EPISKIN
L'ORÉAL RESEARCH & INNOVATION

DFC-PRO019/01
October 2025

BIOLOGICAL EVALUATION OF MEDICAL DEVICES:

STANDARD OPERATING PROTOCOL (SOP) FOR IN VITRO SKIN IRRITATION TEST WITH SKINETHIC™ RHE MODEL

I. PROTOCOL INTRODUCTION

1 - IN VITRO SKIN IRRITATION TEST WITH SKINETHICTM RHE MODEL

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:

EPISKIN
4 rue Alexander Fleming
69366 LYON, FRANCE
Tel: +33 4 37 28 72 00
Email: sales@episkin.com

2 - 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.

3 - 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-1α: Measurement of IL-1α concentration in the culture medium by ELISA is proposed as complementary endpoint to cell viability. The pertinence of this measurement 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 percentage. The MTT reduction percentage 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 from healthy donors are cultured for 17-days on an inert 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
Sample extracts 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 sample is followed by rinsing with phosphate buffer saline without Ca^{2+} and Mg^{2+} (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.
A 1% volume fraction of sodium dodecyl sulfate (SDS) in saline solution of NaCl 0.9% 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 0.9% and sesame oil (exposed 37 +/- 1°C for 72 +/- 2 hrs) will be used as vehicle controls that undergone the ISO 10993612 medical device extraction procedure. For each treated tissue, the cell viability is expressed as the percent relative to negative control tissues (PBS- treated tissues). Values under or equal to 50% are used to classify the test sample medical device or device component as irritant. Quantification of released Interleukin-1 alpha (IL-1α) will be assessed in the supernatant of the epidermis as a complementary biological endpoint.

4 - DATA ANALYSIS/ PREDICTION MODEL

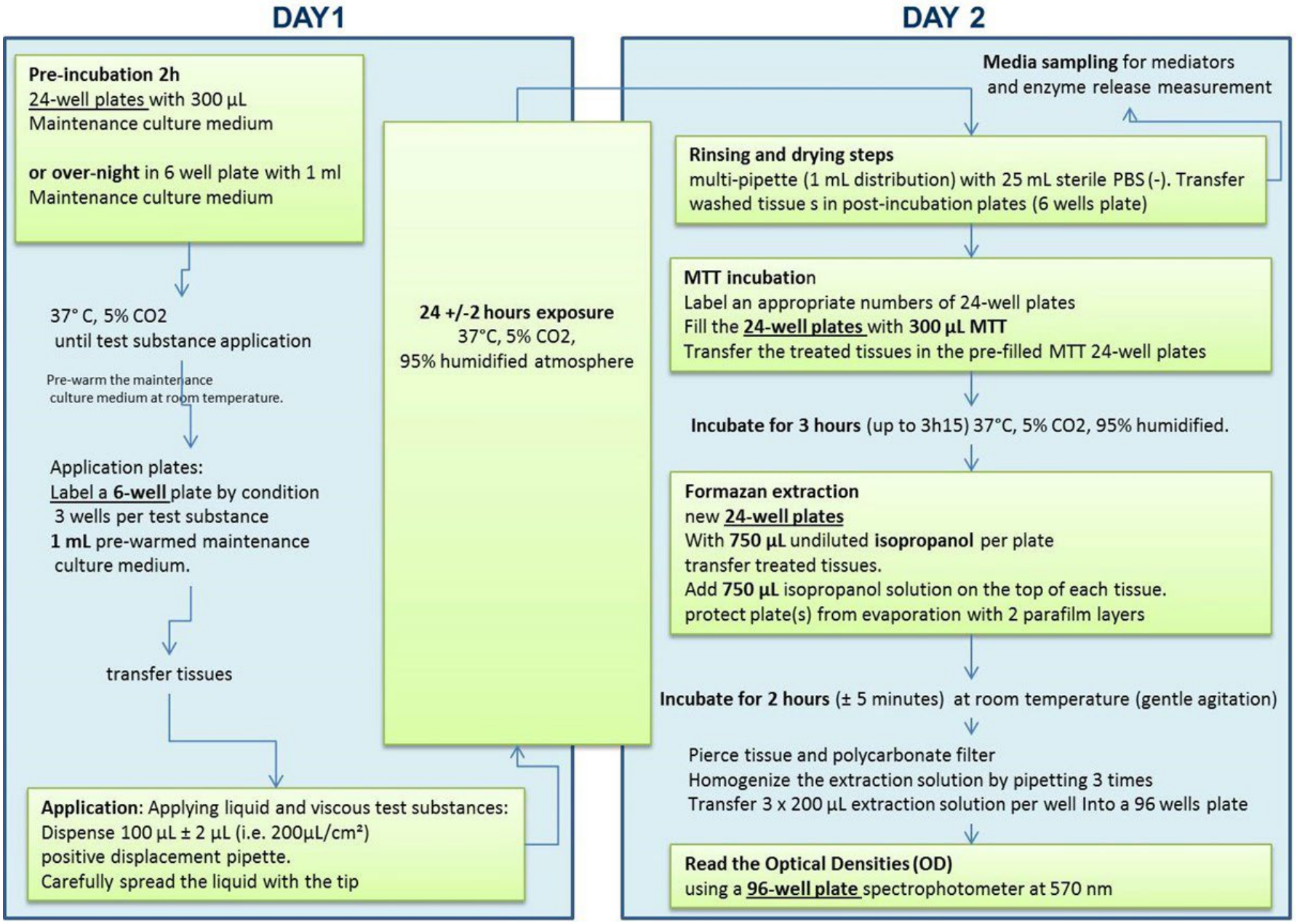
Irritation potential of test sample is determined according to the UN GHS and EU CLP (Category 2: Irritant or "No Category"; UN, 2009; EU, 2008). The prediction model is based on the prediction model of the OECD 439. The prediction model is defined as described below:

***In vitro* results**	***In vivo* classification**
Mean tissue cell viability ≤50%	Irritant
Mean tissue cell viability >50%	Non Irritant

5 - ABBREVIATIONS AND DEFINITIONS

ET50: 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
%NSMTT: Non-Specific reduction of MTT
%NSC_{living}: Non-Specific Color on living tissues

6 - SCHEMA OF THE PROTOCOL



II. MATERIALS AND PREPARATIONS

1 - 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 and to hepatitis B antigens. 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.

2 - 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.

3 - CELL OR TEST SYSTEM

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

4 - 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_2, 95% relative humidity)	For incubating tissues
Laboratory balance (accuracy 0.1 mg)	For pipette verification and test sample 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^- rinses
1 L beaker	For rinsing tissues with PBS^-
1 Funnel	For rinsing tissues with PBS^-
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

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).**

5 - MEDIA, REAGENTS, OTHERS

SkinEthic™ RHE set and media provided by EPISKIN:

Reference	Description	Conditions	Shelf life
SkinEthic™RHE/S/17	Epidermal tissues, small size, day 17	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.

- Document MDS: Skinethic™ RHE set – EPISKIN™ materials receipt (**Appendix 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^{2+} and Mg^{2+} (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

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

Test samples identification

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

N.B.: Non-coded test samples should be handled following material safety datasheet. Unknown and coded test samples 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±2°C, 5±1% CO_2, ≥ 90% humidity for 24±1 h.
3) At the end of the incubation, discard the water.
4) Keep dead epidermis frozen (dry) in freezer ("killed" epidermis can be stored and used up to 6 months).
5) Tissues should be de-frozen before use at room temperature (at least 10 minutes) in 300 µL
   maintenance medium.
6) Further use of thawed "killed" tissues is similar to living tissues.
7) Apply test treatment on "killed" tissues from the same batch than untreated "killed" tissues used as negative control.
8) Proceed similarly to living tissues for application, rinsing, post-soak, etc.
9) Perform this assay only once (at least 3 tissues, usually 3 tissues) per test sample when necessary.

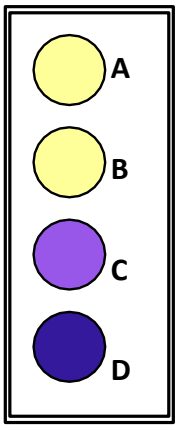
III. METHOD

1 - 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 sample used. Prior to experiments, test sample should be put in contact with the MTT solution as described below.

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



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

Step 1:

- Fill tube with 100 µL of the sample exctract to be evaluated or controled.

- Add 300 µL of MTT ready to use solution (1 mg/mL) and mix.

- Incubate the mixture for 3 hours ± 5 minutes at 37°C protected from light (test conditions).

If the MTT solution color becomes blue or purple, the test sample interacts with the MTT. It is then necessary to evaluate during the future studies the part of OD due to the non-specific reduction of the MTT (i.e. by using "killed" epidermis) to define the non-specific reduction of MTT (%NSMTT) value.

Step 2:

- Use "killed" tissues that exert no metabolic activity but can absorb and bind the test sample like viable tissues.

- Each MTT interacting test sample 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 sample (steps 1 and 2) is performed only on

one occasion (a single run even if additional runs are required to classify the test sample as I or NI).

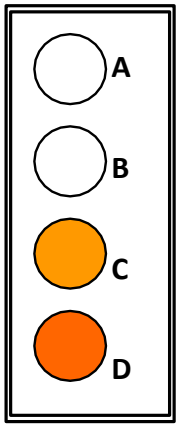
- Report systematically the part of OD due to the non-specific reduction of the MTT (to define the %NSMTT value for a MTT-reducing test sample) for a test sample before calculating the final viability.

2 - CHECKING FOR COLOR TEST SAMPLES ONLY (ANNEX 2)

Colored test sample can generate a remaining Non-Specific Color on living tissues ($\%NSC_{living}$). Therefore, each test samples have to be checked for its colorant properties. Indeed, test sample that appear red, blue, black and green by absorbing light should be potentially considered as intrinsic colorant.

Adapted controls should always be included for colored test sample.

Specific controls must be used in these cases consisting of test sample-treated tissues that followed all the steps of the method except the MTT incubation. $\%NSC_{living}$ is determined after isopropanol extraction and OD reading in similar conditions.



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 sample should be checked (step 2), otherwise no adapted controls are required.

It is then necessary to evaluate during the future studies the part of OD due to the Non-Specific Color (i.e. by using living epidermis tissues without MTT conversion test) to define the $\%NSC_{living}$ value.

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

Step 2:

- The Non-Specific Color ($\%NSC_{living}$) is first quantified by using three living tissues per chemical.

- Coloring test sample controls are treated and handled like normal treated tissues samples except that they do not get into contact with the MTT solution as they are incubated in maintenance medium.

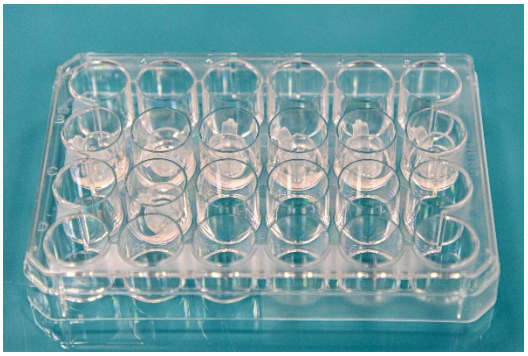
An independent $\%NSC_{living}$ control needs to be conducted with each test performed (concurrently to every testing: i.e. for each time in each run).

- Report **systematically and concurrently to every testing** the part of OD due to the non-specific coloration (to define the $\%NSC_{living}$ value), for a test coloring chemical before calculating the final % viability.

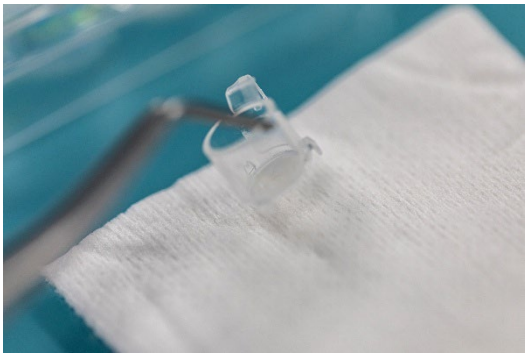
3 - ROUTINE CULTURE PROCEDURE

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 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).*

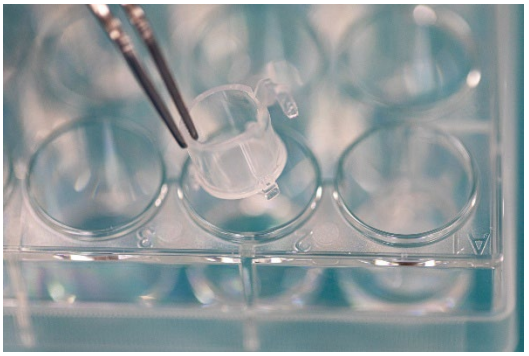


Picture 1



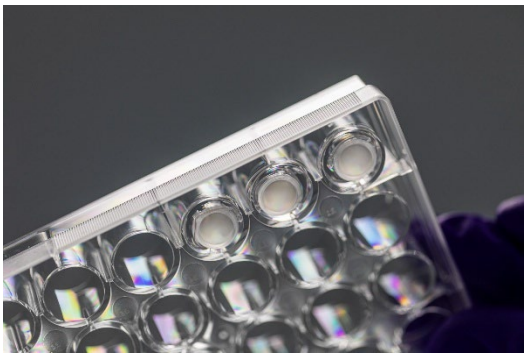
Picture 2

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_2 until test sample application.

4 - 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 sample (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.

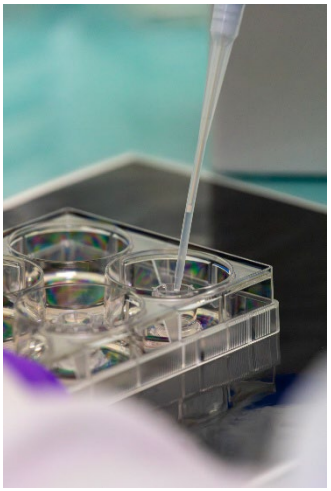
Alternatively, the pre-incubation plates can be used (aspiration of medium followed by the addition of 1 mL of fresh maintenance medium.

Topical applications: 24 ±2 hours treatment

It is recommended to perform this step under sterile conditions.

Three tissues per test sample 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/cm²) of the undiluted test sample on the top of each epidermis tissue (3 per test sample: replicate 1, replicate 2 and replicate 3), using positive displacement pipette. Use the tip to spread the test sample 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 µL 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 sample 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±2 hours at 37°C, 5% CO_2, 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 µL 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.

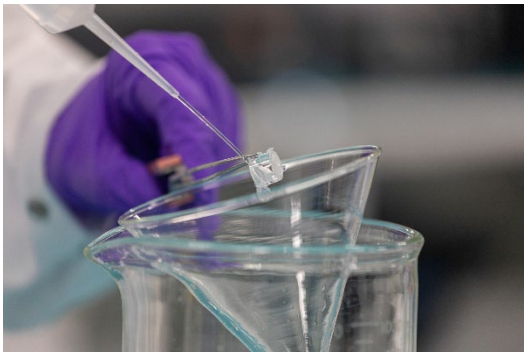
It's recommended to prepare a 24 well plates with 300 µL maintenance medium which will be used to transiently store the rinsed tissues during the rinsing step. As soon as the last tissue is rinsed you can transfer all the tissues to the plate containing the MTT solution.

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^- 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^-
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^- at a 5-8 cm distance from the insert to remove all residual test sample from the epidermal surface *(see Picture 6).*



Picture 6

6) After the last rinsing, empty the insert at the most (for example, knock the forceps on the beaker, insert turned upside down).
7) Dry the insert bottom on a sterile absorbent paper or gauze for 1-2 seconds.
8) Sweep gently the surface of the *stratum corneum* with both ends of double-sided swab (5-6 turns per end) *(see Picture 7).*

Picture 7

5 - 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% CO2, 95% humidified atmosphere.

Media sampling for mediators and enzyme release measurement

The subnatant underneath the treated SkinEthic™ RHE tissues are kept to eventually measure the mediators and/or enzyme releases such as cytokine release (IL-1α, 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 sample 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 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 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 µL 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 µL isopropanol solution on the top of each tissue.
7) Ensure that tissue is completely covered by the isopropanol solution.
8) Consciously protect plate(s) from evaporation 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 (± 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.

6 - ENDPOINT MEASUREMENT

Absorbance / optical density measurements

1) At the end of the 2 hours (± 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 SO	*empty*	*empty*	*empty*
F		PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	PC SO	NACL 1	*empty*	*empty*	
G		NACL 1	NCAL 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			
	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).

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

7 - ACCEPTANCE CRITERIA

Quality controls performed by EPISKIN

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.

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 ≥ 1.2 at 570 nm according to the historical database.

The Standard Deviation value is considered as valid if it is ≤ 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 ≤ 18 %.

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

Test sample 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 sample, if one batch predicts a different class of irritation, the test sample 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.

8 - 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 ≤ 5% relative to negative control.

Blanks:
- Calculate the OD mean from the 6 replicates for each plate 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 OD_{NC}

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 $\%PC = [OD_{PC} / \text{mean } OD_{NC}] \times 100$
- 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 $\%TT = [OD_{TT} / \text{mean } OD_{NC}] \times 100$
- 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 sample

Test sample that interfere with MTT can produce non-specific reduction of the MTT. It is necessary to evaluate the OD or area due to the non-specific reduction (%NSMTT) and to subtract it before calculations of final viability.

OD_{ku}: OD untreated "killed" tissues + MTT incubation

OD_{kt} : OD or test sample treated "killed" tissues + MTT incubation

$\%NSMTT = [(OD_{kt} - OD_{ku})/ OD_{NgC}]x100$

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.

$OD_{TT\text{-}MTT}$: OD treated tissue without MTT incubation

OD_{NgC}: Mean OD Negative Control (living tissues + MTT incubation)

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

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

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 (≤) 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 ≥ 50% of the negative control should be taken with caution

9 - PREDICTION MODEL

Irritation potential of test sample 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 samples is predicted by mean tissue viability of tissues exposed to the test sample.

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

Criteria for *in vitro* interpretation	***In vivo* classification**
Mean tissue cell viability ≤ 50%	Irritant
Mean tissue cell viability > 50%	Non Irritant

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

IV. LITTERATURE

Alépée N., Tornier C., Robert C., Amsellem C., Roux M.-H., Doucet O., Pachot J., Meloni M., de Brugerolle de Fraissinette A., 2010. A catch-up validation study on reconstructed human epidermis (SkinEthic™ RHE) for full replacement of the Draize skin irritation test. Toxicology In Vitro 24, 257-266.

Anon (2003). United Nations (UN). Skin Corrosion/Irritation. UN, Globally harmonized system of classification and labelling of chemicals. UN, New York and Geneva, pp. 123-135.

Casas, J.W.; Lewerenz, G.M.; Rankin, E.A.; Willoughby, J.A. Sr.; Blakeman, L.C.; McKim, J.M. Jr.; and Coleman, K.P. (2013) In Vitro Human Skin Irritation Test for Evaluation of Medical Device Extracts. Toxicology In Vitro 27, 2175-2183

Coquette A., Berna N., Vandenbosch A., Rosdy M., and Poumay Y., (1999). Differential expression and release of cytokines by an in vitro reconstituted human epidermis model following skin irritant and sensitizing compounds. Toxicology in Vitro. 13: 867-877.

Coquette A., Berna N., Vandenbosch A., Rosdy M., De Wever B., Poumay, Y., 2003. Analysis of interleukin -1α (IL-1α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis of the prediction of *in vivo* skin irritation of chemicals and/or sensitization. Toxicology In Vitro 17, 311-321.

De Brugerolle de Fraisinette A., Picarles V., Chibout S., Kolopp M., Medina J., Burtin P., Ebelin ME., Osborne S., Mayer F.K., Spake A., Rosdy M., De Wever B., Ettlin RA., and Cordier A. (1999). Predictivity of an in vitro model for acute and chronic skin irritation (SkinEthic®) applied to the testing of topical vehicles. Cell Biol. Toxicology. 15: 121-135.

De Jong WH, Hoffmann S, Lee M, Kandárová H, Pellevoisin C, Haishima Y, Rollins B, Zdawczyk A, Willoughby J, Bachelor M, Schatz T, Skoog S, Parker S, Sawyer A, Pescio P, Fant K, Kim KM, Kwon JS, Gehrke H, Hofman-Hüther H, Meloni M, Julius C, Briotet D, Letasiova S, Kato R, Miyajima A, De La Fonteyne LJJ, Videau C, Tornier C, Turley AP, Christiano N, Rollins TS, Coleman KP.
(2019) Round robin study to evaluate the reconstructed human epidermis (RhE) model as an in vitro skin irritation test for detection of irritant activity in medical device extracts. Toxicol In Vitro. 2018 Aug;50:439-449. doi: 10.1016/j.tiv.2018.01.001.

De Wever B. and Charbonnier V., (2002). Using tissue engineered skin to evaluate the irritation potential of skin care products. Cosmetics and Toiletries. 119(9): 28-36.

Doucet O., Robert C., and Zastrow L., (1996). Use of a serum-free reconstituted epidermis as a skin pharmacological model. Toxicology in Vitro. 10: 305-313.

Doucet O., Garcia N., and Zastrow L. (1998). Skin culture model: a possible alternative to the use of excised human skin for assessing in vitro percutaneous absorption. Toxicology in Vitro 12: 423-430.

Method B.4 of Annex V to Council Directive 67/548/EEC

ECVAM Scientific Advisory Committee (ESAC) statement (2007). Statement on the validity of *in-vitro* tests for skin irritation, ESAC 26th meeting, 26-27th April, 2007.

ECVAM SIVS: Final draft Performance standards Version 2007-04-13.

ESAC (2007): Statement on the validity of in-vitro tests for skin irritation. ESAC 26th meeting, 26-27th April 2007. Available at: http://ecvam.jrc.ec.europa.eu (accessed on 10.12.2010)

ESAC (2008a): Statement on the scientific validity of *in vitro* tests for skin irritation testing, issued by the ECVAM Scientific Advisory Committee (ESAC29), 5 November 2008. Available at: Available at: http://ecvam.jrc.ec.europa.eu (accessed on 10.12.2010)

ESAC (2009a): Performance Standards for in vitro skin irritation test methods based on Reconstructed human Epidermis (RhE). Available at: http://ecvam.jrc.ec.europa.eu (accessed on 10.12.2010)

ESAC (2009b): Statement on the "Performance under UN GHS of three in vitro assays for skin irritation testing and the adaptation of the Reference Chemicals and Defined Accuracy Values of the ECVAM skin irritation Performance Standards", issued by the ECVAM Scientific Advisory Committee (ESAC30), 9 April 2009. Available at: http://ecvam.jrc.ec.europa.eu (accessed on 10.12.2010)

EU (2008b): Commission Regulation (EC) No 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Annex, part B. Methods for the determination of toxicity and other health effects. *Official Journal of the European Communities L 142, 141 - 443* , Office for Official Publications of the European Communities, L-2985 Luxembourg

EU (2008c): Commission Regulation (EC) No 1272/2008 on classification, labelling and packaging of samples and mixtures (CLP) entered into force on the 20 January 2009 which will stepwise replace the Council Directive 67/548/EEC (samples), Directive 1999/458/EC (preparations), amending Regulation (EC) No 1907/2006 and subsequent amendments and adaptations.
Official Journal of the European Communities L 353, 1-1355, Office for Official Publications of the European Communities, L-2985, Luxembourg

EU (2009c): Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). *Official Journal of the European Communities L 220, 1 - 94*, Office for Official Publications of the European Communities, L-2985 Luxembourg

Fartasch M., and Ponec M.(1994). Improved barrier structure formation in air-exposed human keratinocyte culture systems. J. Invest. Dermatol. **102**: 366-374.

ISO/TC 194: Biological evaluation of medical devices - Part 12: Sample preparation and reference materials (ISO 10993-12:2012).

Kandárová H. (2006). Dissertation: Evaluation and validation of reconstructed human skin models as alternatives to animal tests in regulatory toxicology. Free University of Berlin.

Kandárová H., Liebsch M., Spielmann H., Genshow E, Schmidt E., Traue D., Guest R. Whittingham A., Warren N., Gamer AO., Remmele M., Kaufmann T., Wittmer E., De Wever B., Rosdy M (2006a).Assessment of the human epidermis model SkinEthic RHE for in vitro skin corrosion testing of chemical according to new OECD TG431. Toxicology In Vitro. **20**: 547-59.

Kandárová H., Liebsch M., Schmidt E., Genshow E., Traue D., Meyer K., Spielmann H., Steinoff C., Tornier C., De Wever B., Rosdy M (2006b). Assessment of the skin irritation potential of chemicals by using the SkinEthic reconstructed human epidermal model and the common skin irritation protocol evaluated in the ECVAM skin irritation study. Alternatives to Laboratory Animals. **34**: 393-406.

Method B.4 of Annex V to Council Directive 67/548/EEC, OECD TG 404.

Mosmann, T., (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65**, 55-63.

OCDE (2015), Test No. 404: Acute Dermal Irritation/Corrosion, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris, https://doi.org/10.1787/9789264242678-en.

OCDE (2019), Test No. 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris, https://doi.org/10.1787/9789264264618-en.

OCDE (2019), Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris, https://doi.org/10.1787/9789264242845-en.

Pellevoisin C, Videau C, Briotet D, Grégoire C, Tornier C, Alonso A, Rigaudeau AS, Bouez C, Seyler N. SkinEthic™, 2018, RHE for in vitro evaluation of skin irritation of medical device extracts. Toxicol In Vitro. 2018 Aug;50:418-425. doi: 10.1016/j.tiv.2018.01.008. Epub 2018 Jan 13.

Prunerias M., Régnier M., Woodley D., (1983). Methods for cultivation of keratinocytes with an air-liquid interface. Journal of Investigative Dermatology. **81**(sup n°1): 28s-33s.

Rosdy M., and Clauss LC.(1990) Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. J. Invest. Dermatol. **95**: 409-414.

Rosdy M., (1994). Reconstituted epidermis for testing. Cosmetics and Toiletries Manufacture Worldwide. pp. 223-226.

Spielmann H., Hoffmann S., Liebsch M., Botham P., Fentem J, Eskes S., Roguet R., Cotović J., Cole T, Worth A., Heylings J., Jones P., Robles C., Kandárová H., Gamer A., Remmele M, Curren R., Raabe R., Cokchott A., Gerner I., Zuang V., 2007. The ECVAM international validation study in vitro test for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and

on skin integrity function test. ATLA 35, 559-601.

Tornier C., Rosdy M, Maibach HI. (2006). In vitro skin irritation testing on reconstituted human epidermis: reproducibility for 50 chemicals tested with two protocols. Toxicology In Vitro. **20**: 401-416.

UN (2009): United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Third revised edition, UN New York and Geneva. Available at: http://www.unece.org/trans/danger/publi/ghs/ghs_rev03/03files_e.html (accessed on 10.12.2010)

V. ANNEXES

Annex 1	Main equipment verifications
Annex 2	SkinEthic™RHE tissues receipt
Annex 3	Solutions information and preparation
Annex 4	Methods documentation sheet: Tests samples
Annex 5	Incubation timings
Annex 6	Remarks and comments

ANNEX 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_2 (%)	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 2 .....mg 3 .....mg	1 .....g 2 .....g 3 .....g

Mean	..... mg	.....g
Tolerance	**9.9 mg to 10.1 mg**	**999. 5 mg to 1000. 5 mg**

Date: **ID and signature:**

PIPETTES VERIFICATION

Water temperature: .....

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

Date: **ID and signature:**

ANNEX 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 4: METHODS DOCUMENTATION SHEET: TESTS SAMPLES

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

Test sample name or code	Total weight test sample and vial (g)	Total surface area	Receipt date: (mm/dd/yy)	Physical consistence	Storage conditions Temperature - Place

Date:…… ***ID and signature:……***

APPENDIX 5: INCUBATION TIMINGS

Laboratory: …… *Study N°……* *Batch N°:……*

	Start date and time mm/dd/yyyy hh:mm	**End date and time** mm/dd/yyyy hh:mm
Preincubation		

	Day mm/dd/yyyy	
	Start time (hh:mm)	**End time** (hh:mm)
Treatment 24±2 hours		
MTT incubation 3 hours (up to 3h15)		
Formazan extraction 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:……***