EPISKIN
L'ORÉAL RESEARCH & INNOVATION

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SKINETHIC SKIN IRRITATION

TEST-42bis TEST METHOD FOR THE PREDICTION OF ACUTE SKIN IRRITATION OF CHEMICALS:

42 MINUTES APPLICATION + 42 HOURS POST-INCUBATION

I. PROTOCOL INTRODUCTION

1 - SKINETHIC™ SKIN IRRITATION TEST-42BIS

The SKINETHIC Skin Irritation Test^{-42bis} assay (42 minutes application + 42 hours post-incubation) is designed for the prediction of acute skin irritation of chemicals by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RHE) model.
SKINETHIC Skin Irritation Test^{-42bis} assay is not a kit; SKINETHIC™RHE tissues are commercially available per tissues item.

2 - OBJECTIVES & APPLICATIONS

Tyαpe of Testing:
Replacement

Level of Toxicity Assessment:
Toxic potential, toxic potency, hazard identification

Purpose of Testing:
Rabbit Acute skin irritation potential prediction

Context of Use:
Performance standards for applying human skin models to *in vitro* skin irritation testing were also defined based on the validated test EPISKIN™ test method (ECVAM SIVS, 2007). These performance standards can be then used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

At its 29th meeting, held on 4-5th November, 2008 the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) endorsed that SKINETHIC™RHE test method "measures or predicts the same biological or toxic effect as the fully validated and accepted reference method".

Applicability Domain:
This test is designed for chemicals. The protocol was established for liquids, viscous and solid test substances.

3 - BASIS OF THE METHOD

The relative viability of the treated tissues was measured at the end of the treatment exposure (42 minutes) followed by a post-exposure period (42 hours) using the vital dye MTT. A cut- off value of 50% viability of the negative control value was considered and used to classify test substances as irritant (I) or non irritant (NI). The culture environment might allow the detection of very small quantities of cytokines secreted by the epidermis in response to topical application of test substances.

4 - EXPERIMENTAL DESCRIPTION

Endpoint & Endpoint detection:
Cell viability determination is based on cellular mitochondrial dehydrogenase activity, measured by MTT reduction and conversion into blue formazan salt that is quantified after extraction from tissues (Mosmann T., 1983). The reduction of cell viability in treated tissues is compared to negative controls and expressed as a percentage. The percentage reduction in viability is used to predict the irritation potential.

Endpoint Value:
Quantitative viability is determined as a percentage of negative control.

Test System:
The three-dimensional RHE tissue was first released by Martin Rosdy in 1989, commercialized by SKINETHIC Laboratories in 1992 (Rosdy and Clauss, 1990) and is now produced by EPISKIN. Much of the work with this epidermal tissue engineering has followed the pioneering idea of Michel Prunerias (Prunerias et al., 1983).The RHE model, consists of normal human keratinocytes cultured for 17-days on an inert polycarbonate filter at the air-liquid interface. The RHE model is cultured using a chemically defined growth medium (Rosdy and Clauss, 1990). On day 17, 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, 2006; Kandárová et al., 2006a). The RHE model presents a histological morphology comparable to the *in vivo* human tissue (Doucet et al., 1998). Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability (De Brugerolle et al., 1999; Tornier et al., 2006; Kandárová et al., 2006b). A specific protocol was developed for the testing of finished products applicable for cosmetics formulations (Rosdy, 1994; De Wever and Charbonnier, 1992). The RHE model was validated for skin corrosion testing (Kandárová et al., 2006a) according to the OECD TG 431 (2004).

The quality system of EPISKIN is ISO 9001:2015 certified. Each batch production was provided with quality controls values and recommendations such as storage conditions, RHE instructions for use, lot number and origin, histology, cell viability (MTT OD > 0.7), barrier function integrity (4.0h ≤ ET50 ≤ 10.0h), absence of bacteria, fungi, mycoplasma, HIV-1 and 2

and Hepatitis B, C.

Basic Procedure:
Each test substance (test material, negative and positive controls) is topically applied concurrently on three tissues replicates for 42 minutes at room temperature (RT, comprised between 18°C to 24°C). Exposure to the test substance was followed by rinsing with phosphate buffer saline (PBS) and mechanically dried. Epidermis were then transferred to fresh medium and incubated at 37°C for 42 additional hours. 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. Sodium Dodecyl Sulphate (SDS 5%), and PBS treated epidermis are used as positive and negative controls, respectively. For each treated tissue, the cell viability is expressed as the percentage of the mean negative control tissues. Values under 50% is qualified the test substance as irritant.

5 - DATA ANALYSIS / PREDICTION MODEL

Irritation potential of test substance is determined according to the EU classification (R38 or no label). The mean relative tissue cell viability above 50 % predicts its non-irritancy potential. The prediction model is defined as described below:

***In vitro* results**	***In vivo* classification**
Mean tissue cell viability ≤50%	R38, Irritant (I)
Mean tissue cell viability >50%	Non-irritant (NI)

6 - TEST COMPOUNDS & RESULTS SUMMARY

A total of 20 test substances, consisting of 10 *in vivo* irritants and 10 *in vivo* non-irritants were proposed in the Performance Standards for applying human skin models to *in vitro* skin irritation testing, in the ECVAM Skin Irritation Validation Study (ECVAM SIVS, 2007).

The blind catch up validation study was performed under GLP like conditions in the three participating laboratories. An independent statistical analysis was performed to assess intra and inter-laboratory reproducibility. Regardless the measure (standard deviation, coefficient of variation and 1-way ANOVA), good reproducibility was achieved in the three laboratories. Correct predictions of the skin potential for those twenty test substances were assessed with 90 % sensitivity and 80 % specificity (MTT only) *(see Table)*. Overall accuracy was 85 % and was not improved by IL-1α release measurement for these 20 test substances.

Laboratory	Specificity [%]		Sensitivity [%]	
	All runs	Three valid runs	All runs	Three valid runs
Lab 1	80.0	80.0	90.0	90.0
Lab 2	80.0	80.0	90.0	90.0
Lab 3	80.0	80.0	90.0	90.0

***Table**: Summary of the predictive capacity (specificity and sensitivity) in the three laboratories considering either all chemical runs or only those chemicals, which had three valid runs*

7 - MODIFICATIONS OF THE METHOD

None.

8 - DISCUSSION

- Ethical considerations: *in vitro* test system
- Specific equipment: no specific equipment required (only common cell culture and chemicals equipment)
- Amount of training: one day training session could be made but is optional on complete customer's will and responsibility.

9 - STATUS

Participation in Validation Studies:
The protocol was developed and refined at SKINETHIC Laboratories in compliance with the ECVAM performance standards (ECVAM SIVS, May 2007) requirements.

Regulatory acceptance:
Following the 26th meeting of the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC), performance standards for applying human skin models to *in vitro* skin irritation testing were defined based on the validated test EPISKIN method (ECVAM SIVS, 2007). These performance standards can be then used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.
At its 29th meeting, held on 4-5th November, 2008 the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) endorsed that SKINETHIC™ RHE test method "which measure or predict the same biological or toxic effect as the fully validated and accepted reference method" (Draize full replacement method).

10 - PROPRIETARY AND/OR CONFIDENTIALITY ISSUES

The Reconstructed Human Epidermis technology and associated production methods and media are proprietary to L'Oréal, France.

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

11 - ABBREVIATIONS & DEFINITIONS

ET-50: Exposure time that induce 50% cell viability
I: Irritant
MTT: 3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NC: Negative Control
NI: Non-Irritant
OD: Optical Density
PC: Positive Control
RHE: Reconstructed Human Epidermis
SDS: Sodium Dodecyl Sulphate
SIVS: Skin Irritation Validation Study

II. TECHNICAL DESCRIPTION

1 - HEALTH & SAFETY ISSUES

SKINETHIC™RHE 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 and to hepatitis B antigens. Nevertheless, normal handling procedures for biological materials should be followed:

1) It is recommended to wear gloves during handling;
2) 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.
3) Examine all kit components for integrity.

2 - CELL / TEST SYSTEM

Test system description:
The three-dimensional Reconstructed Human Epidermis (RHE) model, commercialized by EPISKIN (Lyon, France) consists of normal human keratinocytes cultured for 17-days on an inert polycarbonate filter at the air-liquid interface. The RHE model is cultured using a chemically defined growth medium (Rosdy and Clauss, 1990). On day 17, 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, 2006; Kandárová et al., 2006a). The RHE model presents a histological morphology comparable to the *in vivo* human tissue (Doucet et al., 1998). Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability (De Brugerolle et al., 1999; Tornier et al., 2006; Kandárová et al., 2006b).

Quality control:
The RHE model kits are manufactured according to defined quality assurance procedures (ISO9001 certification).
All the 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.

3 - EQUIPMENT

Materials not provided by EPISKIN:

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, 95% relative humidity)	*For incubating tissues*
Laboratory balance (accuracy 0.1mg)	*For pipette verification and test substance weighing*
96-well plate photometer with a 570nm filter	*For Optical Density readings (MTT)*
Plate shaker	*For extraction of formazan*
Stop-watches	*To be used during application of test materials*
Sterile, blunt-edged forceps	*For handling tissue inserts*
Plastic wash bottles	*For collecting PBS rinses*
1 L beaker	*For rinsing tissues with PBS*
1 Funnel	*For rinsing tissues with PBS*
Small glass weight boats	*For weighing powders*
1 gauged flask	*For SDS 5% solution preparation*
Mortar and Pestle	*For grinding granular*
Adjustable Pipette / multi-step Pipette	*For pipetting 1 mL assay medium*
Adjustable Pipette / multi-step Pipette	*For pipetting 300 µL MTT /medium*
Adjustable Pipette	*For pipetting 750 µL propan-2-ol*
Ajustable Pipette	*For pipetting 200 µL formazan extract from 24-well plate into 96-well plate for the plate photometer*
Positive displacement pipette for 10µL and 16 µL delivery	*For application of liquid and viscous test materials*
Multi-pipette + adapter for 25mL tip	*For washing*

Dulbeccos' PBS without Ca^{2+} and Mg^{2+}	*Use as negative control, MTT diluent, and for rinsing tissues.*
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*
MTT – Thiazolyl Blue Tetrazolium Bromide (Sigma, # M-5655, cell culture tested, purity min. 97.5% or M-2128)	*For the MTT assay*
5 % (aq) SDS [151-21-3] (Sigma # L-4509, purity min. 98.5%)	*To be used as positive control with each kit*
Sterile H_2O (distilled or aqua pure)	*For powder applications*
Extra 6-well plates – sterile	*To transfer tissue inserts to fresh media*
Extra 24-well plates – sterile	*For the 42 min application + MTT incubation + formazan extraction steps*
Extra 96-well plates – sterile	*For OD measurements*
Parafilm	*Covering plates during formazan extraction*
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 (**Annex 1**). If the last verification date does not fit the requested specifications, proceed to verification before testing and record it in MDS: Main equipment verification and MDS: Detailed equipment verification (**Annexes 1** and **2)**.

4 - TISSUES AND MEDIA

RHE set and media provided by EPISKIN.

The Reconstructed Human Epidermis (RHE/S/17) and the necessary culture media (maintenance medium SMM, and growth medium SGM) are usually shipped on Monday. They are received one or two day(s) following the shipment (for Europe and USA). Results of the quality controls are supplied with the sets.

The quality system of EPISKIN is ISO 9001:2015 certified. A batch production is delivered only if quality controls criteria correspond to a normal histology (absence of significative alterations), cell viability (MTT OD > 0.7), barrier function integrity (4.0h ≤ ET50 ≤ 10.0h), absence of bacteria, fungi, mycoplasma, HIV-1 and 2 and Hepatitis B, C.

5 - PREPARATIONS

Tissues / Media expiration and storage:

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

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

Receipt of materials supplied by EPISKIN:

1) Document MDS: RHE set- EPISKIN materials receipt (Annex 3).
2) Place the maintenance culture medium in the fridge (2 to 8°C).
3) Keep the RHE tissues and the growth medium at room temperature for the pre-incubation step.

Test substances:

Safety instructions:

- Store test substances in ventilated safety cup boards. Respect special store conditions if necessary (special temperature, protected from light, etc.).
- 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. In accordance with test substance safety guidelines: use safety ventilated cabinet, wear gloves, eye and face protections.

The application is different according to the test substance nature (liquid / viscous, solid or sticky) ***(see section 5).***

Test substances identification:

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

Negative control solution:

PBS will be used as negative control.

Document MDS: Solutions preparation (**Annex 5**).

Positive control solution:

Prepare extemporaneously SDS 5% solution (Positive Control). Document MDS: Solutions preparation (**Annex 5**).

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

MTT solution preparation:
Safety precautions:

- MTT (R26 R22 R36 R37 R38) - MTT solution is light sensitive. Protect it from light using silver paper or appropriate material.
- Isopropanol (R11 R36 R67 S7 S16 S24/25 S26). Work in ventilated cabinets: to prevent accidental contact wear protective gloves, and if necessary a mask and/or safety glasses.

1) MTT stock solution preparation: dissolve MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma M-2128 or M-5655) to a final concentration of 5 mg/mL in PBS (or solution A). Always protect the solution from light. Proceed to 0.22 µm filtration. Discard the MTT solution in 1 mL aliquots in sterile dark 1.5 mL microtubes. Storage: 1 year at –20°C.

   NB: Composition of the solution A: Na_2HPO_4 0.142 g/l, Glucose 1.802 g/l, HEPES 7.149 g/l, KCl 0.224 g/l, NaCl 7.597 g/l. Adjust pH to 7.4 with NaOH 4N. Filtrate 0.22µm and store between 2 to 8°C.

   Document MDS: MTT stock solution (**Annex 10)**.

2) MTT ready to use solution preparation (day of testing): on day of testing, thaw the MTT stock solution (5 mg/mL) and dilute it with pre- warmed maintenance medium at room temperature up to 1 mg/mL.

3) Isopropanol solution: use 2-propanol (CAS N°67-63-0) Sigma-Aldrich ref 190764 Document MDS: Isopropanol (**Annex 10)**.

Check-method for possible direct MTT reduction with test substances:
This verification should be done before starting the experiment (ideally the week before the study/run).

Relative conversion of MTT by the tissue being the parameter evaluated in this test method, it is therefore necessary to assess the non-specific reduction of MTT by the test substance used. Prior to experiments all test substances should be put in contact with the MTT solution as described below:

1) Fill wells of 24-well plate with 300µL of MTT ready to use solution (1 mg/mL)
2) Add 16µL or 16 mg of the test substance to be evaluated or water for control. Mix
3) Protect from light and incubate the mixture for 3 hours (+/- 5 minutes) at 37°C
4) Proceed to visual scoring of MTT interaction as follow:

- Negative control (water): yellow
- Test substances which do not interact with MTT: yellow
- Test substances interacting slightly with MTT: light blue
- Test substances interacting strongly with MTT: dark blue

5) Document MDS: MTT-direct interacting test substances identification (**Annex 6**).

If the MTT solution color becomes blue or purple, the test substance interacts with the MTT. It is then necessary to evaluate the part of optical density (OD) due to the non-specific reduction of the MTT (i.e. by using killed epidermis).

For each MTT-interacting test substance previously detected, and in addition to the normal procedure, 3 killed test substance treated tissues are used for the MTT evaluation following the same protocol as for living tissues *(see paragraph 5.2.3).* Three killed untreated tissues are used as negative controls (untreated killed tissues may exhibit little residual NADH and dehydrogenase associated activity).

Killed epidermis for MTT-interaction substances (if necessary):

1) Transfer living epidermis in 24-well plate and place them at -20°C (or -80°C) for at least 48 hours (3 tissues / MTT-interacting test substance).
2) Thaw killed tissues, before use, on 300µL of maintenance medium for 1 hour (+/- 5 minutes) at room temperature.
3) Further use of killed tissues is similar to living tissues.
4) Document MDS: Killed tissues for MTT-interacting test substances (**Annex 7**).

Adapted controls for coloring test substances:
Since the MTT reduction assay is based on colorimetric measurement it is therefore necessary to assess the non-specific OD of the coloring test substances or of dye test substances able to stain RHE tissues. In addition to classical controls dye test substances should be put in contact with the normal living RHE tissues as described below.

Pre-incubation up to post-incubation steps for coloring test substances:

1) Use living RHE tissues for coloring test substances
2) Proceed to SKINETHIC Skin Irritation Test$^{-42\ bis}$ up to the post-incubation treatment step.
3) Document MDS: Additional control for coloring test substances (**Annex 8**).

Incubation in Maintenance Medium for 3 hours:
Coloring test substance controls must follow a similar treatment to MTT assay but avoiding contact to MTT. Corresponding tissues will thus be incubated in Maintenance Medium.

1) Label an appropriate number of 24-well plates.
2) Fill the 24-well plates with 300 µL Maintenance Medium.
3) At the end of the 42 hours post-incubation period, sweep excess culture medium on the unit bottom of the tissue with absorbent paper and transfer dye test substance control tissues in the pre-filled 24-well plates, by first sloping the insert before complete insert setting at the air-liquid interface.
4) Check the absence of air bubbles.
5) Incubate for 3 hours (+/- 5 minutes) at 37°C, 5% CO_2, 95% humidified atmosphere.
6) Document MDS: Start of 3 hrs incubation time (**Annex 8**).

Formazan extraction:

1) Label an appropriate number of new 24-well plates similarly to those labeled for the previous step.
2) Fill the plate(s) with 800 µL isopropanol (undiluted – *See section 4)*
3) At the end of the 3 hours (± 5 minutes) incubation in Maintenance Medium.
4) Use forceps to transfer treated tissues.
5) Dry the insert bottom of the treated tissue on absorbent paper or gauze.
6) Transfer the tissues in isopropanol solution.
7) Add 700 µL isopropanol solution on the top of each tissue.
8) Ensure that tissue is completely covered by the isopropanol solution.
9) Consciously protect plate(s) from evaporation by stretching parafilm layers over the plate and adding the lid on the plate.
10) Incubate for 2 hours (± 5 minutes) at room temperature with gentle agitation (about 150 rpm) for formazan extraction.
11) Document MDS: Start of isopropanol extraction time (**Annex 8**).

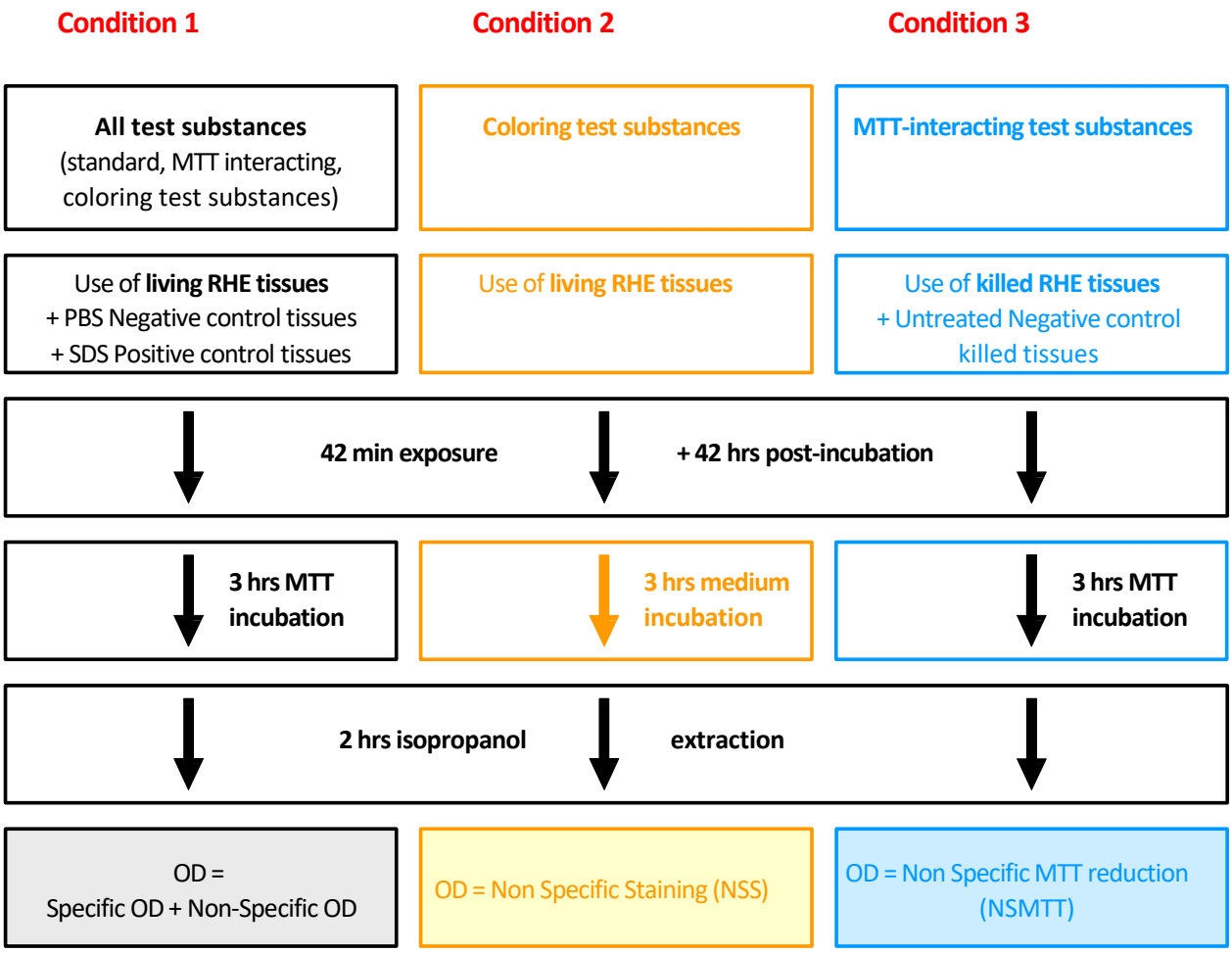
NB: The formazan extraction could be performed overnight (for about 16-18 hours) at + 4°C protected from light. The plate containing the tissues is sealed carefully to prevent evaporation and cross contaminations.

Absorbance / optical density measurements:

1) At the end of the formazan extraction incubation time, open the plate.
2) Remove the parafilm layers.
3) Document in MDS: OD reading (**Annex 8**).
4) Isopropanol solution is used as blank (6 replicates)
5) Maintain the insert with forceps.
6) Pierce tissue and polycarbonate filter with a tip in order to get the whole extraction solution in the corresponding well.
7) Homogenize the extraction solution by pipetting 3 times up and down to complete formazan crystals solubilization.
8) Transfer 3 x 200 µL extraction solution per well into a 96-well plate (labeled appropriately).

9) Read the Optical Densities (OD) using a 96-well plate spectrophotometer: The concentration of formazan is measured by determining the OD at 570 nm using a bandpass of maximum ± 30 nm.
10) All data generated by the 96-well plate spectrophotometer should be printed after each reading and considered as raw data.
11) Identify ODs with conditions and tissues (replicate) studied on the raw data documents.
12) Perform the Quality Control of the raw data.

Guidance FLOWCHART for adapted controls choice based on test substances coloring and/or direct MTT reduction potency.



Case by case test conditions guidance:

	Medium coloration	Tissue staining	MTT interaction	Test conditions
Case 1	-	-	-	**1**
Case 2	+	-	-	**1**
Case 3	-	+	-	**1+2**
Case 4	+	+	-	**1+2**
Case 5	-	-	+	**1+3**
Case 6	+	-	+	**1+3**
Case 7	-	+	+	**1+2+3**
Case 8	+	+	+	**1+2+3**

6 - METHOD

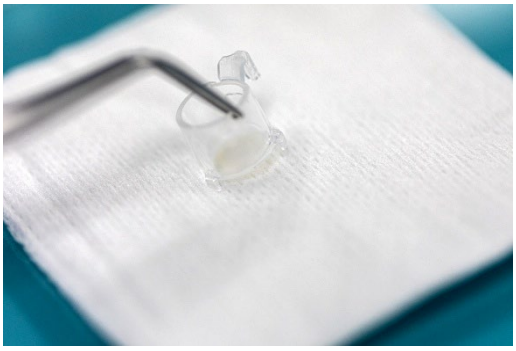
Test System Procurement:
For RHE and media ordering please contact EPISKIN (Tel: +33 4 37 28 22 00, Email: sales@episkin.com).

Pre-incubation step
This step should be conducted under sterile conditions.

Pre-incubation step for tissues receipt at day 18 (usually on Tuesday)

1) Fill an appropriate number of 6-well plates with 1 mL growth culture medium (SGM).
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

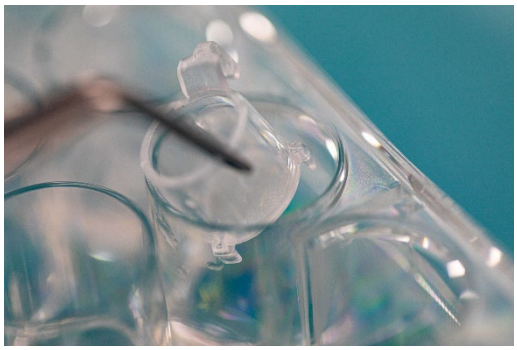
4) Check visually that no agarose is remaining and transfer the tissue on fresh medium by first sloping the insert before complete insert setting.

5) Check the absence of air bubbles by watching underneath the 6-well plate.

6) Place the RHE tissues at 37°C, 5% CO_2 until test substance application.

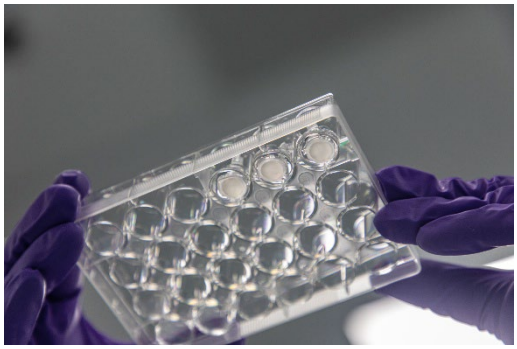
Pre-incubation step for tissues receipt at day 19 (Wednesday):
Proceed to pre-incubation step for at least 2 hours.

1) Fill an appropriate number of 24-well plates with 300 µL growth culture medium (SGM).
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).*
4) Check visually that no agarose is remaining and transfer the tissue on fresh medium by first sloping the insert before complete insert setting at the air-liquid interface *(see Picture 3).*



Picture 3

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



Picture 4

6) Place tissues at 37°C, 5% CO_2 until test substance application.

Application of test substances and rinsing on day 19 (Wednesday):

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.

Use one plate per test substance to prevent any adjacent effects of test substance fumes.

Prepare extemporaneously SDS 5% solution (Positive Control).

Document MDS: Solutions preparation (**Annex 5**).

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

Plates preparation:

Application plates:

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

Post-incubation plates:

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

Topical applications: 42 minutes treatment:

It is strongly 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.

Suggestion: Keep 1 minute interval between each tissue application. We recommend keeping some minutes without test substance application just before washing in order to be ready in time for this latter. Record the exact timings and document the correspondent MDS: Application timing (**Annex 10**).

Due to the application timing of 42 minutes, the application and rinsing phases should be performed in minimum two steps for testing the internal controls (NC and PC) and the 20 test substances *(see example below Tables SET1 and SET2).*

SET 1		APPLICATION Start: 13h30			RINSING Start: 14h13		
Plate 1	Contol PBS	42'	41'	40'	42'	41'	40'
Plate 2	Control SDS	38'30	37'30	36'30	38'30	37'30	36'30
Plate 3	C1	35'	34'	33'	35'	34'	33'
Plate 4	C2	31'30	30'30	29'30	31'30	30'30	29'30
Plate 5	C3	28'	27'	26'	28'	27'	26'
Plate 6	C4	24'	23'	22'	24'	23'	22'
Plate 7	C5	20'30	19'30	18'30	20'30	19'30	18'30
Plate 8	C6	17'	16'	15'	17'	16'	15'
Plate 9	C7	13'30	12'30	11'30	13'30	12'30	11'30
Plate 10	C8	10'	9'	8	10'	9'	8
Plate 11	C9	6'30	5'30	4'30	6'30	5'30	4'30
		End: 14h12			End: 14h55		

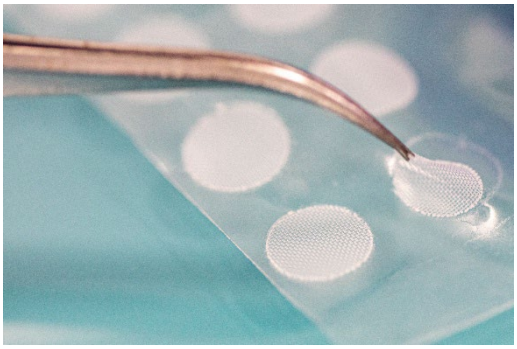
SET 2		APPLICATION Start: 15h30			RINSING Start: 16h13		
Plate 12	C10	42'	41'	40'	42'	41'	40'
Plate 13	C11	38'30	37'30	36'30	38'30	37'30	36'30
Plate 14	C12	35'	34'	33'	35'	34'	33'
Plate 15	C13	31'30	30'30	29'30	31'30	30'30	29'30
Plate 16	C14	28'	27'	26'	28'	27'	26'
Plate 17	C15	24'	23'	22'	24'	23'	22'
Plate 18	C16	20'30	19'30	18'30	20'30	19'30	18'30
Plate 19	C17	17'	16'	15'	17'	16'	15'
Plate 20	C18	13'30	12'30	11'30	13'30	12'30	11'30
Plate 21	C19	10'	9'	8	10'	9'	8
Plate 22	C20	6'30	5'30	4'30	6'30	5'30	4'30
		End: 16h12			End: 16h55		

Liquid and viscous test substances:

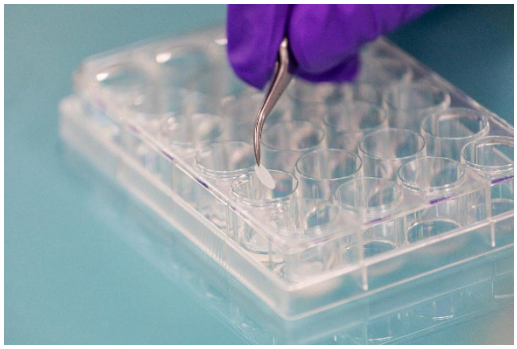
1) Dispense 16 µL ± 0.5 µL of the undiluted test substance on the top of each epidermis tissue (3 per test substance: replicate 1, replicate 2 and replicate 3), Using positive displacement pipette. Use the tip to spread the test substance gently on the epidermis topical surface *(see Picture 5).*

Picture 5

2) Carefully apply a nylon mesh on the whole surface with forceps *(see Pictures 6, 7 and 8).*



Picture 6



Picture 7

Picture 8

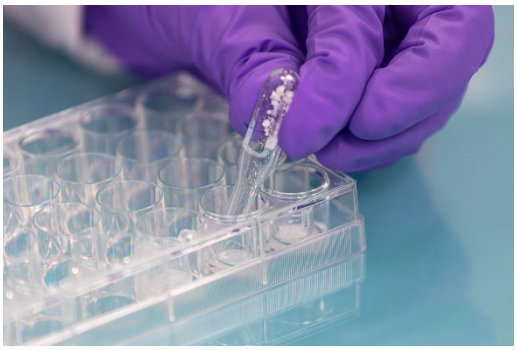
<u>Solid tests substances:</u>

1) If necessary, the test substance should be crushed to a fine powder using a mortar and a pestle.
2) Gently spread 10µL of distilled water using a positive displacement pipette to the epidermal surface in order to improve further contact between the powder and the epidermis.

3) Use special glass weigh boats (or similar tools avoiding electrostatic electricity and allowing a targeted application directly in the insert with no risk of test substance scattering in the medium subnatant) to apply 16 mg ± 2 mg of the powder to the epidermis surface. If necessary, spread it on the epidermal surface *(see Pictures 9, 10 and 11).*

Picture 9

Picture 10



Picture 11

4) Document MDS: Weighing of solids test substances (**Annex 9**).

Sticky test substances:

1) Allow for the tare with a nylon mesh and directly weigh 16 mg ± 2 mg and spread sticky test substance on this latter.
2) Apply the test substance coated side of the nylon mesh on the epidermal surface and spread it gently on the whole surface.
3) Document MDS: Weighing of sticky test substances (**Annex 9**).

Three tissues per test substance should be used. The application order is important since it will be the same for rinsing.

Keep the plate (lids on) containing the treated RHE tissues for 42 minutes

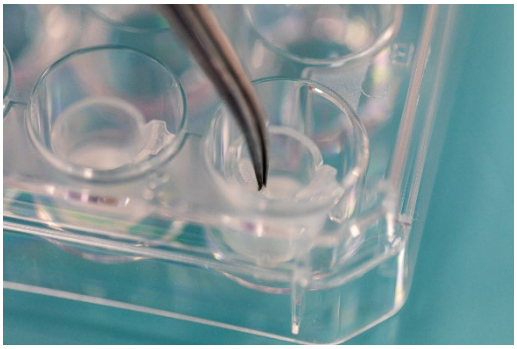
exposure (± 1 minute) in the ventilated cabinet sterile conditions at room temperature.

Rinsing and drying steps:
It is strongly recommended to work in laminar flow hood to prevent contamination.
End of the treatment and removal of the test substance after 42 minutes (± 1 minute) exposure at room temperature.
Strictly respect the application order (time based recorded in **Annex 6**).

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

Liquid, viscous and sticky test substances:

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

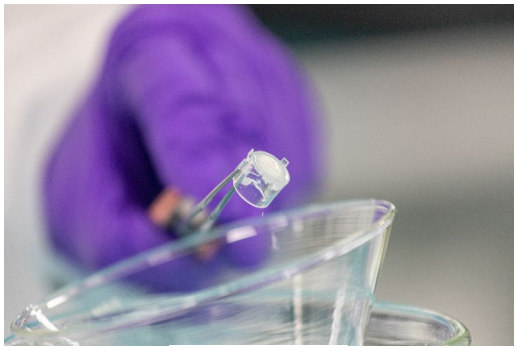


Picture 12

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

Solid test substances**:**

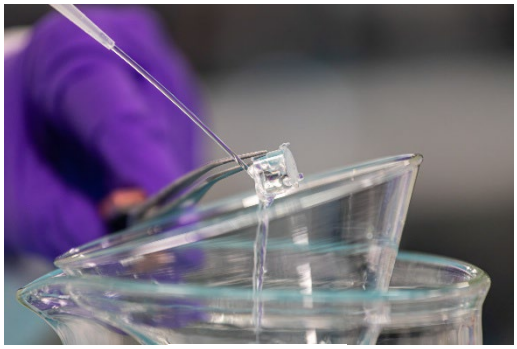
1) Fill a multi-pipette (adjusted for a 1 mL distribution) with 25 mL sterile PBS
2) Open the 24-well plate. Take the solid test substance treated tissue with sterile forceps and close the 24-well plate (to protect the other tissues from washing solution projections).
3) Remove the test substance at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



Picture 13

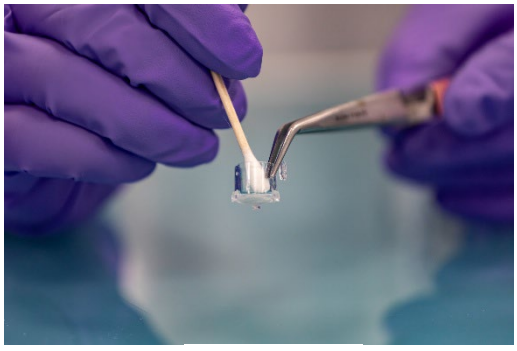
Rinsing step for treated tissues:

1) Place a funnel in a large beaker (to avoid underneath projections/contaminations of the RHE tissues).
2) Maintain the insert over the large funnel and rinse thoroughly 25 times with 1mL PBS 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 a cotton tip (5-6 turns per end) *(see Picture 15).*



Picture 15

6) Transfer the washed tissue on 2 mL growth culture medium (6-well plate designed as post-incubation plates, *see 5.5.1)* by first sloping the insert before complete insert setting.
7) Check the absence of air bubbles.
8) Document MDS: Rinsing timing (**Annex 10**).

Post treatment incubation: 42 hours
Incubate the treated, rinsed and dried epidermis tissues at 37°C, 5% CO_2, 95% humidified atmosphere for 42 hours (± 60 minutes).

Incubation start time corresponds to last tissue rinsing time of each set. See MDS: post-incubation timing (**Annex 10**).

Media sampling for mediators and enzyme release measurement
The subnatant underneath the treated RHE tissues are kept to eventually measure the mediators and/or enzyme releases. The present SOP is only describing the sampling procedure.

This step could be performed during the MTT incubation time (3 hours +/- 5 minutes).

1) Label an appropriate number 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 3 x 500 µL 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 15mL of the growth culture medium that was used as diluent (provided by EPISKIN with the RHE kit – bottle of the SGM).

MTT test:
Tissue viability is assessed by MTT reduction measurement, after the 42 hours (± 60 minutes) incubation at 37°C, 5% CO_2, 95% humidified atmosphere.

Incubation in MTT solution:

1) Prepare MTT ready to use solution according to section 4.
2) Document MDS: MTT stock solution (**Annex 11**).
3) Label an appropriate numbers of 24-well plates

4) Fill the 24-well plates with 300 µL MTT 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.
5) 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.
6) Check the absence of air bubbles.
7) Incubate for 3 hours (+/- 5 minutes) at 37°C, 5% CO2, 95% humidified atmosphere.
8) Document MDS: MTT incubation (**Annex 12**).

Formazan extraction

1) Label an appropriate number of new 24-well plates similarly to those labeled for the previous step (Incubation in MTT solution).
2) Fill the plate(s) with 800 µL isopropanol (undiluted – *See section 4)*
3) At the end of the 3 hours (± 5 minutes) incubation in MTT solution, record MDS: Observations and comments after the 3 hours MTT incubation (**Annex 13**).
4) Use sterile forceps to transfer treated tissues.
5) Dry the insert bottom of the treated tissue on sterile absorbent paper or gauze.
6) Transfer the tissues in isopropanol solution.
7) Add 700 µL isopropanol solution on the top of each tissue.
8) Ensure that tissue is completely covered by the isopropanol solution.
9) Consciously protect plate(s) from evaporation by stretching 3 parafilm layers over the plate and adding the lid on the plate.
10) Incubate for 2 hours (± 5 minutes) at room temperature with gentle agitation (about 150 rpm) for formazan extraction.
11) Document MDS: Isopropanol extraction start time (**Annex 12**).

NB: The formazan extraction could be performed overnight (for about 16-18 hours) at + 4°C protected from light. The plate containing the tissues is sealed carefully to prevent evaporation and cross contaminations.

Absorbance / optical density measurements

1) At the end of the 2 hours (± 5 minutes) or overnight formazan extraction incubation time, open the plate.
2) Remove the 3 parafilm layers.
3) Document in MDS: OD reading (**Annex 12**).
4) Isopropanol solution is used as blank (6 replicates)
5) Maintain the insert with forceps.
6) Pierce tissue and polycarbonate filter with a tip in order to get the whole extraction solution in the corresponding well.
7) Homogenize the extraction solution by pipetting 3 times up and down to complete formazan crystals solubilization.

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

9) Read the Optical Densities (OD) using a 96-well plate spectrophotometer ideally at 570 nm wavelength (eventually between 540 to 600 nm).

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).

PLATE 1	1	2	3	4	5	6	7	8	9	10	11	12
A	empty	blank	blank	blank	blank	blank	blank	empty	empty	empty	empty	empty
B	empty	NC	NC	NC	NC	NC	NC	NC	NC	NC	empty	empty
C	empty	PC	PC	PC	PC	PC	PC	PC	PC	PC	empty	empty
D	empty	C1	C1	C1	C1	C1	C1	C1	C1	C1	empty	empty
E	empty	C2	C2	C2	C2	C2	C2	C2	C2	C2	empty	empty
F	empty	C3	C3	C3	C3	C3	C3	C3	C3	C3	empty	empty
G	empty	C4	C4	C4	C4	C4	C4	C4	C4	C4	empty	empty
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
			Tissue 1			Tissue 2			Tissue 3			

10) All data generated by the 96-well plate spectrophotometer should be printed after each reading and considered as raw data.
11) Identify ODs with conditions and tissues (replicate) studied on the raw data documents.
12) Perform the Quality Control of the raw data.

Remarks and comments – Protocol modifications:

If any, document MDS: Remarks and comments – Protocol modifications (**Annex 14**)

7 - ACCEPTANCE CRITERIA

Negative control (NC) acceptance criteria:
The NC data meet the acceptance criteria if the mean OD value of the 3 tissues is ≥ 1.2 at 570 nm according to the historical database.
The Standard Deviation value is considered as valid if it is ≤ 18%, according to the Performance Standards (ECVAM SIVS, 2007).

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 ≤ 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 replicates / tissue and 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 (4^{th} 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.

8 - DATA ANALYSIS / CALCULATION OF RESULTS

Data calculation step:

Blanks:
- Calculate the OD mean from the 6 replicates for each plates OD_{blank}

Negative PBS-treated controls:
- Calculate the blank corrected value $OD_{NC} = OD_{NCraw} – OD_{blank}$
- Calculate the OD mean per tissue (3 replicates)
- The mean OD for all tissues corresponds to 100% viability =mean O_{DNC}

Positive SDS-treated control:
- Calculate the blank corrected value $OD_{PC} = OD_{PCraw} – OD_{blank}$
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue %PC = [OD_{PC} / mean OD_{NC}] x 100
- Calculate the mean viability for all tissues Mean PC = Σ %PC / number of tissues

Tested compound:
- Calculate the blank corrected value $OD_{TT} = OD_{TTraw} – OD_{blank}$
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue %TT = [OD_{TT} / mean OD_{NC}] x 100
- Calculate the mean viability for all tissues Mean TT = Σ %TT / number of tissues

Standard deviations are calculated on OD and % viabilities.

Data calculations for MTT-interacting substances:
Test substances that interfere with MTT can produce non-specific reduction of the MTT. It is necessary to evaluate the OD due to non-specific reduction and to subtract it before calculations of viability %.

Non-specific MTT reduction calculation (NSMTT)
OD_{Ku}: untreated killed tissues OD
OD_{KT}: test substance treated killed tissues
OD NSMTT = $[(OD_{KT} - OD_{KU}) / OD_{NC}] \times 100$

If NSMTT is > 30% relative to the negative control: additional steps must be undertaken if possible or the test substance must be considered as incompatible with the test.

Treated tissue True MTT metabolic conversion (TOD_{TT}) OD_{TT}: test substance treated viable tissues

$TOD_{TT} = [OD_{TT} – (OD_{KT} – OD_{KU})]$

Relative viability % = $[TOD_{TT} / OD_{NC}] \times 100$

Data calculations for coloring test substances able to stain tissues:
For chemicals detected as able to color the tissues, it is necessary to evaluate the non- specific OD due to the residual chemical staining (unrelated to any mitochondrial activity) and to subtract it before calculations of the "true" viability %.

<u>Non-specific Staining % (NSS %):</u>
OD_{CT}: coloring test substance treated tissue (incubated in Maintenance Medium before isopropanol extraction)
OD_{PBS}: control PBS treated tissue (incubated in Maintenance Medium before isopropanol extraction)
NSS%= $[OD_{CT} / OD_{PBS}] \times 100$

If NSS % is > 30% relative to the negative control: additional steps must be undertaken if possible or the test substance must be considered as incompatible with the test.
<u>True MTT metabolic conversion (TOD_{DT}):</u>
OD_{CT}: coloring test substance-treated tissues (MTT incubation)
TOD_{CT}: true MTT metabolic conversion for coloring test substance treated tissue.

$TOD_{CT} = OD_{TT} – OD_{DT}$

Relative viability % = $[TOD_{CT} / OD_{NC}] \times 100$

Data calculations for coloring test substances which are also MTT- interacting test substances:

Calculate corresponding NSMTT and NSS.

If (NSMTT % + NSS %) is > 30% relative to the negative control: additional steps must be undertaken if possible or the test substance must be considered as incompatible with the test.

True MTT metabolic conversion for dye test substances which are also MTT-interacting test substances (TOD_{DTT}):
OD_{CT}: coloring test substance-treated tissues (MTT incubation)
TOD_{CT}: true MTT metabolic conversion for coloring test substance treated tissue.

$TOD_{CTT} = [OD_{TT} - (TOD_{CT} + TOD_{TT})]$

Relative viability % = $[TOD_{CTT} / OD_{NC}] \times 100$

9 - PREDICTION MODEL

According to EU classification, the irritancy potential of test substances is predicted for distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC. 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 (R38), if the mean relative viability after 42 minutes exposure and 42 hours post incubation is less or equal (≤) to 50% of the negative control. The prediction model (PM) is described below:

Criteria for *in vitro* interpretation	Classification
Mean tissue viability is ≤ 50%	R38, Irritant (I)
Mean tissue viability is > 50%	No label, Non Irritant (NI)

Document all remarks, comments on protocol modifications in **Annex 13**.

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IV. ANNEXES

Annex 1	MDS: Equipment verification
Annex 2	MDS: Detailed equipment verification
Annex 3	MDS: RHE set – Materials receipt
Annex 4	MDS: Test substances
Annex 5	MDS: Solutions preparation (day of testing)
Annex 6	MDS: MTT-Direct interacting test substances identification
Annex 7	MDS: Killed tissues for MTT-interacting test Substances
Annex 8	MDS: Additional control for coloring test substances
Annex 9	MDS: Weighing of solid and sticky test substances
Annex 10	MDS: Application rinsing and post-incubation timing
Annex 11	MDS: Solutions preparation (last day)
Annex 12	MDS: MTT and isopropanol extraction timing
Annex 13	MDS: Observations and comments after 3 hours MTT incubation
Annex 14	MDS: Remarks and comments – Protocol modifications if necessay

ANNEX 1 - METHODS DOCUMENTATION SHEET: EQUIPMENT VERIFICATION

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

Apparatus	Maximum time interval between two verifications	Last verification Date	Verification week of testing if necessary (✓)
Laminar flow hood	2 years		
Non –sterile ventilated Cabinet	2 years		
Laminar flow hood with chemical filter	2 years		
Incubator	3 months		
Refrigerator	3 months		
Freezer (-20°C)	1 year		
Pipettes	6 months		
Spectro-photometer	1 year		
Balance	1 year		

ID:…….. Date: …… Signature: ……..

QC ID: …….. Date: …… Signature:……

ANNEX 2 - METHODS DOCUMENTATION SHEET: DETAILED EQUIPMENT VERIFICATION

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

Identification

- Laminar flow hood: …….
- Non–sterile ventilated cabinet: …….
- Laminar flow hood with chemical filter: …….

Incubator verification

Incubator N°	CO_2 5 ± 0.5%	Temperature (°C) 36.5± 1°	Water bath level (✓)

ID:…… Date: …… Signature: ……

QC ID: …… Date: …… Signature:……

Balance verification

N°: ……		10 mg weight	1 g weight
Weighing	1	… …… mg	… …… g
	2	… …… mg	… …… g
	3	… …… mg	… …… g
Mean		… …… mg	… …… g
Tolerance		9.9 mg to 10.1 mg	999.5mg to 1000.5 mg

ID:…… Date: …… Signature: ……

QC ID: …… Date: …… Signature:……

Pipettes verification

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

ID:…… Date: …… Signature: ……

QC ID: …… Date: …… Signature:……

ANNEX 3 - METHODS DOCUMENTATION SHEET: RHE SET - MATERIALS RECEIPT

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

EPISKIN set receipt:

Date: ……..

Reconstructed Human Epidermal (RHE) tissues:

Quantity: ……..

Batch N°: ……..

Expiration date: ……

Maintenance culture medium:

Quantity: ……..

Batch N°: ……..

Expiration date: ……..

Growth culture medium:

Quantity:

…….. Lot N°:

……..

Expiration date:

……..

Remarks:

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 4 - METHODS DOCUMENTATION SHEET: TEST SUBSTANCES

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

Test substance Name or code	Total weight test substance and vial (g)	Receipt date: *mm/dd/yy*	Expiration Date: *mm/dd/yy*	Physical Consistence[a]	Stocking conditions Temperature - place

[a] *Physical consistence: L = Liquid ; V = Viscous ; S = Solid ; St = Sticky*

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 5 - METHODS DOCUMENTATION SHEET: SOLUTIONS PREPARATION (DAY OF TESTING)

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

SDS 5% solution in distilled sterile water (W/V):
SDS Supplier: ……

Reference: ……

Batch N°: ……..

Expiration date: ……

Weight: ……..

Distilled water volume added: ……..

PBS solution (sterile):
PBS Supplier: ……..

Reference: ……..

Batch N°: ……..

Expiration date: ……..

In case that you have to prepare PBS solution, document the following forms:

Preparation date: ……

Expiration date: ……

Type of sterilization: ……..

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 6 - METHODS DOCUMENTATION SHEET: MTT-DIRECT INTERACTING TEST SUBSTANCES IDENTIFICATION

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

Test substance Name or code	Amount weight (g) or volume (µL)	MTT solution volume (µL)	Start of incubation time	End of incubation time	Interaction Blue color Yes / No

ID:…….. Date: …… Signature: ……

QC ID: …… Date: …… Signature:……

ANNEX 7 - METHODS DOCUMENTATION SHEET: KILLED TISSUES FOR MTT-INTERACTING TEST SUBSTANCES

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

Test substance Name or Code										
Solids weight before application (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Solids weight after application (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Solids weight applied (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Liquids volume (µL)										
Start of incubation time	**Tissue 1**									
	Tissue 2									
	Tissue 3									
End of incubation time	**Tissue 1**									
	Tissue 2									
	Tissue 3									

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 8 - METHODS DOCUMENTATION SHEET: ADDITIONAL CONTROL FOR COLORING TEST SUBSTANCES

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

Test substance Name or Code										
Solids weight before application (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Solids weight after application (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Solids weight applied (mg)	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Liquids volume (µL)										
Start of exposure time	**Tissue 1**									
	Tissue 2									
	Tissue 3									
End of exposure time	**Tissue 1**									
	Tissue 2									
	Tissue 3									
Start of 3 hrs incubation time										
Start of isopropanol extraction time										
OD reading										

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 9 - METHODS DOCUMENTATION SHEET: WEIGHING OF SOLID AND STICKY TEST SUBSTANCES

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

Test substance Name or code	Solids weight before application (mg)			Solids weight after application (mg)			Solids weight applied (mg)		
	Tissue 1	Tissue 2	Tissue 3	Tissue 1	Tissue 2	Tissue 3	Tissue 1	Tissue 2	Tissue 3

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 10 - METHODS DOCUMENTATION SHEET: APPLICATION, RINSING, AND POST-INCUBATION TIMING

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

1st set		NC	PC	Pr 1	Pr 2	Pr 3	Pr 4
Application	Tissue 1						
	Tissue 2						
	Tissue 3						
Rinsing + post-incubation	Tissue 1						
	Tissue 2						
	Tissue 3						

1st set		Pr 5	Pr 6	Pr 7	Pr 8	Pr 9
Application	Tissue 1					
	Tissue 2					
	Tissue 3					
Rinsing + post-incubation	Tissue 1					
	Tissue 2					
	Tissue 3					

2nd set		Pr 10	Pr 11	Pr 12	Pr 13	Pr 14	Pr 15
Application	Tissue 1						
	Tissue 2						
	Tissue 3						
Rinsing + post-incubation	Tissue 1						
	Tissue 2						
	Tissue 3						

2nd set		Pr 16	Pr 17	Pr 18	Pr 19	Pr 20
Application	Tissue 1					
	Tissue 2					
	Tissue 3					
Rinsing + post-incubation	Tissue 1					
	Tissue 2					
	Tissue 3					

ID:……. Date: ……. Signature: …….

QC ID: ……. Date: ……. Signature:……

ANNEX 11 - METHODS DOCUMENTATION SHEET: SOLUTIONS PREPARATION (LAST DAY)

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

MTT stock solution (5mg/mL):
MTT batch N°: ……..

Expiration date: ……..

Stocking place: Freezer N° …….

In case that you do not use any SOP for MTT stock solution preparation, document the following forms:

MTT Supplier: ……

Reference: ……

Batch N°: ……..

Expiration date: ……

Weight: ……..

Solution A / PBS batch N°: ……..

Solution A / PBS volume added: ……

Filtration (0.22µm) (✓)……..

Preparation date: ……

Isopropanol:

Batch N°: ……..

Expiration date: ……

ID:…….. Date: …… Signature: ……..

QC ID: …….. Date: …… Signature:……

ANNEX 12 - METHODS DOCUMENTATION SHEET: MTT AND ISOPROPANOL EXTRACTION TIMING

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

Time	**1st set**	**2nd set**
MTT incubation		
Isopropanol extraction		
OD measurement		

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 13 - METHODS DOCUMENTATION SHEET: OBSERVATIONS AND COMMENTS AFTER 3 HOURS MTT INCUBATION

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

Test substance Name or code	Tissue 1	Tissue 2	Tissue 3
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o
	o	o	o

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 14 - METHODS DOCUMENTATION SHEET: REMARKS AND COMMENTS – PROTOCOL MODIFICATIONS IF NECESSARY

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

ID:…….. Date: …… Signature: ……..

QC ID: …… Date: …… Signature:……