

**Biological evaluation of medical devices:**

**Standard Operating protocol (SOP) for *in vitro* skin  
irritation test with SkinEthic™ RHE model**

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**EPISKIN**

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## PROTOCOL INTRODUCTION

### NAME OF THE METHOD

#### ***In vitro* skin irritation test with SkinEthic™ RHE model**

##### **Abstract:**

The *in vitro* skin irritation test with SkinEthic™ RHE model is designed for the prediction of acute skin irritation of medical devices by measurement of cytotoxic effects of extract (prepared according to ISO10993-12), as reflected in the MTT assay, on the SkinEthic Reconstructed Human Epidermis (RHE) model.

Contacts for In vitro skin irritation test with SkinEthic™ RHE model

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### OBJECTIVE AND APPLICATIONS

#### **Type of Testing:**

Replacement

#### **Purpose of Testing:**

Classification and labelling

#### **Applicability Domain:**

- Liquids (aqueous or non-aqueous)

The method is not designed for testing of gases and aerosols or highly volatile test compounds.

### EXPERIMENTAL DESCRIPTION

#### **Endpoint and Endpoint Measurement:**

**CELL VIABILITY:** Cell viability is determined by a reduction by mitochondrial dehydrogenase activity as measured by formazan salt production from MTT.

**IL-1 $\alpha$ :** Measurement of IL-1 $\alpha$  concentration in the culture medium by ELISA is proposed as complementary endpoint to cell viability. The pertinence of this measure to increase the accuracy of the prediction model will be assessed in a second step.

#### **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 irritation potential. > 50% reduction in cell viability is used to indicate the presence of the significant biological effect.

#### **Experimental System(s):**

SkinEthic™ RHE from EPISKIN: normal human keratinocytes taken from healthy volunteer donors are cultured for 17-days on an inert 0.5 cm<sup>2</sup> polycarbonate filter at the air-liquid

interface to form the three-dimensional SkinEthic™ RHE model comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum.

### Basic Procedure

Samples are prepared according to ISO10993-12 by extraction (37 +/-1°C for 72 +/- 2 hrs with agitation) using polar (0.9% saline solution of NaCl) and non-polar (Sesame Oil of pharmaceutical grade) solvents to be used 24 hours maximum after extraction. Each sample (test materials, negative and positive controls) is topically applied concurrently on three tissues replicates for 24±2 hours at 37°C. Tissue exposure to the test substance is followed by rinsing with phosphate buffer saline (PBS-) and manually dried. Cell viability is assessed by incubating the tissues for 3 hours with 0.3 mL MTT solution (1 mg/mL). The formazan crystals are extracted using 1.5 mL isopropanol for 2 hours at RT and quantified by spectrophotometry at 570 nm wavelength.

SDS 1% (w/v; final concentration) in saline solution of NaCl and in sesame oil treated epidermis are used as positive controls. PBS- treated tissues will be used as negative controls. In addition, saline solution of NaCl and sesame oil (exposed 37 +/-1°C for 72 +/- 2 hrs) will be used as vehicle extraction controls. For each treated tissue, the cell viability is expressed as the percentage of the mean negative control tissues (PBS- treated tissues). Values under or equal to 50% are used to classify the test substance as irritant. Quantification of released Interleukin-1 alpha (IL-1α) will be assessed in the supernatant of the epidermis as a complementary biological endpoint.

### DATA ANALYSIS/ PREDICTION MODEL

Irritation potential of test substance is determined according to the UN GHS and EU CLP (Category 2: Irritant or “No Category”; UN, 2009; EU, 2008). The mean relative tissue cell viability above 50 % predicts its non irritancy potential. The prediction model is defined as described below:

<i>In vitro</i> results	<i>In vivo</i> classification
Mean tissue cell viability ≤50%	Irritant
Mean tissue cell viability >50%	Non Irritant

### ABBREVIATIONS AND DEFINITIONS

ET-50: Exposure time that induce 50% cell viability

I: Irritant

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

MDS: Methods Documentation Sheet

NC: Negative Control

VC: Vehicle Control

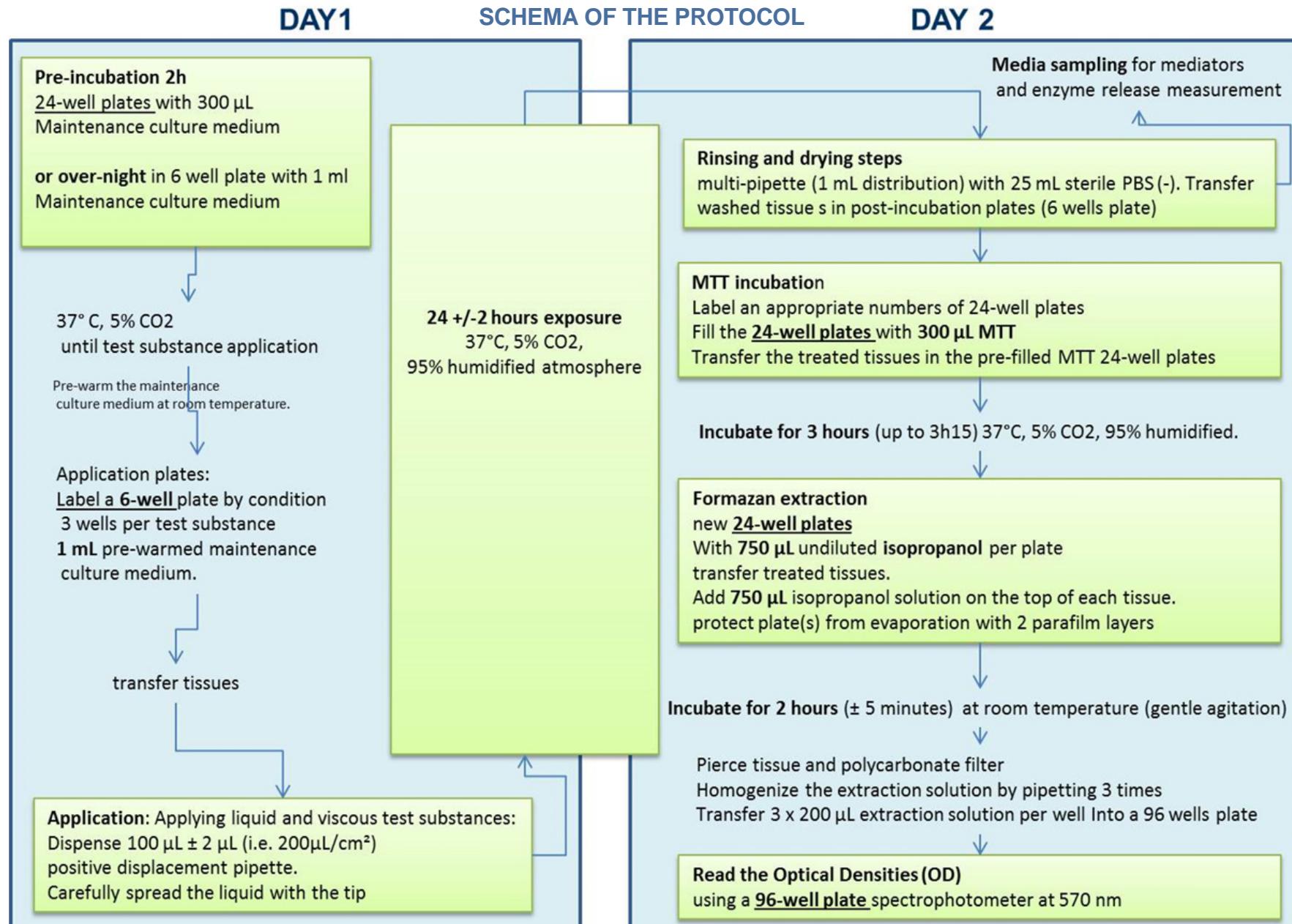
NI: Non Irritant

OD: Optical Density

PC: Positive Control

RHE: SkinEthic™ Reconstructed Human Epidermis

SDS: Sodium Dodecyl Sulphate



## MATERIALS AND PREPARATIONS

### Health and safety issues

EPISKIN 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.

#### Safety precautions:

Irritant materials are dangerous: It is thus recommended to work in laminar flow hood with chemical filter or in ventilated cabinets and wear gloves, coat, as necessary.

### Cell or test system

SkinEthic™ RHE tissues from EPISKIN, 4 rue Alexandre Fleming, 69366 Lyon, France, <http://www.episkin.com>

### Equipment

#### Fixed equipment

ITEM	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 <sub>2</sub> , 95% relative humidity)	For incubating tissues
Laboratory balance (accuracy 0.1 mg)	For pipette verification and test substance weighing
96-well plate photometer with a 570 nm filter	For optical density readings (MTT)
Plate shaker	For extraction of formazan
Stop-watches	To be used during application of test materials
Sterile, blunt-edge forceps	For handling tissue inserts
Plastic wash bottles	For collecting PBS <sup>-</sup> rinses
1 L beaker	For rinsing tissues with PBS <sup>-</sup>
1 Funnel	For rinsing tissues with PBS <sup>-</sup>
1 gauged flask	For SDS 1% solution preparation
Adjustable Pipettes / multi-step Pipettes	For pipetting 1 mL assay medium, 300 µL MTT medium, 750 µL propan-2-ol, 200 µL formazan extract from 24-well plate into 96-well plate for the plate photometer
Positive displacement pipette for 100 µL delivery	For application of liquid and viscous test materials

Multi-pipette + adapter for 25 mL tip	For washing
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### Consumables

Item	Use
Extra 6-well plates – sterile	For the 24h exposure step
Extra 24-well plates – sterile	For the MTT incubation + formazan extraction steps
Extra 96-well plates – sterile	For OD measurements
Parafilm or sealable plastic bag	Covering plates during formazan extraction to avoid solvent
Cotton tip swabs	For drying the tissue surface
Sterile absorbent paper / sterile gauze	To remove agarose fragments or to dry inserts

### Equipment verification

It is strongly recommended to use regularly verified apparatus equipment. Maximum time interval between two verifications is specified for each apparatus necessary in this protocol in Methods Documentation Sheet, MDS: Main equipment verification (**Appendix 1**).

### Media, reagents, others

SkinEthic™ RHE set and media provided by EPISKIN:

Reference	Description	Conditions	Shelf life
SkinEthic RHE/S/17	Epidermal tissues, small size	37°C	7 days
SMM	Maintenance medium	4°C	14 days

N.B.: the SkinEthic maintenance and growth culture media should be pre-warmed only at room temperature (and not at 37°C).

### Reception of materials supplied by EPISKIN:

Examine all kit components for integrity. If there is a concern call EPISKIN (☎ +33 4 37 28 72 00 ; E-mail: [sales@episkin.com](mailto:sales@episkin.com) or [aalonso@episkin.com](mailto:aalonso@episkin.com)).

- 1) Document MDS: Skinethic™ RHE set – EPISKIN™ materials receipt (**Appendix 2**).
- 2) Keep SkinEthic™ RHE tissues and the maintenance medium at room temperature for the pre- incubation step.

### Materials not provided by EPISKIN:

Item (or equivalent if not specified only)	Use
PBS without Ca <sup>2+</sup> and Mg <sup>2+</sup> (PBS-)	Use as negative control, MTT diluent, and for rinsing tissues
Saline (0.9% solution of NaCl)	To be used as polar vehicle
Sesame oil of pharmaceutical grade - CAS Number 8008-74-0	To be used as non-polar vehicle
MTT – Thiazolyl Blue Tetrazolium Bromide (Sigma, # M-5655, cell culture	For the MTT assay

20% SDS [151-21-3], Cat # 05030, Fluka /Sigma	To be used as stock solution for preparing 1% SDS (positive control) in sesame oil and saline
Isopropanol solution (2-propanol (CAS N° 67-63-0), Sigma-Aldrich ref 190764)	For extraction

## Preparations

### Media and Endpoint Assay Solutions

#### MTT stock solution preparation

MTT solution is light sensitive. Protect it from light using silver paper or appropriate material.

- Prepare a 5 mg/mL solution in PBS-.
- Thoroughly mix this stock solution during 15 minutes at RT.
- Keep in the fridge (2 to 8°C) protected from light up to 16 days.

#### MTT ready to use solution preparation

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

Document MDS: MTT stock solution (**Appendix 3**).

#### Isopropanol solution

Use 2-propanol (CAS N°67-63-0).

Document MDS: Isopropanol (**Appendix 3**).

### Test Compounds

#### Positive Controls (1% v/v SDS)

500 µL of 20% SDS (Fluka /Sigma) should be mixed with 9.5 mL of the particular vehicle (saline or sesame oil) and thoroughly vortexed.

#### Test substances identification

Main information concerning the test substances (name or code, total weight, total surface area, reception date, physical consistence, storage conditions) should be registered in MDS: Test substances (**Appendix 4**).

N.B.: Non-coded test substances should be handled following material safety datasheet. Unknown and coded test substances with no or incomplete safety handling information should be considered as irritating and toxic and must be handled with maximum care.

**Water killed epidermis preparation (for MTT-interacting chemicals or MTT pre-check inconclusive due to colour)**

- 1) Place the living epidermis in a 24-well plate pre-filled with 300 µL of distilled water.
- 2) Incubate at  $37\pm 2^{\circ}\text{C}$ ,  $5\pm 1\%$   $\text{CO}_2$ ,  $\geq 90\%$  humidity for  $24\pm 1$  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-soak, etc.
- 9) Perform this assay only once (at least 3 tissues, usually 3 tissues) per test sample when necessary.

## METHOD

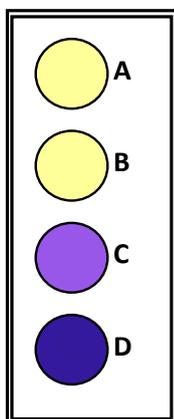
### Checking for direct MTT reduction of test samples (Annex 1).

*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 substance used. Prior to experiments, test substance should be put in contact with the MTT solution as described below.

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

#### Step 1:



- Fill tube with 100  $\mu$ L of the test substance to be evaluated or control.
- Add 300  $\mu$ L of MTT ready to use solution (1 mg/mL) and mix.
- Incubate the mixture for 3 hours  $\pm$  5 minutes at 37°C protected from light (test conditions).

If the MTT solution color becomes blue or purple, the test substance 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.

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

#### Step 2 :

- Use killed tissues that possess no metabolic activity but can absorb and bind the test sample like viable tissues.
- Each MTT interacting test substance 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).

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

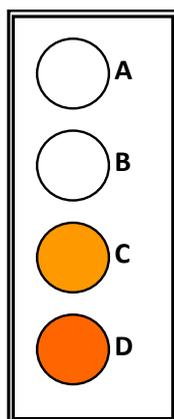
- Report systematically the part of OD due to the non-specific reduction of the MTT (to define the %NSMTT value for a MTT-reducing test substance) for a test substance before calculating the final viability.

### Checking for color test samples only (Annex 2)

Colored test substance can generate a remaining Non-Specific Color on living tissues (%NSC<sub>living</sub>). Therefore, each test substance has to be checked for its colorant properties. Indeed, test substance 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 substance.

Specific controls must be used in these cases consisting of test substance-treated tissues that followed all the steps of the method except the MTT incubation. %NSC<sub>living</sub> is determined after isopropanol extraction and OD reading in similar conditions.



A: control  
B: Test sample 1: no color  
C: slight coloration of an orange Test sample  
D: Strong coloration of an orange Test sample

NB: Orange is an example. A coloring test sample can have of course another color.

### Step 1:

- Fill tubes with **10±1 µL** of the extract of the 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 example on the left).

When a colored solution is observed, the tissue staining ability of the test substance should be checked (step 2), otherwise no adapted controls are required.

It 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<sub>living</sub> value.

The visual **possible interference should be checked** once (step 1).

### Step 2 :

- The Non-Specific Color (%NSC<sub>living</sub>) is first quantified by using three living tissues per chemical.
- Coloring test substance 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<sub>living</sub> control needs to be conducted with each test performed (concurrently to every testing: i.e. for each time in each run).**

- Report **systematically and concurrently to every testing** the part of OD due to the Non-Specific coloration (to define the %NSC<sub>living</sub> value), for a test coloring chemical before calculating the final % viability.

## Routine culture procedure

### Pre-incubation step

This step should be conducted under sterile conditions at least 2 hours before experimentation up to 24 hours).

- 1) Fill an appropriate numbers of 6-well plates with 1 mL SkinEthic Maintenance Medium.
- 2) Remove the adhesive tape from the agarose plate containing epidermal tissues. Open the 24-well plates and remove the absorbent paper.
- 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 inclining the insert to avoid underneath bubble formation. See Picture 3.



Picture 3

- 5) Check the absence of air bubbles by watching underneath the 24-well plate. See Picture 4.



Picture 4

6) Place tissues at 37°C, 5% CO<sub>2</sub> until test substance application.

### Test materials exposure procedures

#### Plates preparation

##### Application plates:

- 1) Pre-warm the maintenance culture medium at room temperature.
- 2) Label a 6-well plate by condition: 3 wells per test substance (code number, 3 replicates), positive controls (PC1, PC2), vehicle controls (VC1, VC2) and negative control (NC), respectively.
- 3) Fill the 3 wells with 1 mL pre-warmed maintenance culture medium.
- 4) Use sterile forceps to transfer tissues by first inclining. See Picture 3.
- 5) Check the absence of air bubbles by watching underneath the 6-well plate.

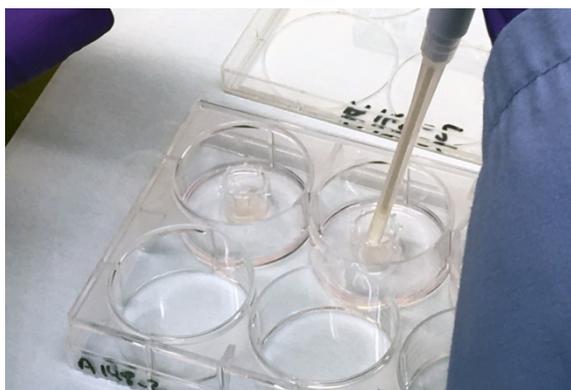
Alternatively, the pre-incubation plates can be used (aspiration of medium followed by the addition of 1 mL of fresh maintenance medium).

#### Topical applications: 24 ±2 hours treatment

It is recommended to perform this step under sterile conditions.

Three tissues per test substance should be used (3 replicates). The application order is important since it will be the same for washing.

- 1) Dispense 100 µL ± 2 µL (i.e. 200 µL/cm<sup>2</sup>) of the undiluted test substance on the top of each epidermis tissue (3 per test substance: replicate 1, replicate 2 and replicate 3), using positive displacement pipette. Use the tip to spread the test substance gently on the epidermis topical surface. See Picture 5.



Picture 5

N.B.: The model surface is hydrophobic and it is important to check that the 100  $\mu$ L are well distributed over the whole surface of the epidermis. Sometimes with polar solvent, because of surface tension mechanisms, the droplet is distributed only to the periphery of the epidermis. In this case, use either a tip to spread the substance or forceps to tap the insert into the bottom of the plate until the entire surface of the epidermis is covered.

Incubate the plate (lids on) containing the treated SkinEthic™ RHE tissues for 24 $\pm$ 2 hours at 37°C, 5% CO<sub>2</sub>, 95% humidified atmosphere.

### **Rinsing and drying steps**

#### Prepare 24 well plates for MTT test:

- 1) Label an appropriate numbers of 24-well plates
- 2) Fill the 24-well plates with 300  $\mu$ L MTT ready to use solution from light by wrapping in aluminum paper until tissues transfer in MTT. Sweep excess culture medium on the unit bottom of the tissue with absorbent paper.

#### Prepare 24 well plates for transitory storage (maintenance medium)

If you have a large number of tissues as is the case for the Round Robin Study (RRS), the rinsing step will require a long time that will lead to a discrepancy in the duration of MTT exposure between the first tissues and the last one rinsed.

It's recommended to prepare a 24 well plates with 300  $\mu$ L maintenance medium which will be used to transiently store the rinsed tissues during the rinsing step. As soon as the last tissue is rinsed you can transfer all the tissues to the plate containing the MTT solution.

#### Rinsing steps:

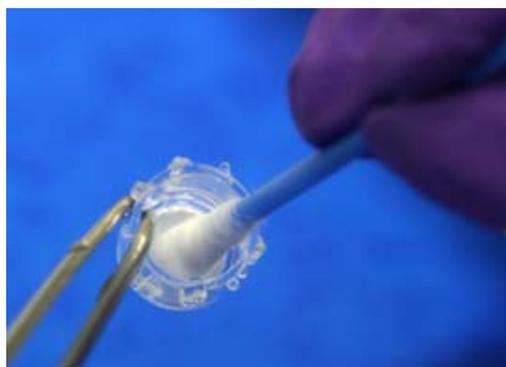
In order to prevent pollution the lids should be put on the plates continuously during the rinsing and drying steps. It is also recommended to put a lid on the sterile PBS<sup>-</sup> container.

- 1) Place a funnel in a large beaker (to avoid underneath projections/contaminations of the SkinEthic™ RHE tissues).
- 2) Fill a multi-pipette (adjusted for a 1 mL distribution) with 25 mL sterile PBS<sup>-</sup>
- 3) Open the 6-well plate.
- 4) Take the treated tissue with sterile forceps and close the 6-well plate (to protect the other tissues from washing solution projections)
- 5) Maintain the insert over the large funnel and rinse thoroughly 25 times with 1mL PBS<sup>-</sup> at a 5-8 cm distance from the insert to remove all residual test substance 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 double-sided swab (5-6 turns per end). See Picture 15.



Picture 15

### MTT test

Tissue viability is assessed by MTT reduction measurement, immediately after rinsing of the tissues

**Note:** Additional specific tissue controls for coloring test samples will be incubated with the **maintenance medium** (not with the MTT solution).

### Incubation in MTT solution

- 1) Remove excess culture medium on the bottom of the tissue insert with absorbent paper.
- 2) Transfer the treated tissues in the pre-filled MTT 24-well plates, by first sloping the insert before complete insert setting at the air-liquid interface. Respect the application order.
- 3) Check the absence of air bubbles.

- 4) Incubate for 3 hours (up to 3h15min) at 37°C, 5% CO<sub>2</sub>, 95% humidified atmosphere.

#### Media sampling for mediators and enzyme release measurement

The supernatant underneath the treated SkinEthic™ RHE tissues are kept to eventually measure the mediators and/or enzyme releases such as cytokine release (IL-1 $\alpha$ , IL-8...) or LDH release.

The present SOP is only describing the sampling procedure.

This step could be performed during the MTT incubation time (3 hours up to 3 hours 15).

- 1) Label an appropriate numbers of polypropylene tubes with caps (three per tissue unit)
  - test substance code or name
  - replicate number
  - assay number (or batch number and date)
- 2) Homogenize the maintenance culture medium, by gentle agitation (about 300 rpm) for 2 minutes. This step helps to homogenize the released mediators in the medium before sampling.
- 3) Transfer 2 x 500  $\mu$ L of incubation medium for each tissue to the pre-labeled tubes.
- 4) Freeze the tubes at -20°C until analyses (for at least a year).
- 5) Freeze also 15 mL of the maintenance culture medium that was used as diluent (provided by SkinEthic with the SkinEthic™ RHE kit – bottle of the SGM).

#### **Formazan extraction**

- 1) Label an appropriate numbers of new 24-well plates similarly to those labelled for the previous step (Incubation in MTT solution or Maintenance Medium).
- 2) Fill the plate(s) with 750  $\mu$ L undiluted isopropanol.
- 3) At the end of the 3 hours (up to 3h15) incubation in MTT solution, Use sterile forceps to transfer treated tissues.
- 4) Dry the insert bottom of the treated tissue on sterile absorbent paper or gauze.
- 5) Transfer the tissues in isopropanol solution.
- 6) Add 750  $\mu$ L 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 by stretching 2 parafilm layers over the plate and adding the lid on the plate (or place the plate cover by the lid into a sealable plastic bag).
- 9) Incubate for 2 hours ( $\pm$  5 min) at room temperature with gentle agitation (about 150 rpm) for formazan extraction.
- 10) Document MDS: Isopropanol extraction start time (**Appendix 5**).

NB: The formazan extraction could be performed overnight (for about 16-18 hours) at RT protected from light. The plate containing the tissues is sealed carefully to prevent evaporation and cross contaminations.

#### **Endpoint measurement**

##### **Absorbance / optical density measurements**

- 1) At the end of the 2 hours ( $\pm$  5 min) or overnight formazan extraction incubation time, open the plate.
- 2) Remove the 2 parafilm layers (or the plate from the sealable plastic bag).
- 3) Isopropanol solution is used as blank (6 replicates)
- 4) Maintain the insert with forceps.
- 5) Pierce tissue and polycarbonate filter with a tip in order to get the whole

extraction solution in the corresponding well.

6) Homogenize the extraction solution by pipetting 3 times up and down to complete formazan crystals solubilisation.

7) Transfer 3 x 200 µL extraction solution per well (= 3 wells per tissue i.e. 3 replicates per tissue) into a 96-well plate (labelled appropriately, accordingly to example below.

8) Read the Optical Densities (OD) using a 96-well plate spectrophotometer ideally at 570 nm wavelength (eventually between 540 to 600 nm) using a bandpass of maximum ± 30 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank	blank	blank	empty	empty	empty	empty	empty	empty
B	NC	NC	NC	NC	NC	NC	NC	NC	NC	empty	empty	empty
C	NACL	NACL	NACL	NACL	NACL	NACL	NACL	NACL	NACL	empty	empty	empty
D	SO	SO	SO	SO	SO	SO	SO	SO	SO	empty	empty	empty
E	PC NACL	PC NACL	PC NACL	PC NACL	PC NACL	PC NACL	PC NACL	PC NACL	PC NACL	empty	empty	empty
F	PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	empty	empty	empty
G	NACL 1	NACL 1	NACL 1	NACL 1	NACL 1	NACL 1	NACL 1	NACL 1	NACL 1	empty	empty	empty
H	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	empty	empty	empty
	Tissue 1			Tissue 2			Tissue 3					

NB: For the reading, use a 570 nm wavelength OD reading program performing a simple OD reading without any additional formula (individual well reading, no programmed blank, and no automatic mean calculation).

9) All data generated by the 96-well plate spectrophotometer should be printed after each reading and considered as raw data.

10) Identify ODs with conditions and tissues (replicate) studied on the raw data documents.

11) Perform the Quality Control of the raw data.

## Acceptance criteria

### Quality controls performed by EPISKIN

All the SkinEthic™ RHE model kits are free of viruses, bacteria and mycoplasma. The quality of the final RHE product is assessed by a MTT cytotoxicity test of untreated tissues, by ET50 with Triton X-100 at the concentration of 1%, and by histological examination. The quality system of the EPISKIN Laboratories is ISO 9001:2008 certified. Each batch production is provided with quality controls values and recommendations such as storage conditions, SkinEthic™ RHE instructions for use, lot number and origin, histology, cell viability (MTT OD > 0.7), barrier function integrity (4.00 > ET50 > 10.00 hrs), absence of bacteria, fungi, HIV, and Hepatitis B, C.

### Test acceptance criteria

Negative control (NC) acceptance criteria: The NC data meet the acceptance criteria if the mean OD value of the 3 tissues is  $\geq 1.2$  at 570 nm according to the historical database. The Standard Deviation value is considered as valid if it is  $\leq 18\%$ .

Positive control (PC) acceptance criteria: The PC data meet the acceptance criteria if the mean viability, expressed as % of the NC, is  $< 40\%$  and the Standard Deviation value is  $\leq 18\%$ .

Batch acceptance criteria: All test substance data from one batch are considered as valid if both the negative and the positive controls data fulfill the above criteria requirements.

Test substance data acceptance criteria: The inter-batch mean viability is calculated from the three independent assays or runs using intra-batch tissue mean (3 tissues per run).

Standard deviation of each intra-batch mean should not be > 18 %. For a given test substance, if one batch predicts a different class of irritation, the test substance must be retested in one additional batch (4th run). In this case, results from all four batches (4 runs) will be considered for the final mean and analyzed, except if technical problems are identified for the batch.

## Data analysis

### Data calculation step

These data calculation step is adapted only if the tested samples have no interaction with the MTT reagent, are non-colored, with a low ability to stain the tissues and measured non-specific color value  $\leq 5\%$  relative to negative control.

Blanks:

- Calculate the OD mean from the 6 replicates for each plate  $OD_{\text{blank}}$

Negative PBS<sup>-</sup>-treated controls:

- Calculate the blank corrected value  $OD_{\text{NC}} = OD_{\text{NCraw}} - OD_{\text{blank}}$
- Calculate the OD mean per tissue (3 replicates)
- The mean OD for all tissues corresponds to 100% viability = mean  $OD_{\text{NC}}$

Positive SDS-treated control:

- Calculate the blank corrected value  $OD_{\text{PC}} = OD_{\text{PCraw}} - OD_{\text{blank}}$
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue  $\%PC = [OD_{\text{PC}} / \text{mean } OD_{\text{NC}}] \times 100$
- Calculate the mean viability for all tissues  $\text{Mean PC} = \Sigma \%PC / \text{number of tissues}$

Tested compound:

- Calculate the blank corrected value  $OD_{\text{TT}} = OD_{\text{TTraw}} - OD_{\text{blank}}$
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue  $\%TT = [OD_{\text{TT}} / \text{mean } OD_{\text{NC}}] \times 100$
- Calculate the mean viability for all tissues  $\text{Mean TT} = \Sigma \%TT / \text{number of tissues}$

Standard deviations are calculated on OD and % viabilities.

### Data calculations for MTT interacting substance

Test substance 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.

$OD_{\text{ku}}$ : OD untreated killed tissues + MTT incubation

$OD_{\text{kt}}$  : OD or test substance treated killed tissues + MTT incubation

$$\%NSMTT = [(OD_{\text{kt}} - OD_{\text{ku}}) / OD_{\text{NgC}}] \times 100$$

$$\text{Viability per tissue} = \%TT - \%NSMTT$$

**Data calculations for dyes and coloring test samples able to stain tissues.**

For test samples 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<sub>TT-MTT</sub>: OD treated tissue without MTT incubation

OD<sub>NgC</sub>: Mean OD Negative Control (living tissues + MTT incubation)

$$\%NSC_{\text{living}} = [(OD_{\text{TT-MTT}} / OD_{\text{NgC}}) \times 100]$$

$$\text{Viability per tissue} = \% \text{ TT1} - \%NSC_{\text{living}}$$

**Remarque :**

- IF the mean of % Non-Specific Color on living tissues (%NSCliving) or % Non-Specific MTT reduction (NSMTT) of the qualified test is less than or equal to ( $\leq$ ) 50%, THEN the test sample is considered to be compatible with the test method.
- IF the mean of %NSCliving or %NSMTT of the qualified test is greater than ( $>$ ) 50% AND their classification (I or NI) remains the same upon correction, THEN the test sample is considered to be compatible with the test method.
- IF the mean of %NSCliving or %NSMTT of the qualified test is greater than ( $>$ ) 50% AND the classification of the qualified test changes upon correction, THEN this test sample 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 sample producing %NSMTT and/or %NSCliving  $\geq$  50% of the negative control should be taken with caution

**Prediction model**

Irritation potential of test substance is determined according to the UN GHS and EU CLP (Category 2: Irritant or “No Category”; UN, 2009; EU, 2008). In the present study, the irritancy potential of test substances is predicted by mean tissue viability of tissues exposed to the test substance.

The test substance is considered to be irritant to skin (Category 2), if the mean relative viability after 24 hours exposure is less or equal ( $\leq$ ) to 50% of the negative control in at least one extraction solvent. The prediction model is defined as described below:

<b>Criteria for <i>in vitro</i> interpretation</b>	<b><i>In vivo</i> classification</b>
Mean tissue cell viability $\leq$ 50%	Irritant
Mean tissue cell viability $>$ 50%	Non Irritant

Document all remarks, comments on protocol modifications in **Appendix 6**.

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### Appendix 1: Main Equipment verifications

Laboratory: .....

Study N°.....

To be done according to internal laboratory procedures (to be recorded in the raw data), or if not available proceed as defined below. Measurements, recordings etc...considered as data and all documentations should be archived in the laboratory.

#### INCUBATOR VERIFICATION

Incubator n°	CO <sub>2</sub> (%)	Temperature (°C)	Water bath level (OK)

**Date:**

**ID and signature:**

#### FRIDGE VERIFICATION

Refrigerator ID	Temperature (°C)	
	Lower: .....	Upper: .....

**Date:**

**ID and signature:**

#### SPECTROPHOTOMETER VERIFICATION (LINEARITY TESTING)\*

Spectrometer n° (96 well plate reader)	Wavelength (nm)	Checking the linearity of response (OD values limit acceptance)	
		Lower: .....	Upper: .....

\*Spectrophotometer verification might also be performed using a verification plate certified by the manufacturer

**Date:**

**ID and signature:**

#### BALANCE VERIFICATION

N° :	10 mg weight	1g weight
Weighing	1 :.....mg	1 :.....g
	2 :.....mg	2 :.....g
	3 :.....mg	3 :.....g

Mean	.....mg	.....g
Tolerance	<b>9.9 mg to 10.1 mg</b>	<b>999. 5 mg to 1000. 5 mg</b>

**Date:**

**ID and signature:**

**PIPETTES VERIFICATION**

Water temperature: .....

Balance N°: .....	Pipette N° .....	Pipette N° .....	Pipette N° .....	Pipette N° .....	Pipette N° .....
mg	1 :	1 :	1 :	1 :	1 :
mg	2 :	2 :	2 :	2 :	2 :
mg	3 :	3 :	3 :	3 :	3 :
Mean					
SD					
CV (%)					
Tolerance	5%	5%	5%	5%	5%

**Date:**

**ID and signature:**

## Appendix 2: SkinEthic™ RHE tissues receipt

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

- ◆ Receipt date: .....
- ◆ Batch number: .....
- ◆ Check :
  - Sending date: .....
  - Expiration date: .....
- ◆ Kit compliance:
  - Control certificate N°: .....
  - Compliance:      YES      NO

### Maintenance medium:

- ◆ Batch N°: .....
- ◆ Expiration date: .....
- ◆ Storage temperature: +4°C.....
- ◆ Storage place: Refrigerator N° .....

### Growth medium:

- ◆ Batch N°: .....
- ◆ Expiration date: .....
- ◆ Storage temperature: +4°C.....
- ◆ Storage place: Refrigerator N° .....

❖ Incubation conditioning start      **Time :**      **Date:**

**Date:** .....

**ID and signature:**.....

### Appendix 3: Solutions information and preparation

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

◆ MTT stock solution preparation at a concentration of 5 mg/mL:

- MTT batch N°: .....
- Weight: .....
- PBS batch N°: .....
- PBS Volume added: .....
- Preparation date: .....
- Expiration date: .....
- Storage place: Refrigerator N°.....

◆ MTT ready to use solution preparation at a concentration of 1 mg/mL

- Time & date preparation.....
- Maintenance medium batch.....
- Maintenance medium Volume.....

◆ Phosphate Buffer Solution (PBS) for negative control and rinsing steps:

- PBS Supplier / Reference: .....
- Batch N°: .....
- Expiration date: .....

◆ Isopropanol solution:

- Isopropanol Supplier / Reference: .....
- Batch N°: .....
- Expiration date: .....

◆ SDS 1% solution in distilled sterile water for positive control:

- SDS Supplier / Reference: .....
- SDS Batch N°: .....
- Expiration date: .....
- Weight: .....
- Distilled water Supplier / Reference: .....
- Distilled water Batch N°: .....
- Distilled water volume added: .....
- Preparation date: .....
- Expiration date: .....
- Sesame seeds oil Supplier / Reference: .....
- Sesame seeds oil Batch N°: .....
- Sesame seeds oil volume added: .....

**Date:** .....

**ID and signature:**.....



### Appendix 5: Incubation timings

Laboratory: .....

Study N°.....

Batch N°:.....

	<b>Start date and time</b> mm/dd/yyyy  hh:mm	<b>End date and time</b> mm/dd/yyyy  hh:mm
<b>Preincubation</b>		

	<b>Day</b> mm/dd/yyyy	
	<b>Start time</b> (hh:mm)	<b>End time</b> (hh:mm)
<b>Treatment</b>  24±2 hours		
<b>MTT incubation</b>  3 hours (up to 3h15)		
<b>Formazan extraction</b>  2 hours ± 5 minutes		

**Date:** .....

**ID and signature:**.....

### Appendix 6: Remarks and comments

Laboratory: .....

Study N°.....

Batch N°:.....

REMARKS AND COMMENTS	
PROTOCOL MODIFICATIONS / DEVIATIONS IF NECESSARY	

Date:.....

ID and signature:.....