EPISKIN
L'ORÉAL RESEARCH & INNOVATION

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INVITTOX PROTOCOL

SKINETHIC™SKIN CORROSIVITY TEST

I. PROTOCOL INTRODUCTION

1 - SKINETHIC™SKIN CORROSIVITY TEST

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect on the SKINETHIC™ Reconstructed Human Epidermis (SKINETHIC™ RHE), as measured by the MTT assay. The test is compliant with the OECD Test Guideline No. 431.

2 - OBJECTIVE AND APPLICATIONS

Type of Testing:
Replacement, screening

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

Purpose of Testing:
Classification and labelling

Context of Use:
The test method was granted regulatory approval as a replacement for the *in vivo* skin corrosivity test (Method B.40 bis, EU 2000, 2008; OECD Test Guideline 431, OECD 2004) and it is used for hazard identification and classification of corrosive potential in order to fulfil the regulatory requirements concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (EU, 2008). Furthermore, the test method is recommended for its use within the context of the sequential skin corrosivity testing strategy (OECD Test Guideline 404, OECD 2002 and Method B.4, EU 2004 and 2008).

The test allows the identification of corrosive chemical substances and mixtures and enables the identification of non-corrosive substances and mixtures when supported by a weight of evidence determination using other existing information (OECD, 2004 and EU, 2008).

Applicability Domain:
The test method allows the hazard identification of mono and multi-component test substances (solids, liquids, semi-solids, soluble or insoluble in water). Gazes and aerosols cannot be evaluated.

Additional Information:
Highly colored chemicals and/or MTT reducers may interfere with cell viability measurements and therefore adapted controls for corrections need to be used.

3 - RATIONALE

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin.
The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals.
The determination of the skin corrosion potential is therefore included in international regulatory requirements for the testing of substances, such as the U.S. Code of Federal Regulations (US DOT, 1991), the updated OECD Test Guideline No 404 (OECD, 2002) and the Method B.4 of the Annex to Commission Regulation 440/2008/EC (EU, 2008). Corrosivity was usually determined *in vivo* using the Draize rabbit skin test (Draize *et al*., 1944).

The present test is based on the experience that corrosive substances show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis.
The test is designed to predict and classify the skin corrosivity potential of a test substance by assessment of its effect on a reconstructed human epidermis.

SKINETHIC™ RHE is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. The test method consists of a topical exposure of the neat test substance onto the SKINETHIC™ RHE, followed by cell viability assessment. Viability decreases in test substance treated tissues is expressed comparatively to negative controls. Percentage viability (%) is used to predict and classify skin corrosion potential following a defined prediction model.

4 - EXPERIMENTAL DESCRIPTION

Endpoint and Endpoint Measurement:
Cell viability determination, used as the endpoint, is based on cellular mitochondrial dehydrogenase activity, measured by tetrazolium salt MTT reduction *[(3-4,5-dimethyl triazole 2-yl) 2,5-diphenyltetrazoliumbromide]*, and conversion into a blue formazan salt that is quantitatively measured after extraction from tissues (Mossman, 1983).

Endpoint Value:
The percentage viability of each treated tissue was calculated from the percentage of MTT conversion in the test substances treated tissues relative to the corresponding negative controls (100% viability).
VIABILITY: reduction in cell viability at different exposure periods is used to indicate the presence of the significant biological effect.

Experimental System(s):
SKINETHIC™ RHE is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Differentiated and stratified epidermis model comprises the main basal, suprabasal, spinous and granular layers and a functional stratum corneum (Rosdy and Clauss, 1990; Fartasch and Ponec, 1994; Doucet *et al*., 1998; Kandárová, 2006*).* A generic description of general and functional conditions that skin models need to comply with can be

found in the OECD test Guideline 431 *In vitro* Skin Corrosion: Human Skin Models (OECD, 2004).

Basic Procedure
Test substances are applied to the stratum corneum of the epidermal model (at least two epidermis units per test substance) for two different exposure periods: 3 min, and 60 min. Exposure to the test substance was terminated by rinsing with phosphate buffered saline (PBS). SKINETHIC™ RHE tissues exposed to the control test substances serve as the controls for all corresponding exposure periods.

The viability of the epidermis is assessed by measuring the mitochondrial activity. The tissues are incubated for 3 hours with MTT solution (1 mg/ml; 0.3 ml per well). MTT, a yellow-coloured tetrazolium salt, is reduced by succinate dehydrogenase into a blue formazan precipitate in the mitochondria of living cells. The precipitated formazan is extracted overnight by using isopropanol (1.5 ml), and is then quantified spectrophotometrically at a wavelength between 540 nm and 600 nm (ideally 570 nm). H_2O (40 µl) and 8N KOH (40 µl) are used as negative and positive controls, respectively. For each treated tissue the viability is expressed as a % relative to negative control tissues (mean).

5 - DATA ANALYSIS/ PREDICTION MODEL

The test results are interpreted on the basis of the exposure time needed to cause cell viability to decrease. The determination of the UN packing groups and GHS classifications is summarized in the Table reported in the **'Prediction Model'** section of the **'Technical Description'**.

6 - TEST COMPOUNDS AND RESULTS SUMMARY

A total of 25 test compounds, consisting of 6 inorganic acids, 4 amines, 3 phenolic derivatives, 3 fatty acids, 2 alkalis, 1 ketone, 1 hydrocarbon, 1 S-containing compound, 1 brominated derivative, 1 chlorinated solvent, 1 surfactant, 1 miscellaneous (Kandárová *et al*., 2006a; Tornier *et al*., 2010). The protocol and Prediction Model (as described in the „Technical Description") was concluded to have a good predictive capacity in the me-too validation study in terms of predictive capacity for distinguishing „corrosive" and „non- corrosive" chemicals (Kandárová *et al*., 2006a).

7 - MODIFICATIONS OF THE METHOD

The ESAC statement of the validated SKINETHIC™ RHE method for skin corrosion testing has been endorsed on November 16th, 2006.

An adaptation of the validated SKINETHIC™ Reconstructed Human Epidermis (RHE) skin corrosion test method was peer-reviewed and published by Tornier *et al*. (Toxicology In Vitro 24:1379–1385,

2010).

The validated protocol presented in the „Technical Description" section complies with the OECD Guideline for the Testing of Chemicals No. 431: *In Vitro* Skin Corrosion.

8 - DISCUSSION

Some highly reactive substances can produce fumes, which may affect adjacent units in the same plate. It is recommended that if there is any suspicion that a material could cause fumes, it should be tested alone in a single plate. It is particularly important that the negative control units are not exposed to fumes from other units, hence it is recommended to routinely incubate positive and negative controls in a separate plate.
Some chemicals can directly reduce the MTT reagent (e.g. reducers). Other chemicals can directly color the tissue or the cells. In these cases, a special procedure allowing the quantification of the "true" MTT mitochondrial reduction should be applied. The use of specific and adapted controls will enable the calculation of true tissues viability after subtracting the unspecific Optical Densities due to direct chemical MTT reduction and/or to chemical residual color extracted from the tissues. Conditions for specific controls use are described in the procedure.

9 - STATUS

Participation in Validation Studies:
ESAC unanimously endorsed the statement that the SKINETHIC™ RHE test method for skin corrosion was scientifically validated for use as a replacement for the animal test and that this test was ready to be considered for regulatory acceptance (ESAC, 2006).

Regulatory Acceptance:
In 2000, the human epidermis model assays, which meet certain criteria, have been included into "Annex V. Part B.40 on Skin Corrosion" of the "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" (EU, 2000).

The EPISKIN™ test method was further recommended to be used as one of the *in vitro* methods for skin corrosivity testing in the OECD Test Guideline 404 (OECD, 2002) and in the Method B.4 of Annex V of the Directive 67/548/EEC (EU, 2004) laying down the step- wise testing strategy for classifying skin corrosives by the sequential application of three alternative methods: structure-activity relationships, pH measurements and a single *in vitro* method (Worth *et al*., 1998).

In 2004, *In Vitro* Skin Corrosion: Human Skin Model Test was adopted as the OECD Test Guideline No 431 which is applicable to the assays for skin corrosion employing reconstituted human skin (i.e. EPISKIN™) models (OECD, 2004). In Guideline TG 431 (paragraphs 9–11) general functional and performance criteria were defined if other (or new) skin or epidermis models are used in the context of this guideline. The current SOP is describing a generally applicable method for Skin Corrosion Testing, here applied to the SKINETHIC™ RHE model.

10 - PROPRIETARY AND/OR CONFIDENTIALITY ISSUES

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

11 - ABBREVIATIONS AND DEFINITIONS

C: Corrosive
ET-50: Exposure Time that reduces 50% cell viability
MDS: Methods Documentation Sheet
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC: Negative Control
N-C: Non-Corrosive **OD**: Optical Density **PC**: Positive Control
RHE: Reconstructed Human Epidermis

II. TECHNICAL DESCRIPTION

1 - HEALTH AND SAFETY ISSUES

SKINETHIC™ RHE tissues are manufactured in compliance with ISO-9001 certification Version 2015. 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.

Safety instructions for working with chemicals:

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

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

2 - MATERIALS AND PREPARATIONS

A. CELL OR TEST SYSTEM

Test system description:
The SKINETHIC™ RHE tissue model (EPISKIN – 4 Rue Alexander Fleming – 69007 LYON, FRANCE) consists of normal human keratinocytes cultured for 17- days (± 1 day) 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, 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 de Fraissinette *et al*., 1999; Tornier *et al*., 2006; Kandárová *et a*l., 2006b).

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

All the SKINETHIC™ RHE model kits are free of viruses, bacteria and mycoplasma. The quality of the final RHE product is assessed by a MTT cytotoxicity test of untreated tissues, by ET-50 with Triton X-100 at the concentration of 1%, and by histological examination.

B. EQUIPMENT

Materials not provided by EPISKIN:

Sterile, blunt-edged forceps	*For handling tissue inserts*
Sterile disposable pipettes, pipettes tips	*For diluting, adding, and removing media and test materials. For topically applying test materials to tissues*
Cell incubator (37°C, 5% CO_2, 95% relative humidity)	*For incubating tissues prior and during assays*
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*

Laboratory balance (accuracy 0.1 mg)	*For pipette verification, test substance weighing*
96-well plate photometer with a 570nm filter	*For Optical Density readings (MTT)*
Adjustable Pipette / multi-step Pipette	*For 1 ml assay medium*
Adjustable Pipette / multi-step Pipette	*For dispensing 300 µl MTT /medium*
Adjustable Pipette	*For dispensing 700+800 µl propan-2-ol*
Plate shaker	*For extraction of formazan*
Adjustable Pipette	*For dispensing 200 µl formazan extract from 24-well plate into 96-well plate for the plate photometer*
Stop-watches	*To be used during application of test materials*
Plastic wash bottles	*For collecting PBS rinses*
Small glass weight boats	*For weighing powders*
Mortar and Pestle	*For grinding granulars*
Positive displacement pipette for 20 µl and 40 µl delivery	*For application of liquid and viscous test materials*
500 ml wash bottle	*For rinsing tissue after test material exposure*
1 l beaker	*For collecting PBS washes*
1 Funnel	*For rinsing tissues with PBS*
Extra 6-well plates – sterile	*To transfer tissue inserts to fresh media and checking of direct MTT interaction with test substances*
Extra 24-well plates – sterile	*For application + MTT incubation + formazan extraction steps*
Extra 96-well plates – sterile	*For OD measurements*

Parafilm	*Covering plates during formazan extraction*
Absorbent paper / gauze	*To remove agarose fragments or to dry inserts*

Equipment verification:
It is strongly recommended to use regularly verified apparatus equipment. **Annex 1** provides examples of possible verification measurements for particularly sensitive equipment concerning this protocol.

C. MEDIA, REAGENTS, SERA, OTHERS

The SKINETHIC™ RHE kit components:
SKINETHIC™ RHE set and media provided by EPISKIN.

Reference	Description
RHE/S/17	Epidermal tissues, small size, day 17
SMM	SKINETHIC™ Maintenance Medium

The SKINETHIC™ RHE (RHE/S/17) and the necessary culture media (SKINETHIC™ Maintenance Medium (SMM)) are received one or two day(s) following the shipment (for Europe and USA). Results of the quality controls for histology and cell viability 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 (4.00h ≤ ET-50 ≤ 10.00h), absence of bacteria, fungi, mycoplasma, HIV-1 and 2 and Hepatitis B, C.

For the SKINETHIC™ Maintenance Medium and SKINETHIC™ RHE tissue models refer to the Technical Data, Safety Sheet and Certificated Analysis, located in the plastic file inside the shipping box.

Store the SKINETHIC™ RHE tissues at room temperature until their transfer into SKINETHIC™ Maintenance Medium.
Store SKINETHIC™ Maintenance Medium at 4°C in the dark.

Reagents not provided with the SKINETHIC™ RHE kit:

Dulbeccos‟ PBS without Ca^{2+} and Mg^{2+}	*Use for diluting MTT, 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) or equivalent	*For the MTT assay*
8N KOH	*To be used as positive control with each kit*
Sterile H_2O (distilled or aqua pure)	*To be used as negative control with each kit and for powder applications*

D. PREPARATIONS

Test substances:
A single testing run composed of at least two replicate tissues should be sufficient for a test substance.

Main information concerning the test substances (name or code, total weight, reception date, expiration date, physical consistence, stocking conditions) should be registered. **Annex 3** proposes an example of MDS: Test substances**.**

Negative control solution:
Distilled H_2O will be used as negative control

Positive control solution:
8N KOH will be used as positive control.

MTT solution preparation
Safety precautions:

- MTT (H315, H319, H335, **H341**, P261, P281, P305+P351+P338) - MTT solution is light sensitive. Protect it from light using silver paper or appropriate material.
- Isopropanol (H225, H319, H336, P210, P261, P305+P351+P338)

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 or equivalent) 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 8)**.

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 SKINETHIC™ 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 or equivalent Document

MDS: Isopropanol (**Annex 8)**.

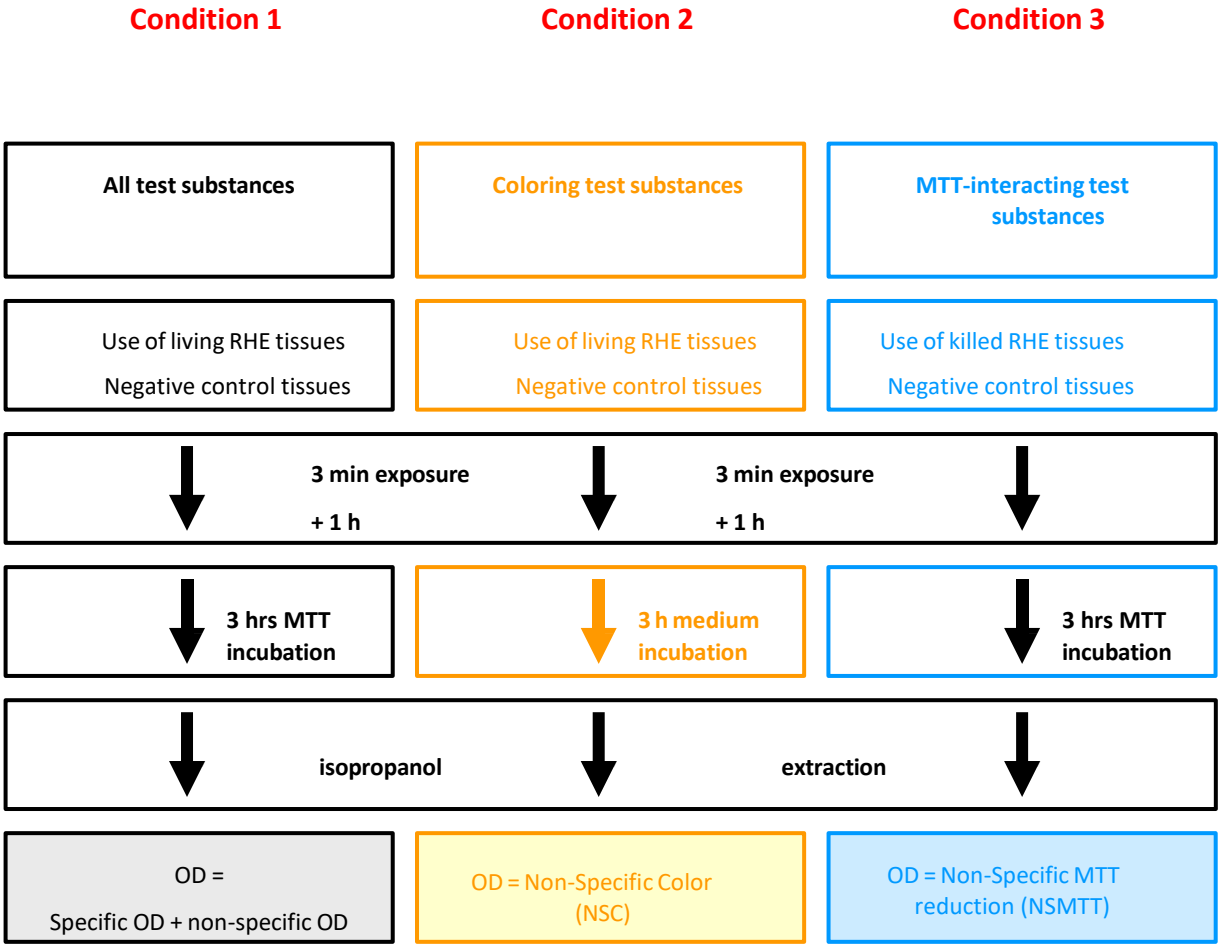
Pre-check method for possible direct MTT reduction with test substances:
Relative conversion of MTT by the tissue being the parameter evaluated in this assay, it is therefore necessary to assess the non-specific reduction of MTT by the test substance used. Prior to experiments all test substances should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

1) Fill wells of 6-well plate with 1 ml of MTT ready to use solution (1 mg/ml)
2) Add 40 ± 3 µl or 20 ± 3 mg of the test substance to be evaluated. Mix. MTT ready to use solution is used as control.
3) Protect from light and incubate the mixture for 3 h ± 15 min at 37°C, 5% CO_2.
4) Proceed to visual scoring of MTT interaction as follow:
   - Negative control (MTT ready to use solution): yellow
   - Test substances which do not interact with MTT: yellow
   - Test substances interacting with MTT: blue
5) Document MDS: MTT-direct interacting test substances identification (An example is given in Annex 4).

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 nonspecific reduction of the MTT (i.e. by using freeze-killed epidermis).

For each MTT-interacting test substance previously detected, and in addition to the normal procedure, 2 freeze-killed test substance treated tissues are used for the MTT evaluation following the same protocol as for living tissues (see paragraph 5.2.3). Two freeze-killed untreated tissues are used as negative controls (untreated freeze-killed tissues may exhibit little residual NADH and dehydrogenase associated activity).

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**

Pre-check method for mesh compatibility with liquid or viscous test substances:
Some chemicals may interact with the mesh ad therefore the compatibility of each liquid or viscous test substance with nylon mesh has to be checked.

To test if a test substance interacts with the mesh, place the mesh on inert surface (for example a slide) and apply 40 ± 3 µl of the test substance. After a 15 min exposure time at room temperature, check visually if an interaction between the test substance and the mesh is noticed.

In case of interaction between the test substance and the mesh, the test substance has to be applied without a mesh as spreading aid.

3 - METHOD

A. TEST SYSTEM PROCUREMENT

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

Tissues/Media expiration and storage:

Reference	Description	Conditions	Shelf life
RHE/S/17	Epidermal tissues, small size, day 17	37°C	7 days
SMM	SKINETHIC™ Maintenance 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).

Reception of materials supplied by EPISKIN:
Examine all kit components for integrity.

1) Document MDS: RHE set- SKINETHIC materials receipt (**Annex 2**).
2) Place the SKINETHIC™ Maintenance Medium in the fridge (2 to 8°C).
3) Keep the SKINETHIC™ RHE tissues and the SKINETHIC™ Maintenance Medium at room temperature for the pre-incubation step.

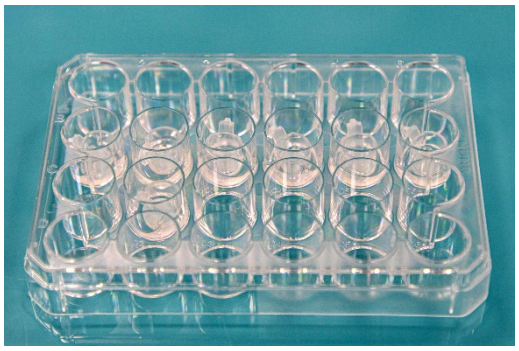
B. ROUTINE CULTURE PROCEDURE

Pre-incubation step:
It is recommended to conduct this step under sterile conditions.

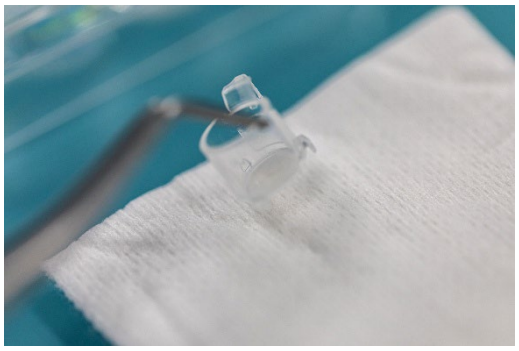
Introductory note:
A minimum of 32 SKINETHIC™ RHE tissues are used for testing 6 (to the exception of inorganic acids) test substances, negative control each of them applied for both 3 min and 1 h to at least two replicates per treatment, as well as the positive control applied for 1 h.

Proceed to a minimum 2 h pre-incubation step. This incubation period can be increased up to 24 h if tissues are not used on day of receipt.

1) Prepare an appropriate number of 6-well plates (1 plate per test substance or control and per application time) for the **3 min** exposure period and the **1 h** exposure period. Fill each well with 1 ml room temperature SKINETHIC™ Maintenance Medium (SMM)
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 absorbent paper or gauze to remove eventual remaining agarose pieces *(see Pictures 1 and 2).*

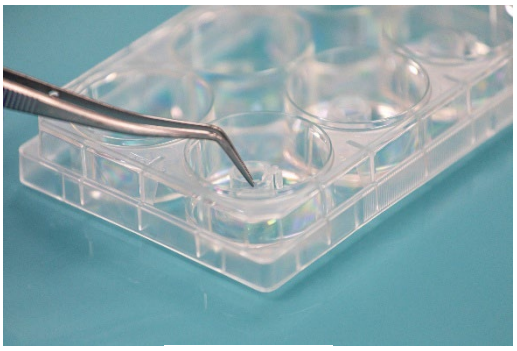


Picture 1



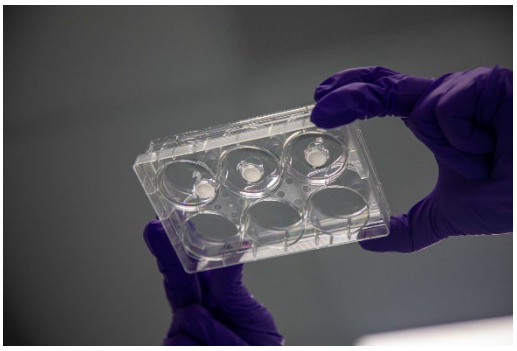
Picture 2

4) Check visually that no agarose is remaining and transfer the tissue on fresh medium by first sloping the insert before complete insert setting *(picture 3)*. For each test substance or control and for each exposure period use one 6-well plate and place tissues in the pre-filled 6-well plate.



Picture 3

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



Picture 4

6) Place the SKINETHIC™ RHE tissues at 37°C, 5% CO_2 for at least 2 hours until test substance application.

7) At the end of the pre-incubation step, transfer tissues into application plates.
8) Record pre-incubation timing in **Annex 7**: MDS pre-incubation, application and rinsing timing.

Freeze-killed epidermis for MTT-interacting substances (if necessary):

1) Prepare freeze-killed tissues by transferring living epidermis at -20°C (or -80°C) for at least 48 hours (2 tissues / MTT-interacting test substance).
2) Further use of freeze-killed tissues is similar to living tissues. Use 2 additional killed tissues as untreated killed controls (The untreated freeze-killed controls show small amount of MTT reduction due to residual reducing enzymes within the freeze-killed tissue).
3) Document MDS: Freeze-killed tissues for MTT-interacting test substances (an example is given in **Annex 5**).

Adapted controls for coloring test substances:
Prior to treatment, test substances should be evaluated for their intrinsic color or ability to become colored in contact with water (simulating a tissue humid environment). Add 20 ± 3 mg (solids) or 40 ± 3 µl (liquids) of the test substance to 300 µl of water in a transparent recipient (a 24-well plate). Cover the plate with an adhesive tape. Shake for 60 ± 15 min (for example 500 rpm) at room temperature. At the end of the shaking period color if solution become colored, living adapted controls should be performed.

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 SKINETHIC™ RHE tissues. In addition to classical controls dye test substances should be put in contact with the normal living SKINETHIC™ RHE tissues as described below.

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

1) Use 2 living SKINETHIC™ RHE tissues for each coloring test substances + 2 additional negative control (H_2O treated) tissues
2) Proceed to SKINETHIC Skin Corrosion Test up to the drying step of the rinsing procedure.
3) Document MDS: Additional control for coloring test substances (**Annex 6**).

C. TEST SUBSTANCE EXPOSURE PROCEDURES

Application of test substances and rinsing:

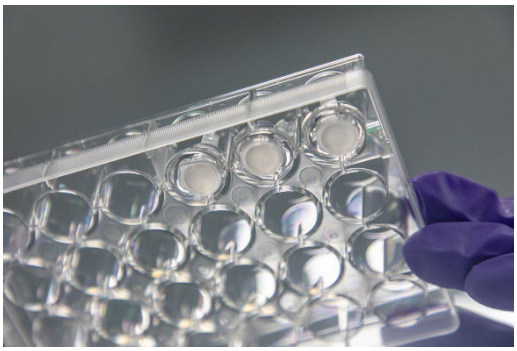
Safety precautions:
Corrosive materials are dangerous: it is thus necessary to work in laminar flow hood with chemical filter or in ventilated cabinets and wear gloves, coat, as necessary.

Since the present test is a short-term test, sterility is not as important as in other applications of the SKINETHIC™ RHE model. Nevertheless, it is important to keep assay media sterile and to keep risk of contamination at a low level.

Plates preparation:

Application plates:

1) Prepare an appropriate number of 24-well plates for the **3 min** exposure period and the **1 h** exposure period. However, it is necessary to use one plate per test substance/control (for all exposure periods) in case of test substances with fume suspicion.
2) Fill wells with 300 µl SKINETHIC™ Maintenance Medium (SMM)
3) Transfer the tissue on fresh medium by first sloping the insert before complete insert setting (Picture 3). After tissue transfer, check the absence of air bubbles by watching underneath the 24-well plate *(see Picture 5).*



Picture 5

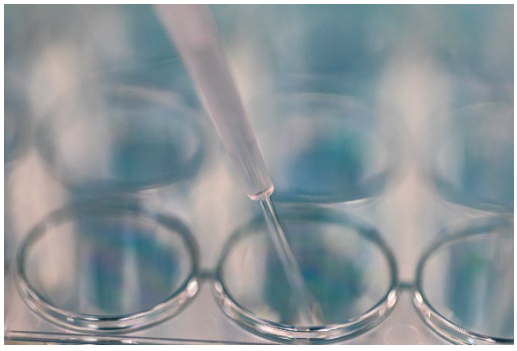
MTT incubation plates:

1) Prepare an appropriate number of 24-well plates for the **3 min** exposure period and the **1 h** exposure period. Prepare MTT solution 1 mg/ml according to section 4. MTT preparation 2).
2) Document MTT preparation. An example is given in **Annex 8** (MDS: solutions preparation)
3) Fill wells with 300 µl MTT solution 1 mg/ml.
4) Protect plates from light (aluminum) until use.

Application volumes/quantities:

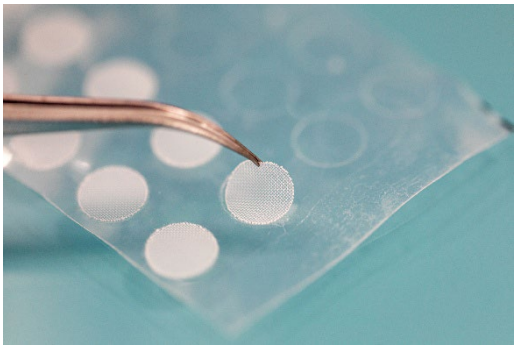
Liquid and viscous test substances:

1) Dispense 40 ± 3 µl of the undiluted test substance on the top of each epidermis tissue (at least two per test substance: replicate 1, replicate 2), using positive displacement pipette (adjustable pipette is acceptable for non-viscous test substances) *(see Picture 6).*



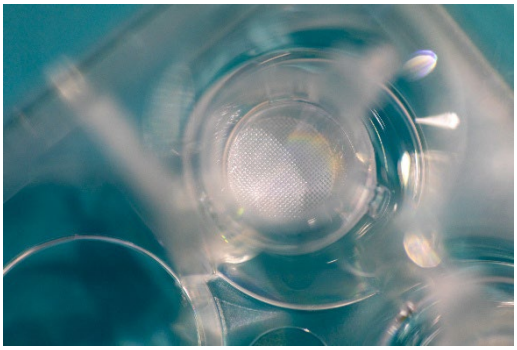
Picture 6

2) Carefully apply a nylon mesh on the whole surface with forceps *(see Pictures 7, 8 and 9).*



Picture 7

Picture 8



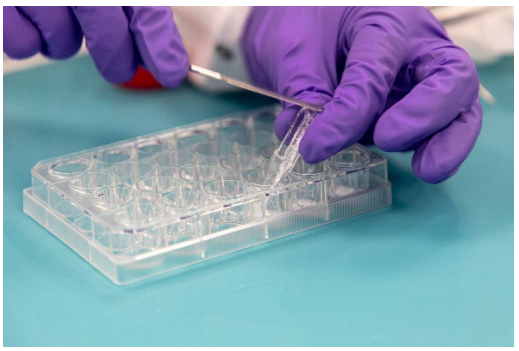
Picture 9

Solid tests substances:

1) If necessary, the test substance should be crushed to a fine powder using a mortar and a pestle.
2) Gently spread 20 ± 2 µl of distilled water using a positive displacement pipette (or adjustable pipette) to the epidermal surface in order to improve further contact between the powder and the epidermis.
3) Use special glass weigh boats (or similar tools avoiding electrostatic electricity and allowing a targeted application directly in the insert with no risk of test substance scattering in the medium subnatant) to apply 20 mg ± 3 mg of the powder to the epidermis surface *(See Pictures 10, 11 and 12).*

Picture 10

Picture 11



Picture 12

4) Document weighing of solids. An example of MDS: Weighing of solids test substances is provided in **Annex 9**.

Waxy (sticky) test substances:

1) Gently spread 20 ± 2 µl of distilled water using a positive displacement pipette (or adjustable pipette) to the epidermal surface in order to improve further contact between the test substance and the epidermis.
2) Allow for the tare with a nylon mesh and directly weigh 20 mg ± 3 mg and spread waxy test substance on this latter.
3) Apply the test substance coated side of the nylon mesh on the epidermal surface and spread it gently on the whole surface.
4) Document weighing of solids test substances. An example of MDS: Weighing of solids test substances is provided in **Annex 9**.

1 hour exposure period (room temperature):
Note: dosing time interval is dedicated by rinsing procedure

At least two tissues per test substance or control should be used (2 replicates). The application order is important since it will be the same for washing.

Apply the test substance quantity described above.

Suggestion: Keep 1 minute interval between each tissue application. Record the exact timing and document the correspondent MDS: Application timing (**Annex 7**).

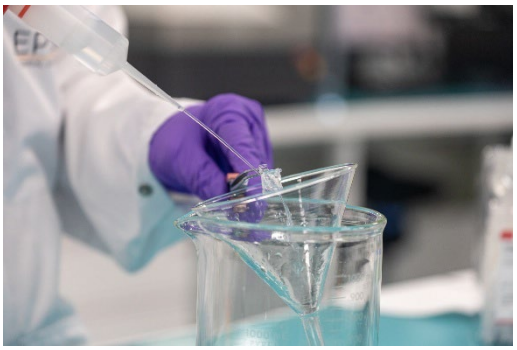
An example of 1 h application is provided in Table 1.

Exposure: 1 h		**Neg C**	**C 1**	**C 2**	**C 3**	**C 4**	**C 5**	**C 6**	**Pos C**
Application	Tissue 1	13h00	13h03	13h06	13h08	13h11	13h14	13h17	13h20
	Tissue 2	13h01	13h04	13h07	13h09	13h12	13h15	13h18	13h21
Rinsing	Tissue 1	14h00	14h03	14h06	14h08	14h11	14h14	14h17	14h20
	Tissue 2	14h01	14h04	14h07	14h09	14h12	14h15	14h18	14h21

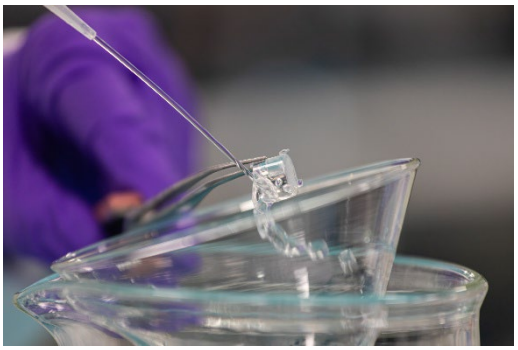
Table 1: Example of 1 h application timing

When the 1 h exposure period is completed remove the first insert with forceps and proceed to rinsing step. Table 1 presents the rinsing timing corresponding to the proposed application timing.

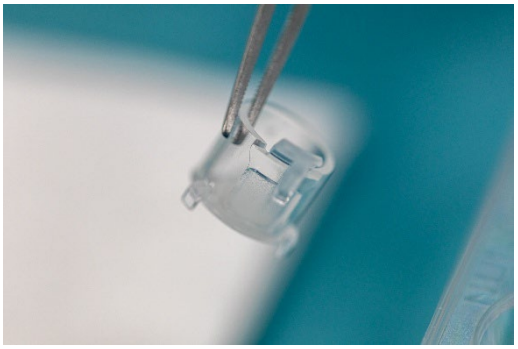
Using a wash bottle or a multi-pipette (adjusted for a 1 ml distribution) gently rinse the tissue with PBS (20 times) over a funnel put on a large beaker to remove any residual of material. See Picture 13. Remove excess PBS by gently shaking the insert *(Picture 14)* and dry bottom insert with absorbent paper or gauze *(Picture 15).*



Picture 13



Picture 14



Picture 15

3 min exposure period (room temperature):
Note: dosing time interval (exactly one minute) is dedicated by rinsing procedure

At least two tissues per test substance or negative control should be used (2 replicates). The application order is important since it will be the same for washing.

Suggestion: Keep 1 minute interval between each tissue application. Record the exact timing and document the correspondent MDS: Application timing (**Annex 7**).

An example of 3 min application is provided in Table 2.

Exposure: 3 min		**Neg C**	**C 1**	**C 2**	**C 3**	**C 4**	**C 5**	**C 6**
Application	Tissue 1	14h40	14h46	14h52	14h58	15h04	15h10	15h16
	Tissue 2	14h41	14h47	14h53	14h59	15h05	15h11	15h17
Rinsing	Tissue 1	14h43	14h49	14h55	15h01	15h07	15h13	15h19
	Tissue 2	14h44	14h50	14h56	15h02	15h08	15h14	15h20

Table 2: Example of 3 min application timing

When the 3 min exposure period is completed remove the first insert with forceps and proceed to rinsing step. Table 1 presents the rinsing timing corresponding to the proposed application timing.

Using a wash bottle or a multi-pipette (adjusted for a 1 ml distribution) gently rinse the tissue with PBS (20 times) over a funnel put on a large beaker to remove any residual of material. See Picture

13. Remove excess PBS by gently shaking the insert (Picture 14) and dry bottom insert with absorbent paper or gauze (Picture 15).

MTT test:
Tissue viability is assessed by MTT reduction measurement at 37°C, CO_2, 95 % humidified atmosphere.

Incubation in MTT solution:

1) Once all tissues have been rinsed and are in the application plate, remove inserts from the application plate, blot bottom and transfer them into the MTT assay 24-well plate.
2) Check the absence of air-bubbles.
3) Incubate for 3 hours ± 15 min at 37°C, CO_2, 95 % humidified atmosphere.
4) Document MDS: MTT incubation (**Annex 10**).

For coloring test substances replace MTT by 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 drying step, 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 ± 15 min at 37°C, 5% CO_2, 95% humidified atmosphere.
6) Document MDS: Start of 3 h incubation time (**Annex 6**).

Formazan extraction:

1) Label an appropriate number of 24-well plates for the **3 min** exposure period and the **1 h** exposure period. Fill the plate with 800 µl isopropanol
2) At the end of the 3 hours ± 15 min incubation in MTT solution, record MDS: Observations and comments after the 3 hours MTT incubation (**Annex 11**).
3) Use forceps to transfer treated tissues.
4) Dry the insert bottom of the treated tissue on absorbent paper or gauze.
5) Transfer the tissues in isopropanol solution.
6) Add 700 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 3 parafilm layers over the plate and adding the lid on the plate.
9) Extract either over night without shaking at room temperature or, alternatively, 2 hours with gentle agitation (about 120 rpm).
10) Document MDS: Isopropanol extraction start time (**Annex 10**) (**Annex 6** for coloring

test substances).

Absorbance / optical density measurements:

1) At the end of the 2 hours or overnight formazan extraction incubation time, open the plate.
2) Remove the 3 parafilm layers.
3) Document in MDS: OD reading (**Annex 10**).
4) Isopropanol solution is used as blank (8 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 aspiring and dispensing 3 times to complete formazan crystals solubilization.
8) Transfer 2 x 200 µl extraction solution per well (= 2 wells per tissue i.e. 2 replicates per tissue) into a 96-well plate (labeled appropriately, accordingly to example presented in Figure 1. Use one plate per exposure time.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NegC	C1	C2	C3	C4	C5	C6	PosC					Tissue 1
B	NegC	C1	C2	C3	C4	C5	C6	PosC					
C	NegC	C1	C2	C3	C4	C5	C6	PosC					Tissue 2
D	NegC	C1	C2	C3	C4	C5	C6	PosC					
E	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank					
F													
G													
H													

Figure 1: Fixed 96-well plate design for OD reading

9) Read the Optical Densities (OD) using a 96-well plate spectrophotometer ideally at 570 nm wavelength (eventually between 540 to 600 nm). No reference filter should be used.
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 Remarks and protocol modifications, **Annex 12** present an example of MDS: Remarks and comments – Protocol modifications.

4 - DATA ANALYSIS / CALCULATION STEPS

Data calculation step:

Blanks:
- Calculate the OD mean from the 8 replicates for each plate OD_{blank}

Negative H_2O-treated controls:
- Calculate the blank corrected value $OD_{NC} = OD_{NCraw} – OD_{blank}$
- Calculate the OD mean per tissue
- The mean OD for all tissues corresponds to 100% viability =mean OD_{NC}

Positive 8N KOH-treated control:
- Calculate the blank corrected value $OD_{PC} = OD_{PCraw} – OD_{blank}$
- Calculate the OD mean per tissue
- 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

Test substance:
- Calculate the blank corrected value $OD_{TT} = OD_{TTraw} – OD_{blank}$
- Calculate the OD mean per tissue
- 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
- Calculate the range between identically treated tissues R % = [1-(%TTmax-%TTmin)] x 100
  NB: %TTmax = higher viability between tissue replicates
  %TTmin = lower viability between tissue replicates

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 freeze-killed tissues OD

OD_{KT}: test substance treated freeze-killed tissues OD

$NSMTT = [(OD_{KT} - OD_{KU}) / \text{mean } OD_{NC}] \times 100$

When NSMTT < 0, consider NSMTT = 0 and do not proceed to TOD and relative viability calculations.

If NSMTT is > 50% relative to the negative control: additional steps must be undertaken if possible

or the test substance must be considered as incompatible with the test. However, if the call is corrosive and the testing of the test substance is valid (from ODTT values) the test is valid since the adapted control has no impact on the final call (Corrosive test substance).

Treated tissue True MTT metabolic conversion (TOD_{TT}) OD_{TT}:

test substance treated viable tissues

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

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

Data calculations for coloring 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 %.

Non-specific Color % (NSC %)

OD_{CT}: coloring test substance treated tissue (incubated in Maintenance Medium before isopropanol extraction)

OD_{H2O}: control H_2O treated tissue (incubated in Maintenance Medium before isopropanol extraction)

$NSC\% = [(OD_{CT} – OD_{H2O}) / \text{mean } OD_{NC}] \times 100$

When NSC < 0, consider NSC = 0 and do not proceed to TOD and relative viability calculations.

If the call is Corrosive, and the testing of the test substance is valid (from OD_{TT} values), the test is valid since the adapted control has no impact on the final call (Corrosive test substance).

If Non-Specific Color (%NSC) is > 50% relative to the negative control, additional steps must be undertaken if possible, for a test substance that gives a Non-Corrosive call. The final viability % is not applicable.

True MTT metabolic conversion (TOD_{DT})

OD_{CT}: coloring test substance-treated tissues (MTT incubation)

TOD_{CT}: true MTT metabolic conversion for coloring test substance treated tissue. TOD_{CT} =

$OD_{TT} – (\text{mean } OD_{CT} - \text{mean } OD_{H2O})$

Relative viability % = [TOD_{CT} / OD_{NC}] x 100

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

Calculate corresponding NSMTT and NSC.

If (NSMTT % + NSC %) is > 50% relative to the negative control, additional steps must be undertaken if possible, for a test substance that gives a Non-Corrosive call. The final viability % is not applicable.

If the call is Corrosive, and the testing of the test substance is valid (from OD_{TT} values), the test is valid since the adapted control has no impact on the final call (Corrosive test substance).

True MTT metabolic conversion for coloring test substances which are also MTT-interacting test substances (TOD_{CTT})

OD_{CT}: coloring test substance-treated tissues (MTT incubation)

TOD_{CTT} = OD_{TT} – (mean OD_{CT} – mean OD_{H2O}) – (mean OD_{KT} – mean OD_{KU}) Relative viability

% = [TOD_{CTT} / mean OD_{NC}] x 100

5 - ACCEPTANCE CRITERIA

The run is qualified (qualified run) if it meets the acceptance criteria for the NC and PC. Otherwise, the run is considered as non-qualified.

A test substance concurrently tested in at least two tissue replicates is called a "testing run".

A “testing run” for a test substance is defined when the cytotoxic effect by using MTT is quantitatively measured. A reported technical issue before the viability measurement is not considered as a “testing run” for the test substance.

Negative control (NC) acceptance criteria: The NC data meet the acceptance criteria if the mean OD value of the 2 tissues is ≥ 0.8 and ≤ 3.0 at 570 nm according to the historical database.

Positive control (PC) acceptance criteria: The PC data meet the acceptance criteria if it is classified as corrosive (% viability < 15% after 1 h exposure).

Test substance data acceptance criteria: The range between identically treated tissues has to be

less than 30%, with the exception of cases with OD ≤ 0.3 and for viabilities out the range 20-100%.

6 - PREDICTION MODEL

Corrosive potential of test substance is determined according to C for Corrosive test substances and N-C for non-corrosive test substances. The prediction model is defined as described below:

Classification	Packing group	EU CLP	Criteria for *In Vitro* interpretation
UN GHS	Corrosive class I, II, III	1A, 1B, 1C	If viability < 50 % after 3 min exposure
			If viability ≥ 50 % after 3 min exposure **and** if viability < 15 % after 1 h exposure
	Non corrosive	Non corrosive	If viability ≥ 50 % after 3 min exposure **and** if viability ≥ 15 % after 1 h exposure

III. LITTERATURE

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IV. ANNEXES

Annex 1	MDS: Detailed equipment verification MDS: RHE set – EPISKIN materials receipt MDS: Test substances
Annex 2	MDS: MTT-direct interacting test substances identification MDS: Freeze-killed tissues for MTT interacting test substances MDS: Additional control for coloring test substances
Annex 3	MDS: Pre-incubation, application, and rinsing timing MDS: Solutions preparation
Annex 4	MDS: Weighing of solid and sticky test substances MDS: MTT and isopropanol extraction timing
Annex 5	MDS: Observations and comments after 3 hours MTT incubation
Annex 6	MDS: Remarks and comments – Protocol modifications if necessary
Annex 7	MDS: Detailed equipment verification MDS: RHE set – EPISKIN materials receipt MDS: Test substances
Annex 8	MDS: MTT-direct interacting test substances identification MDS: Freeze-killed tissues for MTT interacting test substances MDS: Additional control for coloring test substances
Annex 9	MDS: Pre-incubation, application, and rinsing timing MDS: Solutions preparation
Annex 10	MDS: Weighing of solid and sticky test substances MDS: MTT and isopropanol extraction timing
Annex 11	MDS: Observations and comments after 3 hours MTT incubation
Annex 12	MDS: Remarks and comments – Protocol modifications if necessary

ANNEX 1 - 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°	CO2 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 2 - METHODS DOCUMENTATION SHEET: RHE SET – EPISKIN MATERIALS RECEIPT

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

EPISKIN set receipt:

Date: ……..

Day used: ……..

Reconstructed Human Epidermal (RHE) tissues:

Quantity: ……

Batch N°: ……

Expiration date: ……

Maintenance culture medium:

Quantity: ……

Batch N°: ……

Expiration date: ……..

Check integrity of RHE tissues and medium:

Yes □ No □

Remarks:

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……..

ANNEX 3 - 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 ; W = Waxy

ID:……. Date: ……. Signature: …….

QC ID: ……. Date: ……. Signature:…….

ANNEX 4 - 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 5 - METHODS DOCUMENTATION SHEET: FREEZE-KILLED TISSUES FOR MTT- INTERACTING TEST SUBSTANCES

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

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

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……..

ANNEX 6 - METHODS DOCUMENTATION SHEET: ADDITIONAL CONTROL FOR COLOURING TEST SUBSTANCES

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

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

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……..

ANNEX 7 - METHODS DOCUMENTATION SHEET: PRE-INCUBATION, APPLICATION, AND RINSING TIMING

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

Pre-incubation timing: Date: ………………..

Start: ……………….. End: ………………..

Application: timing: Date: ………………..

Exposure:…………..		**Neg C**	**C 1**	**C 2**	**C 3**	**C 4**	**C 5**	**C 6**	**Pos C**
Application	Tissue 1								
	Tissue 2								
Rinsing	Tissue 1								
	Tissue 2								

Exposure:…………..		**Neg C**	**C 1**	**C 2**	**C 3**	**C 4**	**C 5**	**C 6**	**Pos C**
Application	Tissue 1								
	Tissue 2								
Rinsing	Tissue 1								
	Tissue 2								

Exposure:…………..		**Neg C**	**C 1**	**C 2**	**C 3**	**C 4**	**C 5**	**C 6**	**Pos C**
Application	Tissue 1								
	Tissue 2								
Rinsing	Tissue 1								
	Tissue 2								

ID:………………….. Date: ……………….. Signature: ………………..

QC ID: ……………. Date: ……………….. Signature:………………..

ANNEX 8 - METHODS DOCUMENTATION SHEET: SOLUTIONS PREPARATION

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

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

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

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 10 - METHODS DOCUMENTATION SHEET: MTT AND ISOPROPANOL EXTRACTION TIMING

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

Time	3 min	1 h
MTT incubation		
Isopropanol extraction		
OD measurement		

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……

ANNEX 11 - METHODS DOCUMENTATION SHEET: OBSERVATIONS AND COMMENTS AFTER 3 HOURS MTT INCUBATION

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

3 min exposure time

Test substance Name or code	Tissue 1	Tissue 2
	○	○
	○	○
	○	○
	○	○
	○	○
	○	○

1h exposure time

Test substance Name or code	Tissue 1	Tissue 2
	○	○
	○	○
	○	○
	○	○
	○	○
	○	○

ID:..... Date: ..... Signature: .....

QC ID: ..... Date: ..... Signature:.....

ANNEX 12 - METHODS DOCUMENTATION SHEET: REMARKS AND COMMENTS – PROTOCOL MODIFICATIONS IF NECESSARY

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

ID:…….. Date: …….. Signature: ……..

QC ID: …….. Date: …….. Signature:……