan extraction
Solvents HPLC/UPLC grade	HPLC/UPLC measurement
Formazan (CASRN 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

- Prepare a 5 mg/mL solution in PBS^{-}.
- Thoroughly mix this stock solution during 15 minutes at RT.
- Keep in the fridge protected from light up to 16 days or frozen at -18°C to -20°C up to 1 year.

b) MTT ready to use solution preparation

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

Formazan Extraction solution
2-propanol (isopropanol 100%) (CAS N°67-63-0) or equivalent is used as formazan extraction solution.

Test Compounds

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

Proficiency chemicals

Prior to routine use of the SkinEthic RHE test method that adhere to the OECD TG431, laboratories should demonstrate technical proficiency by correctly classifying the twelve Proficiency Chemicals listed in **Table** below. In case of the use of a method for sub-classification, also the correct sub-classification should be demonstrated.

Test chemical	CASRN	Chemical Class	UN GHS Cat. Based on *In Vivo* results	Cat. Based on *In Vitro* results	MTT Reducer	Physical State
Sub-category 1A *In Vivo* Corrosives						
Bromoacetic acid	79-08-3	Organic acid	1A	1A	-	Solid (S)
Boron trifluoride dihydrate	13319-75-0	Inorganic acid	1A	1A	-	Liquid (L)
Phenol	108-95-2	Phenol	1A	1A	-	S
Dichloroacetyl chloride	79-36-7	Electrophile	1A	1A	-	L
Combination of sub-categories 1B-and-1C *In Vivo* Corrosives						
Glyoxylic acid monohydrate	563-96-2	Organic acid	1B-and-1C	1B-and-1C	-	S
Lactic acid	598-82-3	Organic acid	1B-and-1C	1B-and-1C	-	L
Ethanolamine	141-43-5	Organic base	1B	1B-and-1C	Yes	Viscous
Hydrochloric acid (14.4%)	7647-01-0	Inorganic acid	1B-and-1C	1B-and-1C	-	L
***In Vivo* Non Corrosives**						
Phenethyl bromide	103-63-9	Electrophile	Not classified (NC)	NC	Yes	L
4-Amino-1,2,4-triazole	584-13-4	Organic base	NC	NC	-	S
4-(methylthio)-benzaldehyde	3446-89-7	Electrophile	NC	NC	Yes	L
Lauric acid	143-07-7	Organic acid	NC	NC	-	S

Positive Control(s)

8N KOH used as positive control (PC).

Negative Control(s)

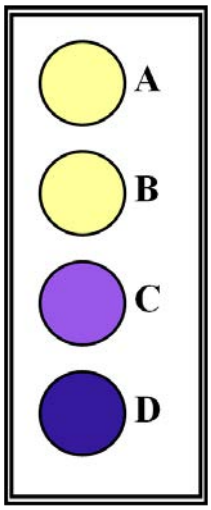
Distilled H_2O used as negative control (NgC).
NgC and PC should be tested concurrently to the test chemicals for each run by the user. The data are not provided by the tissue supplier.

Checking for direct MTT reduction of test chemical s (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 intera ction*
D: *test chemical 3: strong interaction*

Step 1 :

- Fill tube with 300 µL of MTT ready to use solution (1 mg/ml)
- Add 40±2 µL or 20±2 mg of the test chemical to be evaluated or water (or SkinEthic™Maintenance Medium) for control and Mix.
- Incubate the mixture for 3 hours ± 5 minutes at 37°C protected from light (test conditions).

If the MTT solution color becomes blue or purple, the test chemical interacts with the MTT.
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.
- Each MTT interacting test chemical is applied on at least two killed tissues (usually 2 replicates) per time of application. In addition to that, two killed tissues remain treated with sterile distilled water for control (negative killed control).

For details see *Summary of adapted controls (page 13)* 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) (even if additional runs are required to classify the test chemical as **NC, C** or **Sub Cat.**).

- 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** , specific calculation 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 5)

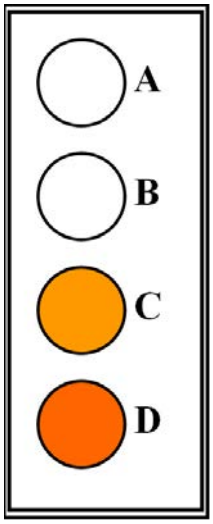
When OD is chosen as endpoint:

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

- Adapted controls should always be included for colored 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 **Data analysis**).



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 Eppendorf tubes with 90 µL of water.
- Add **10 ± 2** mg (solids) or **10 ± 0.5** µL (liquids) of the test chemical to be evaluated.
- Vortex the solution for few seconds.
- Incubate the solution at least 30 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 chemicals hould 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 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 corrosion evaluation of a test chemical (concurrently to every testing for each time).

Step 2 :

The Non-Specific Color (%NSC $_{living}$) is first quantified by using at least two living tissues (usually 2 replicates) per chemical. For details see *Summary of adapted controls depending of test chemical physical properties* (Condition 3), page 13.

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 coloring test chemical before calculating the final % viability (see **Data analysis** , specific calculation for Condition 3, page 26).

When HPLC/UPLC-spectrophotometry is chosen as endpoint:

No pre-check or control are necessary.

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

When OD is chosen as endpoint:

The test chemical intrinsic color can, in some cases, interfere with the MTT formazan extraction readings. Blue, dark purple and black test chemical may be directly tested on colorant controls without additional checking test due to their high probabilities to interfere with the blue MTT (formazan salt). In that case, if the color of the test chemical interferes with the MTT pre-check, an additional 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 *Summary of adapted controls depending of test chemical physical properties* , page 13).

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

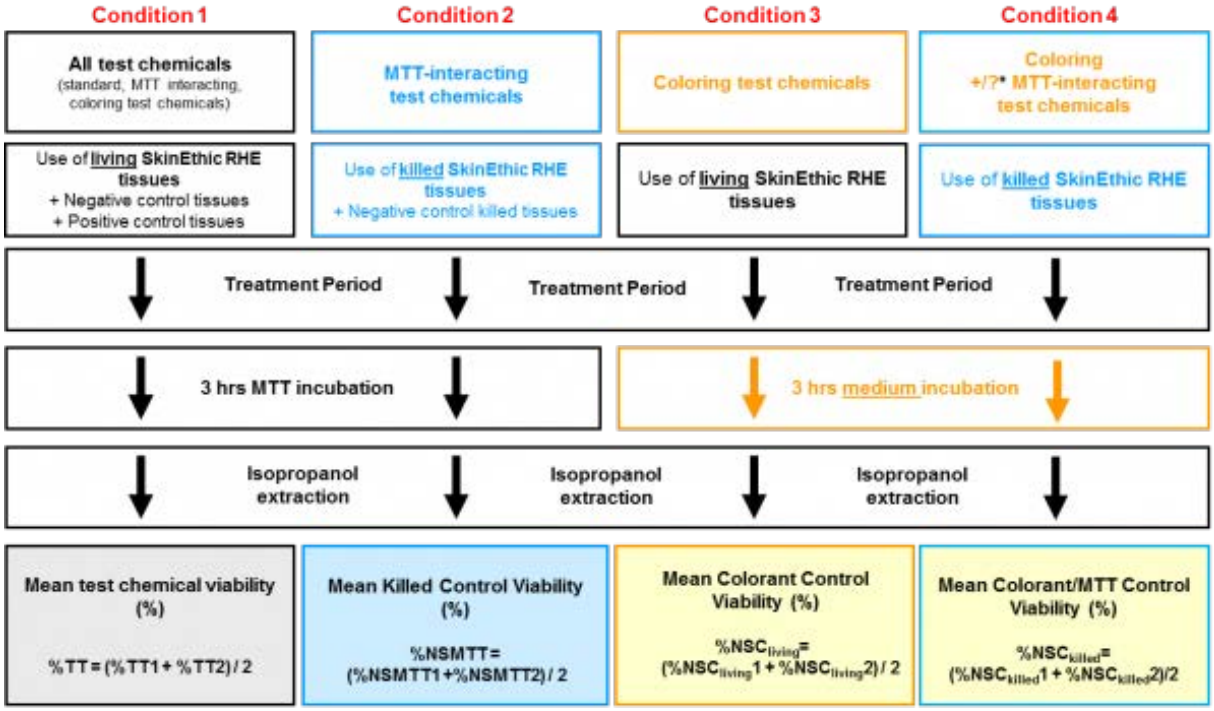
- Evaluation of color test chemicals with possible MTT direct interaction is documented using **Annex 1** and **Annex 2** .
- Report systematically and concurrently to every testing the part of OD due to the non-specific coloration on killed tissues to define the %NSC $_{killed}$ value for a coloring MTT-reducer test chemical before calculating the final % viability (see **Data analysis** , specific calculation 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 $_{living}$ 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 tissues with MTT incubation).

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



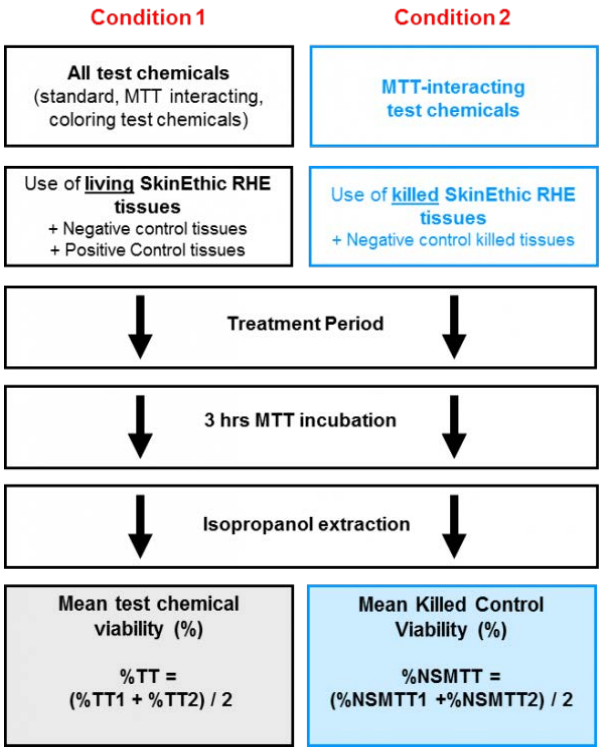
*? : not determinable due to strong colour interference

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)



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.

Method

Test System Procurement

Reconstructed Human Epidermis Epidermis SkinEthic™ RHE model

Tissues/Media Description	*Storage conditions*	*Shelf life*
Epidermal tissues, small size (0.5 cm ^{2}), full maturity	37°C, 5%CO_2, ≥ 90% humidity	see technical data sheet
SkinEthic™ Maintenance Medium	2° C to 8°C	see technical data sheet

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

Reception of materials supplied by Episkin SA :

Examine all kit components for integrity. If there is a question, a concern or something unusual, +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) after use.

2) Keep the SkinEthic™ RHE tissues and the SkinEthic™ maintenance 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 substances (page 10) or MTT pre-check inconclusive due to color (page 11).

1. Place the living epidermis in a 24-well plate pre-filled with 300 µL of distilled water.
2. Incubate at 37°C, 5% CO_2, in a > 90% humidified for 24±1 hrs.
3. At the end of the incubation, discard the water.
4. Keep killed epidermis frozen (dry) in freezer at -18°C to -20°C (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 Negative Control and test treatment on killed tissues from the same batch.
8. Proceed similarly to living tissues for application, rinsing, post-soak, etc.
9. Perform this assay only once (at least 2 tissues, usually on duplicate tissues) per test chemical when necessary.

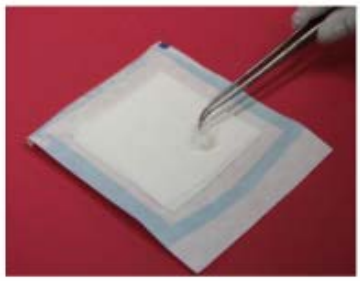
Tissue conditioning prior to testing

It is recommended to conduct this step under sterile conditions.
Proceed to a minimum 3 h pre-incubation step. This incubation period can be increased up to 24 hrs if tissues are not used on day of receipt.

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

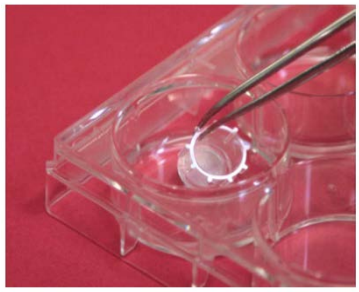
1. Fill an appropriate number of 6-well plates with 1 mL SkinEthic™ maintenance medium at RT.
2. Remove the adhesive tape from the agarose plate containing epidermal tissues. Open the 24-well plates.
3. Use sterile forceps to take off tissues from the agarose, clean the bottom of the insert on sterile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

Picture 1



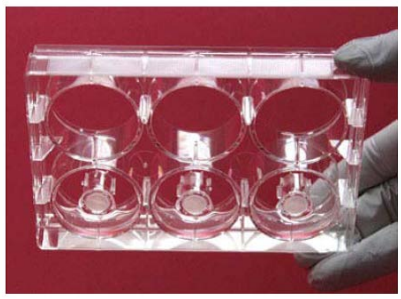
Picture 2

4. Check visually that no agarose is remaining and transfer the tissue on fresh medium by first slopping the insert before complete insert setting (**Picture 3**).



Picture 3

5. Check the absence of air bubbles by watching underneath the 6-well plate. See **Picture 4**.



Picture 4

6. Place the SkinEthic™ RHE tissues at 37±2 °C, 5±1% CO_2, ≥ 90% humidity for at least 3 hours until test chemical application.
7. At the end of the pre-incubation step, transfer tissues into application plate.

Test Material Exposure Procedures

Safety precautions
Corrosive materials are dangerous: it is thus necessary to work in laminar flow hood with chemical filter or in ventilated cabinets and wear gloves, coat, as necessary.
Since the present test is a short term test, sterility is not as important as in other applications of the SkinEthic ™ RHE model. Nevertheless, it is important to keep assay media sterile and to keep risk of contamination at a low level.

Plates' preparation

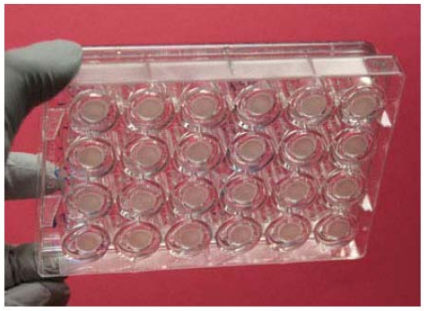
Application plates:

1. Prepare an appropriate number of 24-well plates for the 3 min ± 15 s exposure period at RT, 1 hr ± 1 min exposure period at RT: at least 2 wells per test chemical (usually 2 replicates per exposure period), positive control (PC, 2 replicates) and negative control (NgC, 2 replicates per exposure period).

    - Negative control is concurrently performed for both 3 min ± 15 s and 1 h ± 1 min time treatments to the test chemical, in at least two replicates per time treatment.
    - Positive control is concurrently performed for the 1 h ± 1 min time treatment to the test chemical, in at least two replicates.

    It is recommended to use one plate per test chemical and per control (for all exposure periods).

2. Fill the wells with 300 µL maintenance medium.
3. Transfer the tissue on fresh medium by first slopping the insert before complete insert setting. After tissue transfer, check the absence of air bubbles by watching underneath the 24-well plate. See **Picture 5.**
4. Check the presence of all materials/equipment necessary for test chemicals application, washing, drying and post-incubation steps.



Picture 5

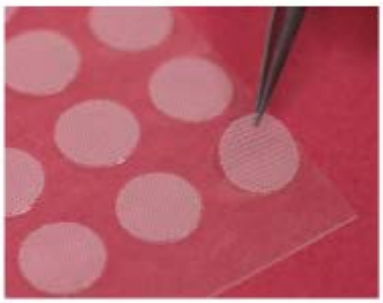
Application volumes/quantities

Liquid and viscous test chemicals:

1. Dispense 40±3 µL (i.e. 80 µL/cm²) of the undiluted test chemical on the top of each epidermis tissue (at least two per test chemical: replicate 1, replicate 2), using positive displacement pipette (note that adjustable pipette is acceptable for non-viscous test chemicals). See **Picture 6**.

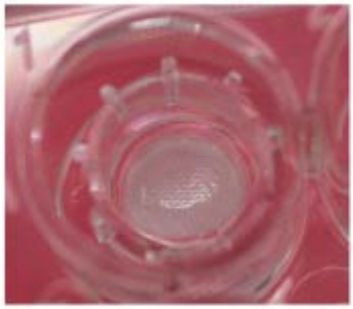
Picture 6

2. Carefully apply a nylon mesh (Ø = 7.5 mm) on the whole surface with forceps. See **Pictures 7, 8** and **9.**



Picture 7

Picture 8



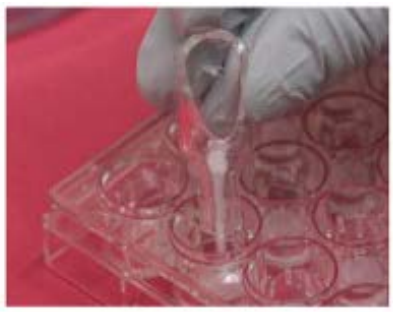
Picture 9

Solid test chemicals:

1. If necessary, the test chemical should be crushed to a fine powder using a mortar and a pestle.
2. Gently spread 20±2 µL of distilled water using a positive displacement pipette (or adjustable 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 20 ± 3 mg (i.e. 40 mg/cm²) of the powder to the epidermis surface. See **Pictures 10, 11** and **12.**
   As an alternative **,** remove the tissue from the plate and place onto a dish of weighting to avoid any contamination of the maintenance medium by the test chemical during its disposal.

Picture 10

Picture 11



Picture 12

Waxy (sticky) test chemicals:

1. Gently spread 20 ± 2 µL of distilled water using a positive displacement pipette (or adjustable pipette) to the epidermal surface in order to improve further contact between the test chemical and the epidermis.
2. Allow for the tare with a nylon mesh and directly weigh 20±3 mg (i.e. 40 mg/cm²) and spread waxy test chemical on this latter.
3. Apply the test chemical coated side of the nylon mesh on the epidermal surface and spread it gently on the whole surface.

Treatment

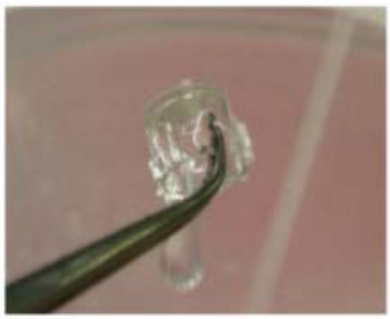
1 hour ± 1 min exposure period (at RT)

Note: *dosing time interval is dedicated by rinsing procedure*
At least two tissues per test chemical or controls should be used (usually 2 replicates). The application order is important since it will be the same for washing. Apply the test chemical quantity described on page 16.
An example of 1 h ± 1 min application treatment and rinsing steps is provided in **Table** below.

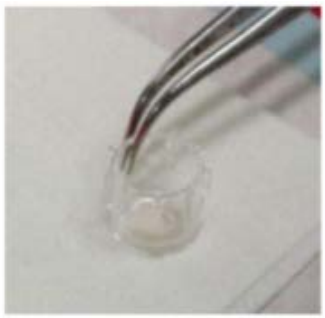
Exposure: 1h		NgC	PC	TT1	TT2	TT3	TT4	TT5	TT6
Application	**Tissues 1**	13h00	13h03	13h06	13h09	13h12	13h15	13h18	13h21
	Tissues 2	13h01	13h04	13h07	13h10	13h13	13h16	13h19	13h22
Rinsing	**Tissues 1**	14h00	14h03	14h06	14h09	14h12	14h15	14h18	14h21
	Tissues 2	14h01	14h04	14h07	14h10	14h13	14h16	14h19	14h22

- When the 1 h ± 1 min exposure period is completed, remove the first insert with forceps and proceed to rinsing step.
- Using an adjustable pipette or a wash bottle, gently rinse the tissue with PBS - (20 x 1 mL) over a funnel put on a large beaker to remove any residual of material. See **Picture 13**.
- Remove excess PBS - by gently shaking the insert (**Picture 14**) and dry bottom insert with absorbent paper or gauze (**Picture 15**).

Picture 13



Picture 14



Picture 15

Suggestion *:* Keep 1-minute interval between each tissue application.
Record starting time of 1 hour ± 1 min exposure timing for each test chemical using **Annex 4**.

3 min ± 15 s exposure period (at RT)

Note: *dosing time interval (exactly one minute) is dedicated by rinsing procedure*

At least two tissues per test chemical or negative control should be used (usually 2 replicates). The application order is important since it will be the same for washing.
An example of 3 min ± 15 s application period and rinsing step is provided in **Table** below.

Exposure: 3min		**NgC**	**TT1**	**TT2**	**TT3**	**TT4**	**TT5**	**TT6**
Application	**Tissues 1**	14h40	14h46	14h52	14h58	15h04	15h10	15h16
	Tissues 2	14h41	14h47	14h53	14h59	15h05	15h11	15h17
Rinsing	**Tissues 1**	14h43	14h49	14h55	15h01	15h07	15h13	15h19
	Tissues 2	14h44	14h50	14h56	15h02	15h08	15h14	15h20

- When the 3 min ± 15 s exposure period is completed remove the first insert with forceps and proceed to rinsing step. The table above presents the rinsing timing corresponding to the proposed application timing.
- Using an adjustable pipette or a wash bottle gently rinse the tissue with PBS $^-$ (20 x 1 mL) over a funnel put on a large beaker to remove any residual of material. See **Picture 13**.
- Remove excess PBS $^-$ by gently shaking the insert (**Picture 14**) and dry bottom insert with absorbent paper or gauze (**Picture 15**).

Suggestion: Keep 1-minute interval between each tissue application. Record the exact timing and document the correspondent Application timing 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 page 10).*

1. Label an appropriate number of 24-well plates for the **3 min ± 15 s** exposure period and **1 h ± 1 min** exposure period.
2. Prepare MTT medium according to ***Preparations*** section, page 8.
3. Dispense **300 µL** of MTT medium in at least 2 wells of the plate (usually 2 replicates) (**1 mg/mL MTT** solution freshly prepared in maintenance medium) (**Conditions 1** and **2**).
4. For the **specific adapted coloring controls,** dispense **300 µL** of maintenance medium instead of MTT medium (**Conditions 3** and **4**).
5. Once all tissues are rinsed for an exposure period, remove remaining maintenance medium below the tissues 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.
6. Incubate tissues for **3 hours ± 15 minutes** at 37±2°C, 5±1% CO_2, ≥ 90% humidity.
7. 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 ussing **Annex 3** and **4**.

Formazan extraction

1. Label an appropriate number of 24-well plates for the **3 min ± 15 s** exposure period and the **1 h ± 1 min** exposure period. 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.

To minimize any potential contamination of the isopropanol extraction solution with test chemical that may have remained on the tissue or with strongly colored test chemical, tissues could be extracted from the bottom only (1.5 mL isopropanol).

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)*** overnight without shaking at room temperature or ***b)*** overnight in the fridge protected from light without shaking. The following day, shake at least 30 minutes at RT on plate shaker (~ 120 rpm), or ***c)*** alternatively, 2 hours protected from light with gentle agitation (about 120 rpm).
   Record Incubation timings using **Annex 3** and **4**.

Endpoint Measurement

At the end of formazan extraction, the following two endpoint mesaurements can be performed

Optical density measurements

1. Remove the parafilm layer(s).
2. Maintain the insert with forceps.
3. Pierce tissue and polycarbonate filter with a tip in order to get the whole extraction solution in the corresponding well (e.g. using a 200 µL tip on a micropipette).
4. Homogenize the extraction solution vigorously up and down through the insert until a homogeneous solution is reached.
5. Remove the empty insert.
6. Use one plate per exposure time. Transfer 2 x 200 µL extraction solution per well (= 2 wells per tissue i.e. 2 replicates per tissue) into a 96-well plate. Be careful to isopropanol evaporation 96 well plates it is recommended-not to fill more than 42 wells/plate before readings.
7. Isopropanol is used as blank (8 replicates).
8. Read the Optical Densities (OD) using a 96-well plate spectrophotometer ideally at 570 nm wavelength (eventually between 540 to 600 nm). No reference filter should be used.
9. All data generated are considered as raw data. Perform the Quality Control of the raw data and adapt archiving upon needs.

HPLC/UPLC-spectrophotometry measurement:

1. **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.
2. Use isopropanol as blank (200 µL / well).
3. **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).
4. Use a validated analytical method (see *Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint* under the **Downloads** section of this protocol) on a qualified HPLC/UPLC-UV/Visible system.
5. Measure peak area at the retention time of the formazan at the wavelength defined in the validated analytical method.
6. All data generated are considered as raw data.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, by e-mail, on the day of delivery of the kit.

Negative control (NgC):

- The absolute OD of the negative control (NgC) tissues (H_2O treated) in the testing run is an indicator of tissue viability in the testing laboratory after shipping and storage procedures and under use conditions.
- A run meets the acceptance criteria if the mean Optical Density (OD NgC) of at least two tissues treated **(usually 2 replicates)** with NgC is ≥ 0.8 at 570nm (± 30nm) with an upper acceptance limit ≤ 3.0, for every exposure time.

- In the range 20-100% viability, and for ODs, difference of viability between the two tissue replicates should not exceed 30%.

Positive control (PC):

- Tissues treated with the PC, i.e. 8N KOH, should reflect the ability of the tissues to respond to a corrosive chemical under the conditions of the test method.
- The positive control (8N KOH) data meet the acceptance criteria if the mean viability of the tissues replicates (usually 2) exposed for 1 h ± 1 min, expressed as % of the NgC, is < 15%. The PC data meet the acceptance criteria if it is classified as corrosive.
- 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
In the range 20-100% viability, and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not exceed 30%.

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 *Validation of an analytical method on a HPLC/UPLC-spectrophotometry endpoint* under the **Downloads** section of this protocol. Once the acceptance criteria defined in the documents in the **Downloads** have been met, the HPLC/UPLC-spectrophotometry system is considered qualified and ready to measure MTT formazan under the experimental conditions described in this procedure.

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

Data Analysis

Data calculation steps

The calculation is described for a testing run composed of two tissue replicates per test chemical (see **Data analysis** and all the paragraphs dedicated to ***Calculation procedures***, pag. 24-26). To be updated upon the need.

Main steps:

(a) Blanks

- Calculate the mean OD of isopropanol 100%.

(b) Negative (H_2O) 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 three tissues).
- Calculate the mean OD for each individual tissue.

(e) Viability %

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

(f) Variability for each test

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

Calculation procedure – For viability tests only (Condition 1)

Calculation for OD reading

- **MEAN OD CALCULATION:**

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

The mean OD of the three Negative Control replicates (H_2O treated) corresponds to 100% reference viability.

*Positive Control (PC) OD Positive Control (PC)	$OD_{PC1} = OD_{PC1\ raw} - OD_{blank\ mean}$ $OD_{PC2} = OD_{PC2\ raw} - OD_{blank\ mean}$
Mean OD Positive Control	$\mathbf{OD_{PC} = [OD_{PC1} + OD_{PC2}]/2}$
*Test chemical Treatment OD Test chemical Treatment (TT)	$OD_{TT1} = OD_{\mathbf{TT1}\ raw} - OD_{blank\ mean}$ $OD_{TT2} = OD_{\mathbf{TT2}\ raw} - OD_{blank\ mean}$
Mean OD Test Treatment	$\mathbf{OD_{TT} = [OD_{TT1} + OD_{TT2}]/2}$

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

% Negative Control 1 % Negative Control 2	$\%NgC1 = [OD_{NgC1} / \text{mean } OD_{NgC}] \times 100$ $\%NgC2 = [OD_{NgC2} / \text{mean } OD_{NgC}] \times 100$
% mean Negative Control	**%NgC= (%NgC1 + %NgC2)/2**
% Positive Control 1 % Positive Control 2	$\%PC1 = [OD_{PC1} / \text{mean } OD_{NgC}] \times 100$ $\%PC2 = [OD_{PC2} / \text{mean } OD_{NgC}] \times 100$
% mean Positive Control	**%PC= (%PC1 + %PC2)/2**
% Test Treatment 1 % Test Treatment 2	$\%TT1 = [OD_{TT1} / \text{mean } OD_{NgC}] \times 100$ $\%TT2 = [OD_{TT2} / \text{mean } OD_{NgC}] \times 100$
% Mean Test Treatment	**%TT = (%TT1 + %TT2)/2**

Calculation for HPLC/UPLC- spectrophotometry endpoint

- **MEAN AREA CALCULATION:**

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

The mean Area of the three Negative Control replicates (H_2O treated) corresponds to 100% reference viability.

*Positive Control (PC) **Mean Area Positive Control**	$\mathbf{Area_{PC} = [Area_{PC1} + Area_{PC2}]/2}$
*Test Treatment (TT) **Mean Area Test Treatment**	$\mathbf{Area_{TT} = [Area_{TT1} + Area_{TT2}]/2}$

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

% Negative Control 1 % Negative Control 2	$\%NgC1 = [Area_{NgC1} / \text{mean } Area_{NgC}] \times 100$ $\%NgC2 = [Area_{NgC2} / \text{mean } Area_{NgC}] \times 100$
% mean Negative Control	**%NgC= (%NgC1 + %NgC2)/2**
% Positive Control 1 % Positive Control 2	$\%PC1 = [Area_{PC1} / \text{mean } Area_{NgC}] \times 100$ $\%PC2 = [Area_{PC2} / \text{mean } Area_{NgC}] \times 100$
% mean Positive Control	**%PC= (%PC1 + %PC2)/2**

% Test Treatment 1	%TT1 = [Area $_{TT1}$ / mean Area $_{NgC}$] x 100
% Test Treatment 2	%TT2 = [Area $_{TT2}$ / mean Area $_{NgC}$] x 100
% mean Test Treatment	**%TT = (%TT1 + %TT2)/2**

The mean relative viability is used for classification according to the ***Prediction Model*** *(page 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
% 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 $_{CNSMTT1}$ +%FV $_{C\ NSMTT2}$)/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
%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 $_{CNSMTT1}$ +%FV $_{CNSMTT2}$)/2**

The mean final viability is used for classification according to the ***Prediction Model*** *(page 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 %.
Note: *This calculation procedure is not applicable to HPLC/UPLC-spectrophotometry.*

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

- **NON-SPECIFIC COLOR CALCULATION ($\%NSC_{Living}$)**

% Non-Specific Color tissue 1	$\%NSC_{living\ 1} = [(OD_{TT1\text{-}MTT} / OD_{NgC}] \times 100$
% Non-Specific Color tissue 2	$\%NSC_{living\ 2} = [(OD_{TT2\text{-}MTT} / OD_{NgC}] \times 100$
Mean % Non-Specific Color	$\mathbf{\%NSC_{living} = (\%NSC_{living1} + \%NSC_{living2})/2}$

- **CORRECTED FINAL VIABILITY (FV_C)**

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

The mean final viability is used for classification according to the ***Prediction Model*** *(page 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' ($\%NSC_{killed}$).
Note: *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	$\%NSC_{killed2} = (OD_{kt\text{-}MTT2} / OD_{NgC}) \times 100$
Mean %Non-Specific Color without MTT	$\mathbf{\%NSC_{killed} = (\%NSC_{killed1} + \%NSC_{killed2})/2}$

- **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	$\%FV_{C\ TT2} = \%\ TT2 - \%NSMTT - \%NSC_{living} + \%NSC_{killed}$
% Mean Final Viability	$\mathbf{\%FV_{C\ TT} = (\%FV_{C\ TT1} + \%FV_{C\ TT2})/2}$

The mean final viability is used for classification according to the ***Prediction Model*** *(page 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:

- ***IF*** the mean of % Non-Specific Color on living tissues(%NSC_{living}) or % Non-Specific MTT reduction (%NSMTT) of the qualified test is less than or equal to (≤) 50%, ***THEN*** the test chemical is considered to be compatible with the test method.
- ***F*** the mean of %NSC_{living} or %NSMTT of the qualified test is greater than (>) 50% AND their classification remains the same upon correction, ***THEN*** the test chemical is considered to be compatible with the test method.
- ***IF*** the mean of %NSC_{living} or %NSMTT 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 corrosion should be considered.

Results for test chemicals 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	*24*	*25 (condition 2)*	*25 (condition 3)*	*26*		

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	*24*	*25*		

Prediction Model

The test method allows the identification of non-corrosive and corrosive test chemicals and further supports the sub-categorisation of corrosive test chemicals into optional sub-category 1A, as well as a combination of sub-categories 1B and 1C. The test method does not allow discriminating between skin corrosive sub-category 1B and sub-category 1C due to the limited set of well-known *in vivo* corrosive sub-category 1C (OECD, 2016).

The prediction model for the SkinEthic™ skin corrosion test method, associated with the UN GHS classification system is described below:

Viability measured after exposure time points (3 and 60 minutes)	**Prediction to be considered**
Step 1	
< 50% after 3 min exposure	Corrosive (C)
≥ 50% after 3 min exposure AND < 15% after 60 min exposure	Corrosive (C)
≥ 50% after 3 min exposure AND ≥ 15% after 60 min exposure	Non Corrosive (NC)
Step 2 for substances/mixtures identified as Corrosive in step 1	
< 18 % after 3 min exposure	Optional Sub-category 1A (Cat. 1A)
≥ 18 % after 3 min exposure	Optional Sub-categories 1B-and-1C (Cat. 1B-and-1C)

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

For a full evaluation of 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 (3hrs ± 15min)

Study N°…….....

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Interaction Blue Color Yes / No
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Date: …….. ID and signature:……

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

Study N°…….....

Test chemical Name or code number	Start of Incubation Time:	End of incubation Time:	Ability to Color Yes / No
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Date: …….. ID and signature:……

Annex 3: Incubation timings for 3 min ± 15 s exposure period

Study N°…..... Batch N°:……..

Test chemical Name Or code*	Tissue	Treatment 3 min ± 15 s		MTT incubation 3 hrs ± 15min		Formazan extraction	
		Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)
……	1						
……	2						
……	1						
……	2						
……	1						
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* A run consists of one or more test chemicals tested concurrently with NgC.

Date: …….. ID and signature:……

Annex 4: Incubation timings for 1 hrs ± 1 min exposure period

Study N°…..... Batch N°:……..

Test chemical Name Or code *	Tissue	Treatment 1 hrs ± 1 min		MTT incubation 3 hrs ± 15min		Formazan extraction	
		Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)	Start time (hh:mm)	End time (hh:mm)
……	1						
……	2						
……	1						
……	2						
……	1						
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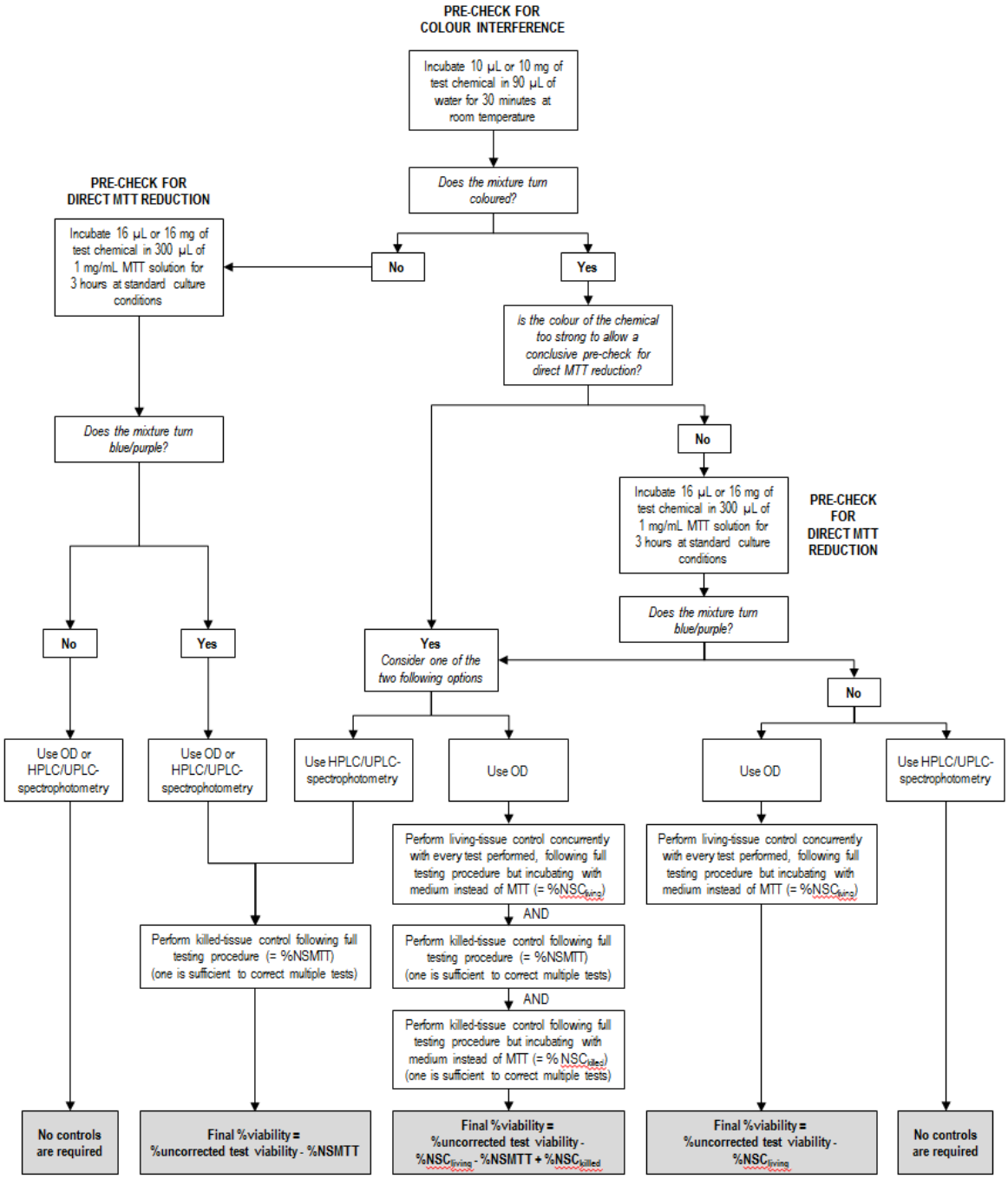
* A run consists of one or more test chemicals tested concurrently with NgC and PC.

Date: …….. ID and signature:……

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



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