SKIN IRRITATION TEST FOR THE PREDICTION OF ACUTE SKIN IRRITATION OF CHEMICALS WITH STERLAB'S RHE: 42 MINUTES APPLICATION + 42 HOURS POST-INCUBATION

Standard Operating Procedure (SOP)

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1. Protocol Introduction

Sterlab Reconstructed Human Epidermis (RHE) are used for the classification of chemicals concerning their skin irritating properties by measurement of its cytotoxic effect, as reflected in the MTT assay.

OBJECTIVES & APPLICATIONS

Type of Testing:
Replacement

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

Purpose of Testing:
Prediction of skin irritation potential

Context of Use:
In vitro skin irritation tests are regulatory accepted.
After the update of the ECVAM performance standards in 2009 the OECD guideline for the testing of chemicals, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method 439" was adopted.

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

BASIS OF THE METHOD

Dermal irritation is induced by chemicals which penetrate the stratum corneum and lead to damages and cell loss of the underlying cells layers.
The relative viability of the treated tissues is measured at the end of the treatment exposure (42 minutes) followed by a post-exposure period (42 hours) using a cell viability test.
A cutoff value of 50% viability of the negative control value was used to classify test substances as irritant **(I)** or non classified as irritant **(NI).**

ABBREVIATIONS & DEFINITIONS

I: Irritant
ID: identity
MDS: Methods Documentation Sheet
MTT: 3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NC: Negative Control
NI: Non Irritant
OD: Optical Density
PBS: Phosphate Buffer Saline
PC: Positive Control
QC ID: Quality Control Identity
RHE: Reconstructed Human Epidermis
RT: Room Temperature
SDS: Sodium Dodecyl Sulfate
Triton X100: Triton

EXPERIMENTAL DESCRIPTION

Endpoint & Endpoint detection:
Cell viability is measured by dehydrogenase conversion of MTT, present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues (Mosmann T., 1983). The reduction of viability of tissues exposed to chemicals in comparison to negative controls is used to predict skin irritation potency.

Endpoint Value:
The quantitative cell viability is determined as a percentage of the negative control.

Test System:
The Sterlab RHE consists of normal human-derived keratinocytes cultured for 17 days at the air-liquid interface. The RHE model is cultured using a chemically defined growth medium. On day 17, a highly differentiated and stratified epidermis model is obtained comprising the basal, spinous and granular layers, and a multilayered stratum corneum. The RHE model presents a histological morphology comparable to the in vivo human tissue.
A generic description of general and functional conditions that skin models need to comply with can be found in the OECD Test Guideline 439.
The Sterlab RHE are cultured on inserts 0.5 cm^2 polycarbonate filters and shipped world-wide on agarose together with Sterlab Maintenance Medium.
The quality system of the Sterlab Laboratories is ISO 9001:2008. Each batch production is provided with quality controls values and recommendations such as storage conditions, RHE instructions for use, batch number and origin, histology, cell viability (MTT OD > 0.8), barrier function integrity performed at day 17 ($4.0 \leq ET_{50}$ (hr) ≤ 9.0), absence of bacteria, fungi, HIV, and Hepatitis B, C.

Basic Procedure:

On day of receipt, Sterlab RHE are conditioned by incubation at 37°C for release of transport-stress related compounds and debris at least 2 hours or overnight.

Each test substance (test chemicals, negative and positive controls) is topically applied concurrently on three tissues replicates for 42 minutes at room temperature (RT comprised between 18°C to 24°C). Exposure to the test substance is followed by rinsing with phosphate buffer saline (PBS) and mechanically dried. RHE are then transferred to fresh medium and incubated at 37°C for 42 additional hours. Cell viability is assessed by incubating the tissues for 3 hours with 0.3 mL MTT solution (0.5 mg/mL). The formazan crystals are extracted using 1.5 mL isopropanol for 2 hours at RT and quantified by spectrophotometry at 550 ± 30 nm wavelength.

Sodium Dodecyl Sulphate (SDS 5%), and PBS are used as positive and negative controls, respectively. For each treated tissue, the cell viability is expressed as the percentage of the mean negative control tissues.

Data analysis

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

***In vitro* results**	***In vivo* classification**
Mean tissue cell viability >50%	No Category (No Cat.)
Mean tissue cell viability ≤50%	Category 2 (Cat. 2)

Test compounds and Result summary

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

The blind catch up validation study was performed under GLP like conditions in the three participating laboratories, good reproducibility was achieved in these three laboratories.

Correct predictions of the skin potential for those twenty test substances were assessed with 100 % sensitivity, 70 % specificity (MTT only) and 85 % accuracy (see Table).

Laboratory	**Specificity [%]**	**Sensitivity [%]**
	All runs	**All runs**
Lab 1	70	100
Lab 2	70	100
Lab 3	70	100

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

METHOD

Modification of the method

Non

Discussion

In contrast to the use of laboratory animals or excised human skin this method offers a high reproducibility due to standardized materials and processes during the production. Furthermore the Sterlab RHE is based on human cells to predict effects on humans and serves as a complete replacement of the *in vivo* acute skin irritation test in rabbits.

Known laboratory use

Sterlab RHE is used in industry and academia for research, efficacy and toxicology testing.

Proprietary and confidential issues

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

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

2. Technical Description

Version 1.0, June 2014

STERLAB RHE IN OECD TEST GUIDELINE 439

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CELL / TEST SYSTEM

Health and Safety issues

Sterlab tissues are manufactured using epidermal cells taken from healthy volunteer donors negative to anti-HIV-1 and 2, to hepatitis C antibodies, to hepatitis B antigens.

Nevertheless, normal handling procedures for biological materials should be followed:

(a) Wear gloves during handling with the skin and kit components;
(b) After use, the epidermis, the material and all media in contact with it, should be decontaminated (e.g. using a 10% solution of bleach or appropriate containers).
(c) Examine all kit components for integrity.

Please contact Sterlab Laboratories, if you have any questions or concerns.

Test system description:

The three-dimensional Reconstructed Human Epidermis (RHE) model, commercialized by Sterlab (Vallauris, France) consists of normal human keratinocytes cultured for 17-days on an insert 0.5 cm^2 polycarbonate filter at the air-liquid interface. The RHE model is cultured using a chemically defined growth medium.

Quality control:

The RHE models are manufactured according to defined ISO9001v2008 quality assurance procedures. All the RHE models are free of viruses, bacteria and mycoplasma. The quality of the final RHE product is assessed by a MTT cytotoxicity test of untreated tissues, barrier function integrity with Triton X100 1% ($4.0 \leq ET_{50}$ (hr) ≤ 9.0) and by histological examination.

TISSUES AND MEDIA

RHE set and media are provided by Sterlab.

The Reconstructed Human Epidermis (205EPID05) and the necessary culture media (Maintenance medium MILM, and Growth medium MILC) are usually shipped on Monday. They are received one or two day(s) following the shipment (for Europe). Results of the quality controls are supplied with the sets. A batch production is delivered only if quality controls criteria correspond to a normal histology (absence of significative alterations), cell viability (MTT OD > 0.8), barrier function integrity with Triton X100 1% ($4.0 \leq ET_{50}$ (hr) ≤ 9.0) and absence of bacteria, fungi, HIV, and Hepatitis B, C.

Expiration and storage

Reference	Description	Storage condition	Shelf life
205EPID05	RHE, small size (0.5 cm^2), day 17	37 ± 0.5°C / 5% CO_2	10 days
205MILM125	Maintenance medium	2 - 8°C	10 days
205MILC125	Growth medium	2 - 8°C	10 days

Receipt of materials supplied by Sterlab

Examine all kit components for integrity. If there is a concern call Sterlab.

Document MDS: RHE set - Sterlab materials receipt (**Annex 1**).

EQUIPMENT

Consumables:

Extra 6-well plate - sterile	*To transfer tissue inserts to fresh medium*
24-well plate - sterile	*For the 42 min application + MTT incubation + formazan extraction steps*
96-well plate - sterile	*For reading formazan extract*
Sterile absorbent paper / sterile gauze	*To remove agarose fragments or to dry inserts*
Circular nylon meshes Ø = 7.5mm	*Use as a spreading aid for liquid test materials*
Adhesive tape or Parafilm M	*Covering plates during formazan extraction*
Tips	*For adjustable pipette*

Fixed Equipment:

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 prior to and during assays*
Laboratory balance (accuracy 0.1mg)	*For pipette verification and test substance weighing*
96-well plate spectrophotometer (550 ± 30 nm)	*For reading OD*
Plate shaker	*For extraction of formazan*
Timers	*To be used during application of test materials*
Sterile forceps	*For handling tissue inserts*
Plastic wash bottles	*For collecting PBS rinses*
1 L beaker	*For rinsing tissues with PBS*
Small glass weight boats	*For weighing powders*
1 gauged flask	*For SDS 5% solution preparation*
Mortar and Pestle	*For grinding granular solids*
Adjustable Pipette / multi-step Pipette	*For pipetting 1 mL assay medium*
Adjustable Pipette / multi-step	*Pipette For pipetting 300 µL MTT / medium*
Adjustable Pipette	*For pipetting 750 µL propan-2-ol*
Adjustable Pipette	*For pipetting 200 µL formazan extract from 24-well plate into 96-well plate*
Positive displacement pipette for 30µL	*For application of liquid and viscous test materials*
Multi-pipette + adapter for 25mL tip	*For washing*
Vacuum source/trap	*For aspirating media and solutions*

Equipment verification

It is strongly recommended to use regularly verified equipment. Maximum time interval between two verifications is specified for each apparatus necessary in this protocol in Methods Documentation Sheet, MDS: Main equipment verification (**Annex 2**).

If the last verification date does not fit the requested specifications, proceed to verification before testing and record it in MDS: Main equipment verification and MDS: Detailed equipment verification (**Annexes 2 and 3**).

OTHER MEDIA AND REAGENTS

Dulbeccos' PBS without Ca^{2+} and Mg^{2+} (Biowest Absyc, L0615)	*Use as negative control, MTT diluent, and for rinsing tissues*
MTT – Thiazolyl Blue Tetrazolium Bromide (Sigma, M5655, cell culture tested, purity min. 97.5%)	*For the MTT assay*
5 % (aq) SDS [CAS N°151-21-3] (Sigma, L4509, purity min. 98.5%)	*To be used as positive control*
Sterile H_2O (distilled or aqua pure)	*For powder applications*
Isopropanol [CAS N°67-63-0] (Sigma, 190764)	*For extraction of the formazan crystals*

MTT solution preparation

Safety precautions
▪ MTT (R26 R22 R36 R37 R38) - MTT solution is light sensitive. Protect it from light using silver paper or appropriate material.
▪ Isopropanol (R11 R36 R67 S7 S16 S24/25 S26)

Work in ventilated cabinets: to prevent accidental contact wear protective gloves, and if necessary a mask and/or safety glasses.

1) MTT stock solution preparation

Dissolve MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich M-5655) to a final concentration of 5 mg/mL in PBS. Always protect the solution from light. The solution is filtered with 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.
Document MDS: MTT stock solution (**Annex 4**).

2) MTT ready to use solution preparation (day of testing)

On day of testing, thaw the MTT stock solution (5 mg/mL) and dilute it with pre-warmed maintenance medium at room temperature up to 0.5 mg/mL.

TEST SUBSTANCES

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

Test substances identification

Main information concerning the test substances (name or code, total weight, reception date, expiration date, physical consistence, stocking conditions) should be registered in MDS: Test substances (**Annex 5**).

Negative control solution

PBS is used as negative control.

Document MDS: Solutions preparation (**Annex 4**).

Positive control solution

Prepare extemporaneously SDS 5% solution (Positive Control). Storage: 1 month at 2 - 8C°.

Document MDS: Solutions preparation (**Annex 4**).

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

3. METHOD

Before starting the 3 independant runs, verifications should be done with the 20 chemicals (ideally the week before the study).

First the coloration of each substance has to be identified : document MDS : Test substance identification (**Annex 5**). Thus an additional condition will be performed with the coloring test substances (named condition 2 in the Flowchart page 12)

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

3.1 Determination of the non specific reduction of MTT by test substance

3.1.1 Check-method for possible direct MTT reduction with test substances

1. Fill wells of 24-well plate with 300µL of MTT ready to use solution (0.5 mg/mL)
2. Add 16µL or 16 mg of the test substance or water for control. Mix.
3. Protect from light and incubate the mixture for 3 hours ± 5 min at 37 ± 0.5°C.
4. Proceed to visual scoring of MTT interaction as follow:
    - Negative control (water): yellow
    - Test substances which do not interact with MTT: yellow
    - Test substances interacting slightly with MTT: light blue
    - Test substances interacting strongly with MTT: dark blue
5. Document MDS: Test substance identification (**Annex 5**).

If the MTT solution color becomes blue or purple, the test substance interacts with the MTT.
It is then necessary to evaluate the part of optical density (OD) due to the non-specific reduction of the MTT (i.e. by using killed epidermis).
For each MTT-interacting test substance previously detected, and in addition to the normal procedure, 3 killed test substance treated tissues are used for the MTT evaluation following the same protocol as for living tissues (named condition 3 in the flowchart page 12). Three killed untreated tissues are used as negative controls (untreated killed tissues may exhibit little residual NADH and dehydrogenase associated activity).

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

1. Transfer living epidermis in 24-well plate and place them at -20°C or -80°C for at least 24 hours before test (3 tissues / MTT-interacting test substance).
2. Thaw killed tissues, before use, on 300µL of maintenance medium for 1 hour ± 5 min at room temperature.
3. Further use of killed tissues is similar to living tissues (see 3.3).
4. Document MDS: Killed tissues for MTT-interacting test substances (**Annex 6**).

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

CONDITION 1	**CONDITION 2**	**CONDITION 3**
All test substances (standard, MTT interacting, coloring test substances)	Coloring test substances	MTT-interacting test substances
Use of living RHE tissues + PBS Negative control tissues + SDS Positive control tissues	Use of living RHE tissues	Use of killed RHE tissues + Untreated Negative control killed tissues
42 min exposure + 42 hours post-incubation		
3 hours MTT incubation	3 hours Maintenance Medium incubation	3 hours MTT incubation
2 hours isopropanol extraction		
OD = Specific OD + Non-Specific OD	OD = Non-Specific Staining (NSS)	OD = Non-Specific MTT reduction (NSMTT)

3.3 Routine Protocol

3.3.1 Pre-incubation step

This step should be conducted under sterile conditions.

Before starting pre-incubation step, the maintenance and growth media should be pre-warmed only at room temperature (and not at 37°C).

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

1. Fill an appropriate numbers of 6-well plates with 1 mL growth culture medium (MILC).
2. Remove the adhesive tape from the agarose plate containing epidermal tissues and open the 24-well plates. See Picture 1.
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.
4. Check visually that no agarose is remaining and transfer the tissue on fresh medium by first slopping the insert before complete insert setting. See Picture 2.
5. Place the RHE tissues at 37°C, 5% CO_2 until test substance application.

Pre-incubation step for tissues receipt at day 19 (Wednesday)

1. Proceed to pre-incubation step for at least 2 hours.
2. Fill an appropriate numbers of 24-well plates with 300 µL growth culture medium (MILC).
3. Remove the adhesive tape from the agarose plate containing epidermal tissues and open the 24-well plates. See Picture 1.
4. Use sterile forceps to take off tissues from the agarose, clean the bottom of the insert on sterile absorbent paper to remove eventual remaining agarose pieces.
5. Check visually that no agarose is remaining and transfer the tissue on fresh medium by first slopping the insert before complete insert setting at the air-liquid interface. See Picture 2.
6. Place tissues at 37°C, 5% CO_2 until test substance application.

	
Picture 1	Picture 2

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

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

3.3.2 Plates preparation

Application plates

1. Pre-warm the maintenance culture medium at room temperature.
2. Label a 24-well plate by condition: 3 wells per test substance (code number, 3 replicates), positive control (PC), negative control (NC), substances for killed tissues (code number, 3 replicates) and coloring test substances (code number, 3 replicates) respectively.
3. Fill the 3 wells with 300 µL pre-warmed maintenance culture medium.
4. Use sterile forceps to transfer tissues by first slopping the insert before complete insert setting at the air-liquid interface.

Post-incubation plates

1. Pre-warm the growth culture medium at room temperature.
2. Label a 6-well plate by condition: 3 wells per test substance (code number, 3 replicates), positive control (PC), negative control (NC), substances for killed tissues (code number, 3 replicates) and coloring test substance (code number, 3 replicates) respectively.

Fill the 3 wells with 2 mL pre-warmed growth culture medium.

3.3.3 Topical applications: 42 minutes treatment

It is strongly recommended to perform this step under sterile conditions.
Three tissues per test substance should be used (3 replicates). The application order is important since it will be the same for washing.
Note: Keep 1 minute interval between each tissue application. We recommend keeping some minutes without test substance application just before washing in order to be ready in time for this latter.
Record the exact timings and document the correspondent MDS: Application timing (**Annex 9**).
Due to the application timing of 42 minutes, the application and rinsing phases should be performed in minimum two steps for testing the internal controls (NC and PC), the 20 test substances, the coloring test substances and the substances for killed tissues.

Liquid and viscous test substances

1. Dispense 16 µL ± 0.5 µL (i.e. 32 µL/cm²) of the undiluted test substance on the top of each epidermis tissue (living tissue and killed tissue if necessary): 3 per test substance, using

positive displacement pipette. Use the tip to spread the test substance gently on the epidermis topical surface. See picture 3.

2. Carefully apply a nylon mesh (Ø = 7mm) on the whole surface with forceps.
   See pictures 4 and 5.
3. Document MDS: Killed tissues for interacting test substances (**annex 6**), MDS: Additional coloring test substances (**annex 7**) and MDS: weighing of solid and sticky test substances (**annex 8**)

Picture 3

Picture 4

Picture 5

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 10µL of distilled water using a positive displacement pipette to the epidermal surface in order to improve further contact between the powder and the epidermis.
3. Use special glass weigh boats to apply 16 mg ± 2 mg (i.e. 32 mg/cm²) of the powder to the epidermis surface. If necessary, spread it on the epidermal surface.
4. Document MDS: Killed tissues for interacting test substances (**annex 6**), MDS: Additional coloring test substances (**annex 7**) and MDS: weighing of solid and sticky test substances (**annex 8**)

See Pictures 6 and 7.

	
Picture 6	Picture 7

Sticky test substances

1. Allow for the tare with nylon mesh and directly weigh 16 mg ± 2 mg (i.e. 32 mg/cm²) and spread sticky test substance on this latter.
2. Apply the test substance coated side of the nylon mesh on the epidermal surface and spread it gently on the whole surface.
3. Document MDS: Killed tissues for interacting test substances (**annex 6**), MDS: Additional coloring test substances (**annex 7**) and MDS: weighing of solid and sticky test substances (**annex 8**)

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

Keep the plate (lids on) containing the treated RHE tissues for 42 minutes exposure (± 1 min) in the ventilated cabinet sterile conditions at room temperature.

3.3.4 Rinsing and drying steps

It is strongly recommended to work in laminar flow hood to prevent contamination.
End of the treatment and removal of the test substance after 42 minutes (± 1 min) exposure at room temperature.
Strictly respect the application order (time based recorded in **Annex 9**).
In order to prevent pollution the lids should be put on the plates continuously during the rinsing and drying steps. We recommend also to put a lid on the sterile PBS container.

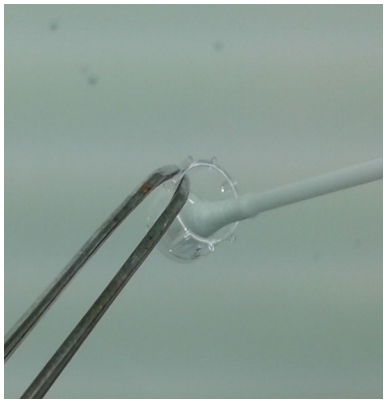
Liquid, viscous, sticky and solid test substances

1. Fill a multi-pipette (adjusted for a 1 mL distribution) with 20 mL sterile PBS
2. Open the 24-well plate.
3. Remove the nylon mesh with fine forceps from the epidermal surface of a treated tissue.
4. Take the treated tissue with sterile forceps and close the 24-well plate (to protect the other tissues from washing solution projections).
   See Picture 8.

Picture 8

Rinsing step for treated tissues

1. Place a funnel in a large beaker (to avoid underneath projections/contaminations of the RHE tissues).
2. Maintain the insert over the large funnel and rinse thoroughly 20 times with 1mL PBS at a 5-8 cm distance from the insert to remove all residual test substance from the epidermal surface.
3. After the last rinsing, empty the insert at the most (for example, knock the forceps on the beaker, insert turned upside down).
4. Dry the insert bottom on a sterile absorbent paper or gauze for 1-2 seconds.
5. Sweep gently the surface of the stratum corneum with both ends of a cotton tip (5-6 turns per end). See Picture 9.
6. Transfer the washed tissue on 2 mL growth culture medium (6-well plate designed as post-incubation plates, see 3.3.2) by first sloping the insert before complete insert setting.
7. Document MDS: Rinsing timing (**Annex 9**).



Picture 9

3.3.5 Post treatment incubation: 42 hours

Incubate the treated, rinsed and dried epidermis tissues at 37°C, 5% CO_2, 95% humidified atmosphere for 42 hours (± 60 min) in the post-incubation plates pre-warm with growth culture medium.
Incubation start time corresponds to last tissue rinsing time of each set.
See MDS: post-incubation timing (**Annex 9**).

3.3.6 MTT test

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

Incubation in MTT solution

1. Prepare MTT ready to use solution according to page 9.
2. Label an appropriate numbers of 24-well plates
3. Fill the 24-well plates with 300 µL MTT from light by wrapping in aluminum paper until tissues transfer in MTT. Sweep excess culture medium on the unit bottom of the tissue with absorbent paper.
4. Transfer the treated tissues in the pre-filled MTT 24-well plates, by first slopping the insert before complete insert setting at the air-liquid interface. Respect the application order.
5. Incubate for 3 hours (+/- 5 min) at 37°C, 5% CO_2, 95% humidified atmosphere.
6. Document MDS: MTT incubation (**Annex 9**).

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 numbers of 24-well plates.
2. Fill the 24-well plates with 300 µL Maintenance Medium.
3. At the end of the 42 hours post-incubation period, sweep excess culture medium on the unit bottom of the tissue with absorbent paper and transfer dye test substance control tissues in the pre-filled 24-well plates, by first sloping the insert before complete insert setting at the air-liquid interface.
4. Check the absence of air bubbles.
5. Incubate for 3 hours ± 5min at 37°C, 5% CO_2, 95% humidified atmosphere.
6. Document MDS: Start of 3 hours incubation time (**Annex 9**).

Formazan extraction

7. Label an appropriate numbers of new 24-well plates similarly to those labeled for the previous step.
8. Fill the plate(s) with 800 µL isopropanol (undiluted) at the end of the 3 hours ± 5 min incubation in MTT solution (**Annex 9**). Use forceps to transfer treated tissues.
9. Dry the insert bottom of the treated tissue on absorbent paper.
10. Transfer the tissues in isopropanol solution.
11. Add 700 µL isopropanol solution on the top of each tissue.
12. Ensure that tissue is completely covered by the isopropanol solution.
13. Consciously protect plate(s) from evaporation by stretching Parafilm over the plate and adding the lid on the plate.
14. Incubate for 2 hours ± 5 min at room temperature for formazan extraction.
15. Document MDS: Start of isopropanol extraction time (**Annex 9**).

Absorbance / optical density measurements

16. At the end of the formazan extraction incubation time, open the plate.
17. Remove the Parafilm.
18. Document in MDS: OD reading (**Annex 9**).
19. Isopropanol solution is used as blank (6 replicates)
20. Maintain the insert with forceps.
21. Pierce tissue and polycarbonate filter with a tip in order to get the whole extraction solution in the corresponding well.
22. Homogenize the extraction solution by pipetting 3 times up and down to complete formazan crystals solubilization.
23. Transfer 3 x 200 µL extraction solution per well into a 96-well labeled plate.
24. Read the Optical Densities (OD) using a 96-well plate spectrophotometer: the concentration of formazan is measured by determining the OD at 550 ± 30 nm.
25. All data generated by the 96-well plate spectrophotometer should be printed after each reading and considered as raw data.
26. Identify ODs with conditions and tissues (replicate) studied on the raw data documents.
27. Perform the Quality Control of the raw data.

4. ACCEPTANCE CRITERIA

Negative control (NC) acceptance criteria: The NC data meet the acceptance criteria if the mean OD value of the 3 tissues is ≥ 0.8 at 550 ± 30 nm according to the historical database.

The **Standard Deviation** value is considered as valid if it is ≤ 18%, according to the Performance Standards (ECVAM SIVS, 2007). The SD value is calculated over replicates for a given run.

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

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

Test substance data acceptance criteria: The inter-batch mean viability is calculated from the three independent assays or runs using intra-batch tissue mean (3 replicates / tissue and 3 tissues per run). Standard deviation of each intra-batch mean should not be > 18%. For a given test substance, if one batch predicts a different class of irritation, the test substance must be retested in one additional batch (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.

5. DATA ANALYSIS / CALCULATION OF RESULTS

5.1 Data Calculation Step

Blank

- Calculate the OD mean from the 6 replicates for each plate: **OD_{blank}**
  Use the blank value to calculate corrected value for substances contained in the same 96-well plate

Negative PBS-treated controls

- Calculate the OD mean per tissue (3 replicates) **OD_{NC}**
- The mean OD for all tissues corresponds to 100% viability = **mean OD_{NC}**

Positive SDS-treated control

- Calculate the OD mean per tissue (3 replicates) **OD_{PC}**
- Calculate the viability per tissue **%PC = [OD_{PC} / mean OD_{NC}] x 100**

Tested compound

- Calculate the OD mean per tissue (3 replicates) **OD_{TT}**
- Calculate the viability per tissue **%TT = [OD_{TT} / mean OD_{NC}] x 100**

For each test substance (including PC) mean viability and standard deviations are calculated.

5.2 Data calculations for MTT-interacting substances

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

Non-specific MTT reduction calculation (NSMTT)

- OD_{KU}: untreated killed tissues OD
- OD_{KT}: test substance treated killed tissues OD
- NSMTT = [(OD_{KT}- OD_{KU}) / mean OD_{NC}] x 100

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

Treated tissue True MTT metabolic conversion (TODTT)

- OD_{TT}: test substance treated viable tissues
- TOD_{TT} = [OD_{TT} – (OD_{KT} – OD_{KU})]
- Relative viability % = [TOD_{TT} / mean OD_{NC}] x 100

5.3 Data calculations for coloring test substances able to stain tissues

For chemicals detected as able to color the tissues, it is necessary to evaluate the non-specific OD due to the residual chemical staining (unrelated to any mitochondrial activity) and to subtract it before calculations of the “true” viability %.

Non-specific Staining % (NSS %)

- OD_{CT}: coloring test substance treated tissue (incubated in Maintenance Medium before isopropanol extraction)
- OD_{PBS}: control PBS treated tissue (incubated in Maintenance Medium before isopropanol extraction)
- **NSS%= [OD_{CT} / OD_{NC}] x 100**

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

True MTT metabolic conversion (TOD_{CT})

- OD_{CT}: coloring test substance-treated tissues (MTT incubation)
- TOD_{CT}: true MTT metabolic conversion for coloring test substance treated tissue.
- TOD_{CT} = OD_{TT} – OD_{CT}
- Relative viability % = [TOD_{CT} / mean OD_{NC}] x 100

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

Calculate corresponding NSMTT and NSS.

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

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

- OD_{CT}: coloring test substance-treated tissues (MTT incubation)
- TOD_{CT}: true MTT metabolic conversion for coloring test substance treated tissue.
- $TOD_{CTT} = [OD_{TT} – (TOD_{CT} + TOD_{TT})]$
- Relative viability % = $[TOD_{CTT} / OD_{NC}] \times 100$

6. PREDICTION MODEL

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

***In vitro* results**	***In vivo* classification**
Mean tissue cell viability >50%	No Category (No Cat.)
Mean tissue cell viability ≤50%	Category 2 (Cat. 2)

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

7. ANNEXES

Annex 1 - Methods Documentation Sheet:

RHE SET - STERLAB MATERIALS RECEIPT

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

STERLAB set receipt date: ……

	Quantity	**Batch N°**	**Expiration date**
Sterlab RHE	……	……	……
Maintenance culture medium	……	……	……
Growth culture medium	……	……	……

Remarks: ……..

……..

……..

ID: …….. Date: …….. Signature: ……

QC ID: …….. Date: …….. Signature: ……

Annex 2 - Methods Documentation Sheet:

EQUIPMENT VERIFICATION

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

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

ID: …….. Date: …….. Signature: ……

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

Annex 3 - Methods Documentation Sheet:

DETAILED EQUIPMENT VERIFICATION

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

Identification

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

Incubator verification

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

ID: …….. Date: …….. Signature: ……

Balance verification

N°: ……..	10 mg weight	1 g weight
Weighing 1	…….. mg	…….. g
Weighing 2	…….. mg	…….. g
Weighing 3	…….. mg	…….. g
Mean	…….. mg	…….. g
Tolerance	10 ± 0.1 mg	1000 ± 0.5 mg

ID: …….. Date: …….. Signature: ……

Pipettes verification

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

ID: …….. Date: …….. Signature: ……

QC ID: …….. Date: …….. Signature: ……

Annex 4 - Methods Documentation Sheet:

SOLUTIONS PREPARATION

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

Positive control: SDS 5% solution in distilled water (w/v)

	Reference	**Batch N°:**	**Expiration date**	**Weight**	**Vehicle volume**
SDS solution in distilled water	……	……	……	……	……
MTT solution in PBS	……	……	……	……	……

ID: …….. Date: …….. Signature: ……

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

Annex 5 - Methods Documentation Sheet:

TEST SUBSTANCES IDENTIFICATION

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

MTT interaction

Start of incubation time : …… End of incubation time : ……

Test substance Name or code	Storage Temperature	Physical Consistence*	Colour	MTT Interaction Blue color Yes / No	Remarks

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

ID: …….. Date: …….. Signature: ……

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

Annex 6 - Methods Documentation Sheet:

KILLED TISSUES FOR MTT-INTERACTING TEST SUBSTANCES

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

Test substance Name or Code		N°……..	N°……..	N°……..	N°……..	N°……..	N°……..	N°……..
Solids Weight before application (mg) W1	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weight (mg) W2	Tissue 1							
	Tissue 2							
	Tissue 3							
Solids weight after application (mg) W3	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weight applied (mg) W=(W1+W2)-W3	Tissue 1							
	Tissue 2							
	Tissue 3							
Liquids volume (µL)								
Test Protocol								
Start of incubation time	Tissue 1							
	Tissue 2							
	Tissue 3							
End of incubation time	Tissue 1							
	Tissue 2							
	Tissue 3							

ID: …….. Date: …….. Signature: ……

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

Annex 7 - Methods Documentation Sheet:

ADDITIONAL CONTROL FOR COLORING TEST SUBSTANCES

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

Test substance Name or Code		N°……..	N°……..	N°……..	N°……..	N°……..	N°……..	N°……..
Solids Weight before application (mg) W1	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weight (mg) W2	Tissue 1							
	Tissue 2							
	Tissue 3							
Solids weight after application (mg) W3	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weight applied (mg) W=(W1+W2)-W3	Tissue 1							
	Tissue 2							
	Tissue 3							
Liquids volume (µL)								
Test Protocol								
Start of incubation time	Tissue 1							
	Tissue 2							
	Tissue 3							
End of incubation time	Tissue 1							
	Tissue 2							
	Tissue 3							

ID: …….. Date: …….. Signature: ……

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

Annex 8 - Methods Documentation Sheet:

WEIGHING OF SOLID AND STICKY TEST SUBSTANCES

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

Code substance		N°……..	N°……..	N°……..	N°……..	N°……..	N°……..	N°……..
Solids weigh before aplication (mg) **W1**	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weigh (mg) **W2**	Tissue 1							
	Tissue 2							
	Tissue 3							
Solids weigh after application (mg) **W3**	Tissue 1							
	Tissue 2							
	Tissue 3							
Test substance weigh applied (mg) **W4=(W1+W2)-W3**	Tissue 1							
	Tissue 2							
	Tissue 3							

ID: …….. Date: …….. Signature: ……

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

Annex 9 - Methods Documentation Sheet:

TIME PROTOCOLE

Note: Keep 1 minute interval between each tissue application. We recommend keeping some minutes without test substance application just before washing in order to be ready in time for this latter

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

Step	**Date**	**Set 1**		**Set 2**		**Remarks**
		Start	**Stop**	**Start**	**Stop**	
Pre-incubation						
Application (42min ± 1min)						
Rinsing						
Post-incubation (42h ± 1h)						
MTT test (3h ± 5min)						
Extration (2h ± 10min)						
OD reading (570 ± 30nm)						

ID: …….. Date: …….. Signature: ……

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

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