DB-ALM Protocol n° 131 : EpiSkin™ Skin Irritation Test15min - 42 hours

Skin Irritation and Corrosivity

The irritation potential of a chemical is predicted through its cytotoxic effect on the Reconstructed Human *Epidermis* (RHE) model EpiSkin™, which is measured by the MTT cell viability assay. The test is compliant with the OECD Test Guideline No. 439 - In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method.

Objective & Application

TYPE OF TESTING	:	Replacement, screening
LEVEL OF ASSESSMENT	:	Toxic potential, hazard identification
PURPOSE OF TESTING	:	Classification and labelling

Context of Use:
The test method is based on the Reconstructed Human *Epidermis* (RHE) model, which was granted regulatory approval as a full replacement for the rabbit *in vivo* skin irritation test under the EC Test Method Regulation (Method B.46, EU, 2009) and under conditions laid down in OECD Test Guideline 439 (OECD, 2010).The test method can be used for the hazard identification of irritant chemical substances and mixtures in accordance with the **United Nations Globally Harmonized System of Classification and Labelling of Chemicals** (UN GHS, UN, 2009) and **European Commission Regulation on the classification, labelling and packaging of substances and mixtures** (CLP, EC No.1272/2008, EU, 2008). Positive results obtained with the test method enable the classification of test substances as "Category 2: Irritant". If the regulatory authorities do not require the optional UN GHS classification of "Category 3: Mild irritant", remaining test substances are labelled as “No Category” (Method B.46, EU 2009; OECD TG 439, 2010).The test method can be used within the sequential dermal irritation/corrosion testing strategy for the full characterization of skin irritation potential (OECD TG 404, 2002; Method B.4, EC No. 440/2008, EU 2008).The EpiSkin ™ SKIN IRRITATION TEST 15min - 42 hours was the first protocol based on the Reconstructed Human *Epidermis* (RHE) to be validated and is therefore designated as Validated Reference Method (VRM) in OECD TG439.

Applicability Domain:

- Solids (soluble or insoluble in water)
- Liquids (aqueous or non-aqueous)
- Semi-solids
- Waxes

The method is not designed for testing of gases and aerosols or highly volatile test compounds.Highly coloured chemicals and/or MTT reducers may interfere with cell viability measurements and therefore adapted controls for corrections need to be used.The Validated Reference Method EpiSkin ™ SKIN IRRITATION TEST 15min - 42 hours represents a performance standard for the predictive capacity of other RHE-based methods. The performance of any novel method must be comparable or better than that of VRM (EpiSkin™) against a defined test set of 20 reference test chemicals (OECD TG 439, 2010).

Résumé

Skin irritation refers to the reversible damage to the skin following the application of a chemical for up to 4 hours, as defined by the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS, UN, 2009). The potential to induce skin irritation is an important consideration for procedures for the safe handling, packing and transports of chemicals.

Skin irritation *in vivo* is determined by a modification of the original Draize rabbit skin irritation test (Draize *et al.*, 1944) as described in the OECD TG 404 (OECD, 2002).

In 1998 an ECVAM workshop made an inventory of the various methods for *in vitro* assessment of the irritant potential of chemicals (van de Sandt *et al.*, 1999) and a scientific validation of related methods was undertaken (Botham *et al.* 1998). During the pre-validation steps, several methods and subsequent refinements were proposed (Zuang *et al.*, 2002; Portes *et al.*, 2002; Cotovio *et al.*, 2005) and finally three methods were accepted for further validation process. To facilitate future regulatory acceptance, a common Prediction Model and protocol, differing only by model-specific details, were agreed to be used with both EpiSkin ™ (EPISKIN SNC) and EpiDerm ™ (MatTeK Corp.).

The test consists of a topical exposure of the neat test chemical to a reconstructed human *epidermis* (RHE) model, followed by a cell viability test. Cell viability is measured by the mitochondrial dehydrogenase conversion of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a blue formazan salt that is quantitatively measured after extraction from tissues (Mossman, 1983). The reduction of the viability of tissues exposed to chemicals, in comparison to negative controls (treated with Dulbecco's D-PBS), is used to predict the skin irritation potential.

Experimental Description

Endpoint and Endpoint Measurement:

TISSUE VIABILITY: determined by a reduction in mitochondrial dehydrogenase activity, measured by formazan salt production from MTT. It is expressed as % of the negative control

Endpoint Value:

≤ 50% VIABILITY: ≤ 50% reduction in cell viability is a cut-off value for the presence of a significant biological effect

Experimental System(s):

RHE EpiSkin™: Small Model (EpiSkin™-SM, EPISKIN SNC Lyon, France) is a three-dimensional human *epidermis* model. Human-derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen. A highly differentiated and stratified *epidermis* model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum (Tinois *et al.*, 1994).

Basic Procedure

Test chemicals are applied topically to the epidermal model for 15 minutes at room temperature. Three *epidermis* units are used per each test chemical, positive and negative controls. Exposure to the test chemical is terminated by rinsing with phosphate buffered saline (PBS). *Epidermis* is then incubated at 37°C for 42 additional hours. For each test chemical, three independent tests with three different batches of EpiSkin™ are normally used. Aliquots of culture media may be collected and kept frozen (-20°C) for cytokine (IL-1alpha) measurements.

The cell viability is assessed by a subsequent incubation of the tissues for 3 hours with MTT solution in a 12 well plate. The precipitated formazan is then extracted using acidified isopropanol and quantified spectrophotometrically at 570 nm using 96 well plates.

Optional step: the protein levels of IL-1alpha in the supernatant may be determined and reported in pg/ml, as an additional information to the TG439's viability endpoint.

SDS 5% and PBS-treated *epidermis* are used as positive and negative controls respectively. For each treated tissue the viability is expressed as a % relative to negative control tissues (mean).

Data Analysis/Prediction Model

Irritation potential of a test chemical is determined according to the UN GHS and EU CLP as "Category 2: Irritant or “No Category” (UN, 2009; No. 1272/2008, EU, 2008). Category 2: Irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test chemical is reduced below 50% of the mean viability of the negative controls. The prediction model is defined as described below:

In vitro **results**	***In vivo*** **classification**
Mean tissue cell viability ≤ 50%	Category 2:Irritant
Mean tissue cell viability > 50%	No Category

Test Compounds and Results Summary

A total of 58 test compounds, consisting of 25 *in vivo* irritants and 33 *in vivo* non irritants, according to the European classification system based on the Dangerous Substance Directive (EU DSD, EU, 2001), have been tested in the ECVAM Skin Irritation Validation study (SIVS, 2003 - 2007). The results were interpreted on the basis of a fixed 15 min exposure-time and 42 hours post-exposure incubation time

(ESAC, Meeting 30, 2009), according to the EU DSD classification, and the UN GHS (UN, 2009) and its EU implementation (CLP, No. 1272/2008 , EU, 2008).

	EU DSD	UN GHS / EU CLP
	EpiSkin (MTT)*	EpiSkin (MTT)*
Sensitivity	72.0%	84.6%
Specificity	81.8%	71.1%
Accuracy	77.6%	74.1%

* Data from the "Explanatory Backgound Document to the OECD Draft Test Guideline on in vitro Skin Irritation Testing", 2009, available at:
/cm_esac/ESAC31_skin-irritation-statement_20090922.pdf

Acceptance Criteria and Proficiency Testing

A generic description of general and functional conditions that human skin models need to comply with, can be found in the OECD Test Guideline 431 *In vitro* Skin Corrosion: Human Skin Model test (OECD, 2004).

In OECD TG 439 (OECD, 2010), it is recommended, prior to a routine use of the test, to check the technical proficiency of the laboratory using ten Proficiency Chemicals listed in the Table below:

Chemical	**CAS NR**	***In vivo* score OECD TG 404**	**Physical state**	**UN GHS Category**
naphthalene acetic acid	86-87-3	0	Solid	No Cat.
isopropanol	67-63-0	0.3	Liquid	
methyl stearate	112-61-8	1	Solid	
heptyl butyrate	5870-93-9	1.7	Liquid	No Cat. (*Optional Cat. 3**)
hexyl salicylate	6259-76-3	2		
cyclamen aldehyde	103-95-7	2.3		Cat. 2
1-bromohexane	111-25-1	2.7		
potassium hydroxide (5% aq.)	1310-58-3	3		
1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	Solid	
heptanal	111-71-7	3.4	Liquid	

** Classification of a test chemical under the optional UN GHS Category 3: "Mild Irritant" is currently not possible with the EpiSkin ™ test and not required under EU CLP regulation (No. 1272/2008 EU, 2008).*

Discussion

The EpiSkin ™ test relies on the MTT reduction as a quantitative indicator of cell viability. The protocol includes several check methods developed to detect and compensate for the properties of a test chemical, which may interfere with the accuracy of the test. Some chemicals can directly reduce the MTT reagent, other chemicals can directly color the tissue or the cells. These properties of test chemicals can interfere only if sufficient amounts of the chemical are still present in (or onto) the tissue at the end of the 42 hours post-treatment incubation period. In these cases, a special procedure for the quantification of the genuine MTT mitochondrial reduction should be applied. The use of specific controls enables the quantification of the *bona fide* tissue viability. After subtracting the unspecific Optical Densitiy due to direct chemical MTT reduction and/or to chemical residual color extracted from the tissues, the remaining OD_{570nm} is used to calculate the cell viability %. Conditions for performing specific control steps are described in the Procedure Details section of this protocol.

Status

Participation in Validation Studies:

Upon review of existing information by the ECVAM Skin Irritation Task Force and an ECVAM Workshop both EpiSkin™ and EpiDerm™ skin irritation (SIT) tests were regarded as sufficiently promising predictors for skin irritancy potential and ready to enter the formal validation study (Portes *et al*., 2002 and Cotovio *et al.*, 2005).

Following the successful conclusion of the ECVAM Skin Irritation Validation Study (SIVS, 2003 – 2007), the ECVAM Scientific Advisory Committee (ESAC) endorsed the scientific validity of EpiSkin™ method as a stand-alone replacement for the Draize Skin Irritation test for the purpose of distinguishing between skin irritating and non-skin irritating substances (ESAC, 2007 and ESAC, Meeting 30, 2009).

Following the 26th meeting of the non-Commission members of the ECVAM Scientific Advisory Committee, performance standards for applying human skin models to *in vitro* skin irritation testing were defined based on the validated test EpiSkin™ method (ESAC, 2007). These performance standards were revised after implementation of UN GHS (ESAC, Meeting 31, 2009) and can be used to evaluate the accuracy and reliability of other analogous test methods (also referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

Regulatory Acceptance:

In July 2009, the reconstructed human *epidermis* test methods for skin irritation testing which meet certain criteria (such as EpiSkin™ , EpiDerm™ SIT and SkinEthic™ RHE) have been included as Method B.46 of the Annex to 440/2008/EC (EU Test Methods Regulation; EU, 2008) during its 1st adaptation to technical progress (Commission Regulation No 761/2009/EC; EU, 2009).

In 2010, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method was adopted as the OECD Test Guideline No 439 which is applicable to the assays for skin irritation employing reconstructed human *epidermis* models (EpiSkin™ , EpiDerm™ SIT and SkinEthic™ RHE) (OECD, 2010). OECD TG 439 defines functional and performance criteria for other (or new) skin or *epidermis* models used in the context of this guideline (OECD, 2010).

Health and Safety Issues

General Precautions

The epidermal cells are taken from healthy volunteer donors negative to anti-HIV 1 and 2, and to hepatitis C antibodies, and to hepatitis B antigens. Nevertheless, normal handling procedures for biological chemicals should be followed:
(a) It is recommended to wear gloves and glasses during handling;
(b) After use, the epidermis, the chemical 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.

Examine all kit components for integrity. If there is a question, a concern or something unusual call: EPISKIN SNC at: +33 (0)4 37 28 72 00 for technical support, and for future users: **www.skinethic.com**.

Safety instructions for working with chemicals:

- Store chemicals in ventilated safety cup boards. Respect special store conditions if necessary (special temperature, protected from light etc.)
- Non-coded chemicals should be handled following chemical safety datasheet.
- Unknown and coded chemicals with none or incomplete safety handling information should be considered as irritating 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(R26 R68 R22 R36 R37 R38), isopropanol (R11 R36 R67), and HCl (R34 R37) are dangerous. Work in ventilated cabinets, to prevent accidental contact wear protective gloves, and if necessary a mask and/or safety glasses.

Abbreviations and Definitions

CLP: Classification, Labelling and Packaging
DSD: Dangerous Substance Directive
D-PBS: Dulbecco's Phosphate-Buffered Saline
ECVAM: European Centre for the Validation of Alternative Methods. As from 2011, ECVAM has been established as the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), hosted by the Joint Research Centre, Institute for Health and Consumer Protection (IHCP).

ESAC: ECVAM Scientific Advisory Committee
EU: European Union
EC: European Comission
IC50: Concentration that induce 50% decrease in cell viability
I: Irritant
IL-1alpha: Interleukin-1alpha
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC: Negative Control
NI: Non Irritant
OD: Optical Density
OECD: Organization for Economic Co-operation and Development
PBS: Phosphate Buffered Saline
PC: Positive Control
RHE: Reconstructed Human *Epidermis*
SDS: Sodium Dodecyl Sulphate
SIVS: Skin Irritation Validation Study
SIT: Skin Irritation Test
TG: Test Guideline
UN GHS: United Nations Globally Harmonized System of Classification and Labelling of Chemicals

Last update: 09 June 2012

PROCEDURE DETAILS, 20 June 2011

EpiSkin™ Skin Irritation Test$^{15min - 42 hours}$

DB-ALM Protocol n° 131

The protocol is compliant with **OECD TG 439** *In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method.*

Some figures in this protocol are snapshots of the actual calculation sheets used for data analysis and are provided here for illustration only. Online version of the protocol includes editable version of MDS sheets, to be filled in when compiling the experiment records. Go to DB-ALM webite (http://ecvam-dbalm.jrc.ec.europa.eu/), section related to protocol No. 131 and select Related information: **Downloads**.

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Materials and Preparations

Cell or Test System

The EpiSkin™-SM kit and biopsy punch can be obtained from EPISKIN SNC, 4 Rue Alexander Fleming – 69366 Lyon cedex 07 France.
Phone: +33 4 37 28 72 00
Fax: +33 4 37 28 72 28
http://www.skinethic.com/order.asp

Equipment

Fixed Equipment

Item	Use
Microbiological safety cabinet (laminar flow hood)	For safe work under sterile conditions
Non-sterile ventilated cabinet	For safe work with chemicals, applications, washes
Cell incubator (37°C, 5% CO_2, 95% relative humidity)	For incubating tissues
Plate reader (96 well) with a 570 nm filter	For optical density readings (MTT)
Water bath at 37°C	For warming media
Vacuum source/trap and Pasteur pipettes	For aspirating media and solutions
Laboratory balance (accuracy 0.1 mg)	For checking chemicals weight and spatula weight
Shaker for 12 well plates	For shaking tissues before media sampling

1 glass funnel	For dropping wash fluids in the bottle
Wash bottle (500 ml)	For collecting wash fluids
1 gauged flask	For SDS 5% solution preparation
200 ml beakers	For PBS (washes)
Adjustable micro-pipette – 0 to 200 µl	For pipetting 200 µl formazan extracts (96 well plate)
Adjustable multi-step pipette, 25 ml combitips	For assay and maintenance media (2 ml), washing
Adjustable positive displacement micro-pipette - 0 to 25 µl	For application of liquids and semi-solids 10 µl
Mortar and Pestle	For grinding granulars
Stop-watches/Timers	For controlling contact and step times
Small sterile blunt-edged forceps	For handling tissue inserts
Small sterile flat-ended forceps	For separating the *epidermis* from the collagen
Vortex mixer	For shaking MTT solution and SDS 5% solution
Small curved flats spatula	For weighing and spreading solids and/or sticky chemicals
1x EpiSkin™-SM biopsy punch (EPISKIN SNC)	For separating the tissue from the insert, prior to isopropanol extraction

Consumables

Item	**Use**
Sterile bottle (eg. 250 ml)	For diluting MTT in the assay medium
Conical opaque centrifuge tube - 50 ml	For MTT stock solution preparation
Small glass weight boats	For weighing powders
Conical “safelock” micro-tubes (1.5 ml) or equivalent	For formazan extraction
96-well flat-botom plates	For reading Optical Density
12-well sterile plates	For tissues incubation and application steps (1 plate per test-chemical)
Nylon mesh (e.g., Sefar Nitex 6 mm, cat.No. 03-150/38)	For weighing and spreading sticky chemical
Cryovials- polypropylene	For collecting and freezing media samples for each tissue
Cotton-tipped swabs	For drying the tissue surface

Media, Reagents, Sera, others

The EpiSkin™-SM kit components:

Description	**Use/Comment**
1 x EpiSkin™-SM plate containing 12 reconstructed *epidermis* units (area: 0.38 cm^2 each)	Each reconstructed *epidermis* is attached to the base of a tissue culture insert with an O-ring set and maintained on nutritive agar for transport
1 x 12-well assay plate	For assays
1 x flask of sterile maintenance medium	Basic medium for incubations
1 x flask of sterile assay medium	Basic medium for use in MTT assays

Materials not provided with the EpiSkin™-SM kit:

Item	Use
12N HCl	For pH adjustment of isopropanol
Isopropanol	For formazan extraction
MTT reagent [3-(4,5-Dimethylthiazol-2-yl)--2,5-diphenyltetrazolium bromide] (e.g., Sigma cat.No. M2128)	For viability measurements
5 % SDS [151-21-3] (w/v; aq; e.g., Sigma, cat.No. L4509, purity 99%) in sterile distilled water	To be used as positive control in each assay
Dulbecco's D-PBS, GIBCO 14040-091 or equivalent	For rinsing tissues and negative control
Sterile distilled H_2O	For powder applications

Preparations

Media and Endpoint Assay Solutions

MTT solution preparation:
Note: MTT solution is light sensitive. Protect it from light using silver paper.

(a) MTT stock solution preparation

- Prepare a 3 mg/ml solution in saline buffer (PBS)
- Vortex 15 minutes
- Keep at 4°C protected from light for up to 15 days in the refrigerator.

(b) MTT ready to use solution preparation

- Pre-warm assay medium to 37°C
- Dilute stock solution 1v+9v, with *assay medium* (final concentration 0.3 mg/ml)
- Protect from light until use (do not exceed 3 hours storage before use)

Fill MDS Annex 8

5% (W/V) SDS (aq) preparation

- Weigh SDS and add distilled water in order to reach the final concentration of 5% (w/v), i.e., 1 g of pure SDS qsp 20 ml water using a gauged flask.
- Preparation can be stored at 4°C for one month.

Fill MDS Annex 8

Acidic isopropanol

- Prepare acidic isopropanol (final concentration: 0.04 N HCl)
- Dilute 1.8 ml of 12 N HCL in 500 ml of isopropanol.
- Preparation can be stored at 4°C protected from light (silver paper) for one month.

Fill MDS Annex 8

Water- killed *epidermis* preparation (for MTT-interacting substances)

- Place the living *epidermis* in a 12 well plate with 2 ml of distilled water (replacing the culture medium).
- Incubate at 37°C, 5% CO_2, in a >95% humidified atmosphere for 48 +/- 1 hours.
- At the end of the incubation, discard the water.
- Keep dead *epidermis* frozen (dry) in freezer at -18°C to -20°C (dead *epidermis* can be stored and used up to 6 months).
- Tissues should be de-frozen before use at room temperature (1 hour in 2 ml of "maintenance" medium).

- Further use of thawed tissues is similar to living tissues.

Test Compounds

Main information concerning the chemicals (codes or numbers, physical consistence, volumes or weight, expiration date, storage conditions, etc.) should be registered in ***the MDS Annex 3*.**

Application of Test Chemicals
This is a detailed description of how to apply test chemicals onto the test system. For complete experimental setup see the Test Material Exposure Procedures section (p.12).

Liquids

- Apply **10 µl** (26.3 µl/cm^2) on the top of each *epidermis*, using a positive displacement pipette.
- Gently spread it with the pipette onto the *epidermis* surface. Ensure to cover all the surface. Record in the MDS.

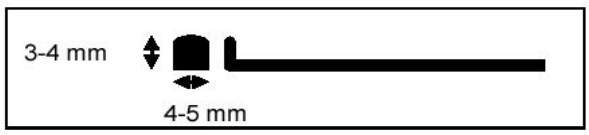
Solids

- The chemical should be crushed to a fine powder, if necessary, using a mortar and a pestle.
- Apply first 5 µl of distilled water using a positive displacement pipette to the epidermal surface in order to improve further contact between the powder and the *epidermis*. Gently spread with the pipette.
- Apply **10 mg +/- 2 mg** (26.3 mg/cm^2) of the powder to the *epidermis* surface. (use special glass weigh boats or similar tools to avoid electrostatic electricity).
- Gently spread on the epidermal surface with a curved flat spatula.
- Record details in ***MDS Annexes 5, 6, 7*** if necessary (grinding, spreading and volume of PBS used)

Viscous and sticky chemicals

- Use a curved flat spatula and weigh directly the chemicals sticked on the curved edge of spatula. In order to spread **10 mg +/- 2 mg** (26.3 mg/cm^2) onto the *epidermis* surface, weigh a higher amount (12 + 2 mg) to compensate for the product remained on the spatula. Gently spread onto the surface by repeated circular movements in order to cover the all surface. A nylon mesh can be used for spreading sticky chemical.
- Record details: note spatula weight before application and after spreading in the ***MDS 5 and 6.***

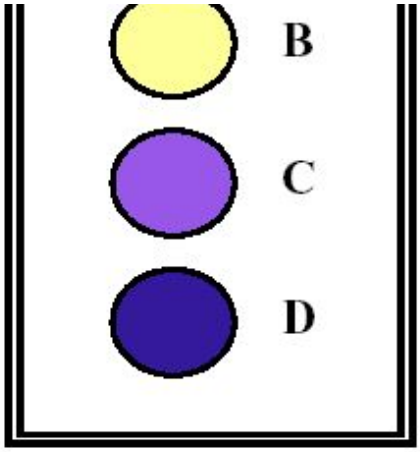
Curved flat micro spatula



Normal supplied stainless steel spatulas can be “home adapted” by twisting the end of the rounded part of the spatula.
Maximum and minimum size of the flat (see below) should be respected. Edges should be blunt in order to avoid tissue damage during spreading.

Check-method for possible direct MTT reduction with test substances
This verification should be done before starting the experiment.

Relative conversion of MTT by the tissue is the key parameter evaluated in this assay. Therefore, it is 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.



- Fill each well of a 12 well plate with 2 mL of MTT solution (0.3 mg/mL).
- Add 10 µl or 10 mg of the substance to be evaluated or water for control and mix.
- Incubate the mixture for 3 hours at 37° C protected from light (test conditions).

If the MTT solution color becomes blue or purple, the substance interacts with the MTT. It is then necessary to evaluate during the future studies the part of OD due to the non specific reduction of the MTT (i.e. by using killed *epidermis* or water killed *epidermis*).

A: control
B: chemical 1 - no interaction
C: chemical 2 - slight interaction
D: chemical 3 - strong interaction
Fill the MDS annex 4

Check-method to detect the coloring potential of test-substances
This verification should be done before starting the experiments

Prior to treatment, chemicals should be evaluated for their intrinsic color or ability to become colored in contact with water, simulating a humid environment of the epidermal tissue. Add 10 mg (solids) or 10 µl (liquids) of the test-chemical to 90 µl of water in a transparent recipient (micro-tube). Mix for 15 minutes. At the end of the shaking period color check is performed by the operator (unaided eye assessment). If a colored solution is detected, the tissue staining ability of the test-chemical needs further check (go to the next step: Check-method to detect coloring test-substances ability to stain tissues). Otherwise, the test-chemical is evaluated as a common chemical. See *Test Material Exposure Procedures* section for further instructions.

Check-method to detect coloring test-substances ability to stain tissues
This verification should be done before starting the experiments

Prior to treatment, colored chemicals should be evaluated for their ability to color and stain the tissues in the test conditions. The non specific color (NSC) is first quantified by using one living tissue unit per chemical. Apply 10 mg (solids) or 10 µl (liquids) of the test-chemical onto the tissue and run a PBS control in parallel. Follow all steps described in chapters *Preparation and pre-incubation (Day 0)* to *Absorbance / Optical Density measurements (Day 3 or 6),* but with only one live tissue unit for each test-chemical. Replace the MTT incubation period by incubation with the assay medium without MTT.

If the test chemical-linked optical density (unspecific OD_{570}) is >5% and ≤ 30% relative to the PBS-negative control OD_{570}, the chemical can be evaluated in further trials by using additional controls in all runs in order to correct final values (subtract viability unrelated OD).

Test-chemicals with NSC ≤ 5% relative to the negative control should be evaluated by using the common procedure. If non specific color NSC is > 30% relative to the negative control, additional steps must be undertaken or the chemical must be considered as incompatible with the test.

Positive Control(s)

5% SDS (w/v; aq) is used as positive control in each assay.

Negative Control(s)

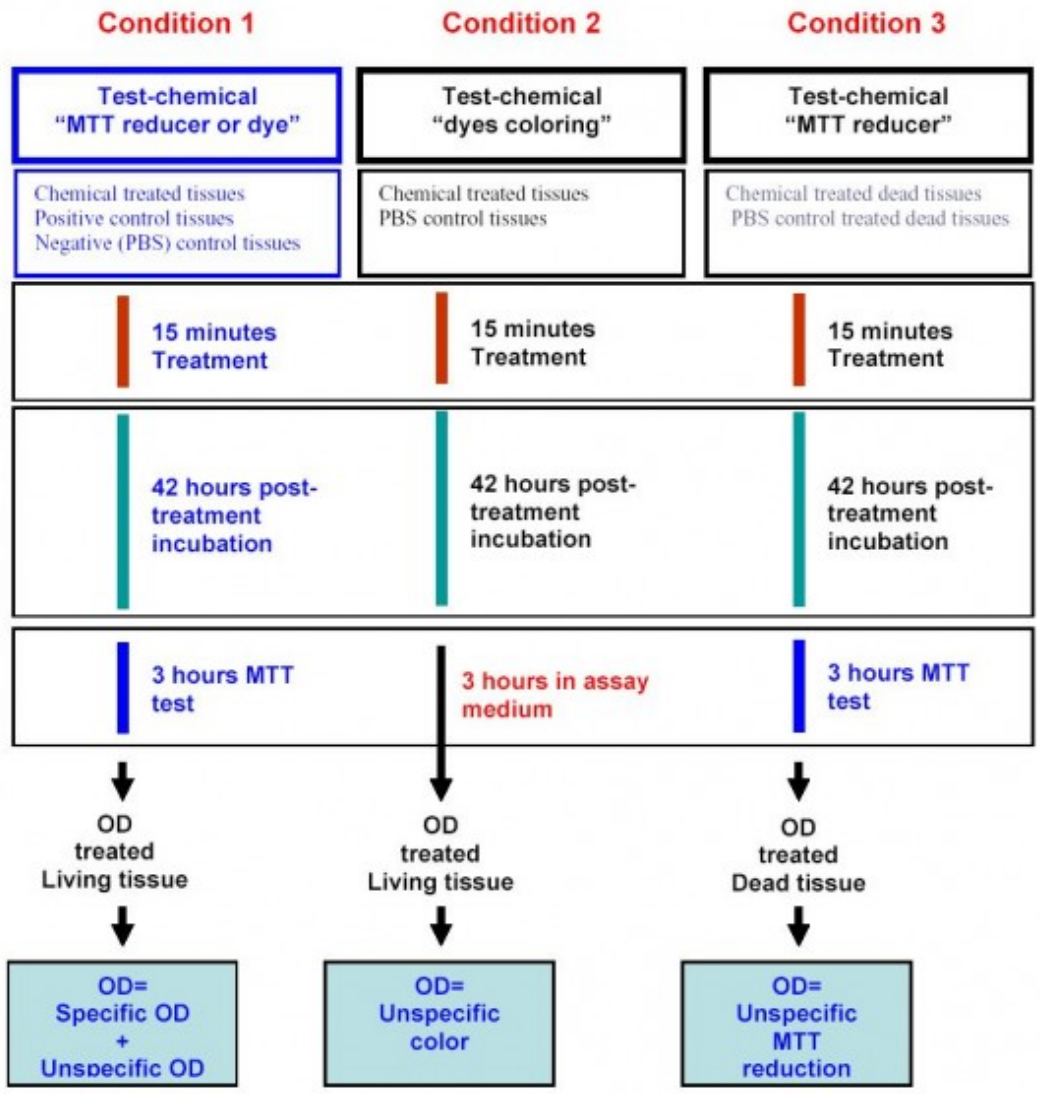
Dulbecco's D-PBS is used as negative control.

Method

TEST METHOD QUICK WORK FLOWCHART

1. **Check test-chemicals for their potential direct MTT reducing activity**
2. **Check the presence of dyes and chemicals able to color the *epidermis***
3. **Receipt: Transfer *epidermis* from transport medium to maintenance medium**

4. **Incubate for 24 hours (37°C, 5% CO_2, humidified atmosphere)**
5. **Apply topically 10 µl or 10 mg of Test Chemical or PC or NC**
6. **Keep at room temperature for 15 minutes**
7. **Stop treatment by rinsing with PBS**
8. **Transfer tissues to fresh maintenance medium**
9. **Incubate 42 hours (37°C, 5% CO_2, humidified atmosphere)**
10. **Sample medium for mediators release (optional)**
11. **Transfer tissues to MTT solution**
12. **Incubate tissues for 3 hours (37°C, 5% CO_2, humidified atmosphere)**
13. **Make biopsy punch of the tissues**
14. **Transfer and immerse in acidified isopropanol**
15. **Formazan extraction: 4 hours (room temperature) or 48-70 hours (at 4°C) one weekend**
16. **Shake and homogenise**
17. **Transfer extracted solution in 96 well plate**
18. **Read OD using a plate spectrophotometer**



Case by case test conditions guidance

	Water coloration	Tissue Binding/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

Test System Procurement

- For contact details on purchasing the kit -see Materials and Preparations section.
  The EpiSkin™-SM kit contains 12 reconstructed *epidermis* units and the necessary culture media (maintenance and assay media) and additional 12 well plates. Each EpiSkin™-SM batch is controlled by the manufacturer. Results of quality controls are supplied with the kits.

Routine Culture Procedure

Receipt of the EpiSkin™-SM kits (Day 0)

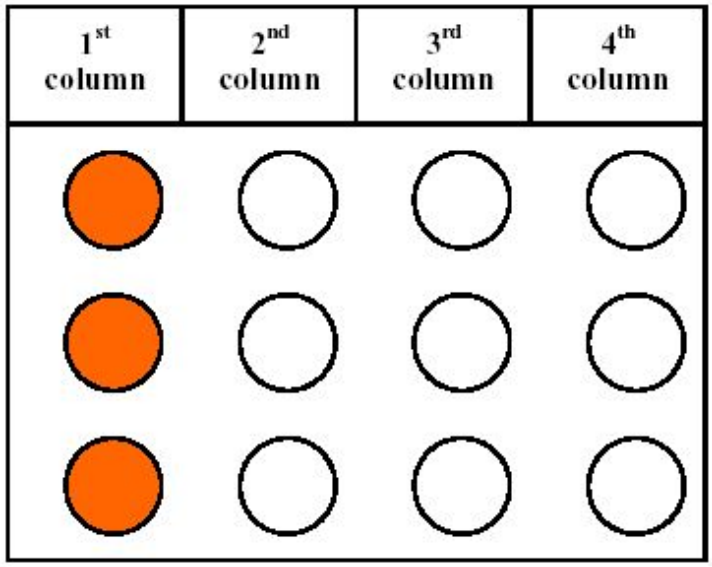
Upon arrival, register kit details (reception etc.) and assay procedures on the MDS form. ***(in MDS Annex 2)***
Check the date of sending written on the package as well as some critical points before opening the EpiSkin™-SM kit:

- inspect the color of the agar medium used for transport and check that its pH is acceptable:
  orange color - acceptable; **yellow or violet color** - not acceptable;
- inspect the color of the temperature indicator to verify that the kit has not been exposed to a temperature above 40°C:
  pale grey - acceptable; **dark grey** - not acceptable;
- Place the *assay medium* supplied with the kits at 2-8°C (refrigerator).
- Pre-warm the maintenance medium supplied to 37°C (water bath).
- Leave the EpiSkin™-SM kits in their packaging at room temperature in the microbiological safety cabinet until the next step.

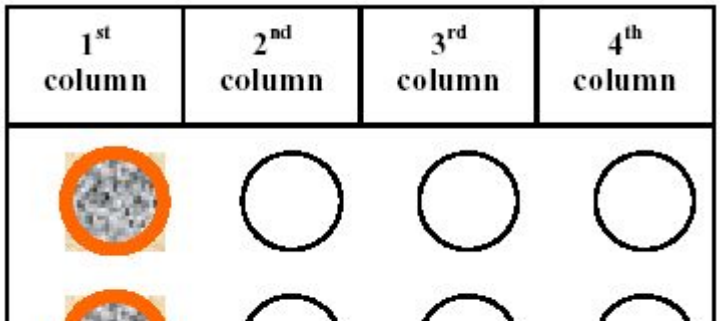
Preparation and pre-incubation (Day 0)
This step should be conducted in sterile conditions (Microbiological safety cabinet)

Note: One sterile 12 wells plate is used for one test-chemical. For a given chemical, the same plate will be used for all steps of the protocol. Prior to *epidermis* transfer from their transport packaging, prepare plates labeling. Mark all plate lids with the code number of the test-chemical (3 wells per chemical), or negative control (3 wells) or positive control (3 wells).

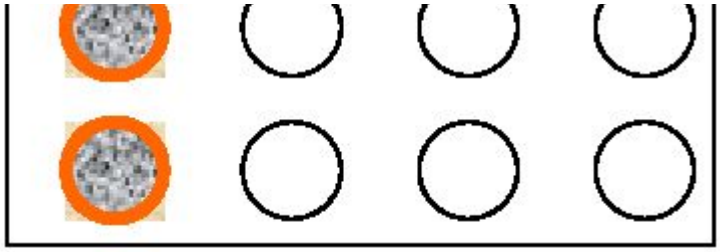
(a) Fill 3 wells of the first column of a 12 well assay plate with pre-warmed maintenance medium (**2 ml per well,** adjustable multi-step pipette).



(b) Open the EPISKIN™-SM kits and transfer the *epidermis* units into maintenance medium filled wells, using sterile forceps. Use 3 units per plate.
Avoid air bubbles formation under the tissue.
Label inserts by replicate treatment order (to be followed in the next step) :



Ex: Rep 1; rep 2; rep 3



(c) Incubate at 37°C, 5% CO_2, in a >95% humidified atmosphere for at least 24 hours.

Test Material Exposure Procedures

Important: Some highly reactive chemicals can produce fumes which may affect adjacent units in the same plate. It is strongly recommended to test all unknown chemicals alone in separate plates. It is also recommended to routinely incubate positive and negative controls in separate plates. Never use cotton buds for spreading the substance onto tissues.

Application of the test substance and rinsing (Day 1)

Controls for MTT direct-interacting chemicals

- For each MTT-interacting substance previously detected, and in addition to the normal procedure, 3 killed treated tissues and 3 killed negative control tissues should be used for the MTT evaluation in one run (untreated killed tissues may exhibit little residual NADH and dehydrogenase associated activity).
- All killed tissues must be both from the same batch. The batch of killed tissues can be different than the batches of the living tissues. These tissues should follow the same treatment steps than the living tissues.

Controls for dyes and chemicals able to color the tissue

- For each substance previously detected as being able to color the tissue, and in addition to the normal procedure, at least 1 additional chemical-treated tissue and at least 1 additional negative control per run should be used for the non specific OD evaluation.
- These tissues should follow the same treatment steps than other tissues except for the MTT step: MTT incubation is replaced by incubation with fresh assay medium. OD readings are made following the same conditions then other tissues.

Fill MDS Annex 6

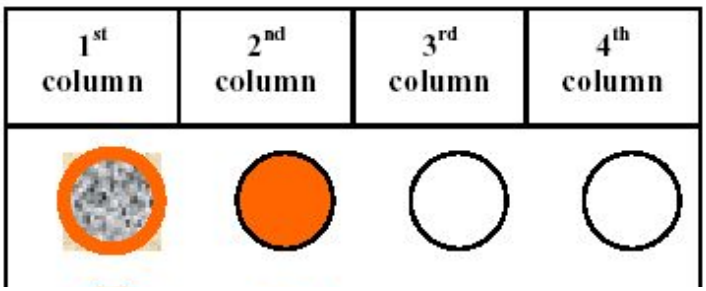
Specific procedure for the positive control (5%SDS)

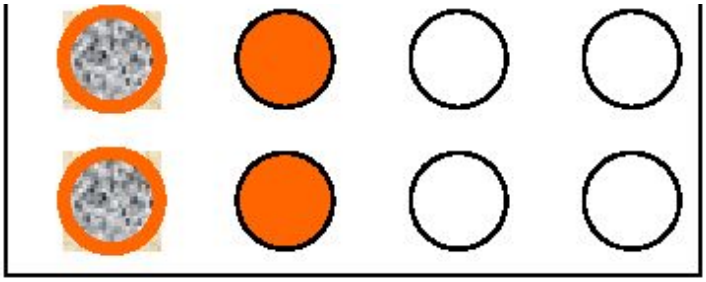
- Apply 10 µl with the positive displacement pipette. To ensure a correct contact with the entire *epidermis* (particularly the center), spread the solution directly with the pipette tip.
- Continue spreading for approximately 30 seconds, to ensure that the SDS covers all the *epidermis,* in particular the center.

Preparation

(a) Pre-warm the maintenance medium to 37°C (water bath).

(b) Fill the second column (3 wells) of a 12 well assay plate with pre-warmed maintenance medium (**2 ml per well**, adjustable multi-step pipette). Verify the plate lids labeling (code number of the test-chemical (3 wells per chemical), or negative control (3 wells) or positive control (3 wells).





Topical applications: 15 minutes treatment
Note: chemicals are applied undiluted (neat)

- Apply 10 µl or 10 mg of the undiluted chemical (or negative control or positive control) on the top of the *epidermis*. 3 tissues per chemical should be used and follow the defined application order (replicate1, replicate 2, replicate 3).
- Keep 1 minute (example) interval between each tissue application, and do not test more than 4 chemicals (=12 tissues) in a block in order to be ready to wash off the test chemical in time (15 minutes contact).
- Record on the plate lid the exact timings during the experiment and fill the corresponding ***MDS Annex 7*** at the end of each run.

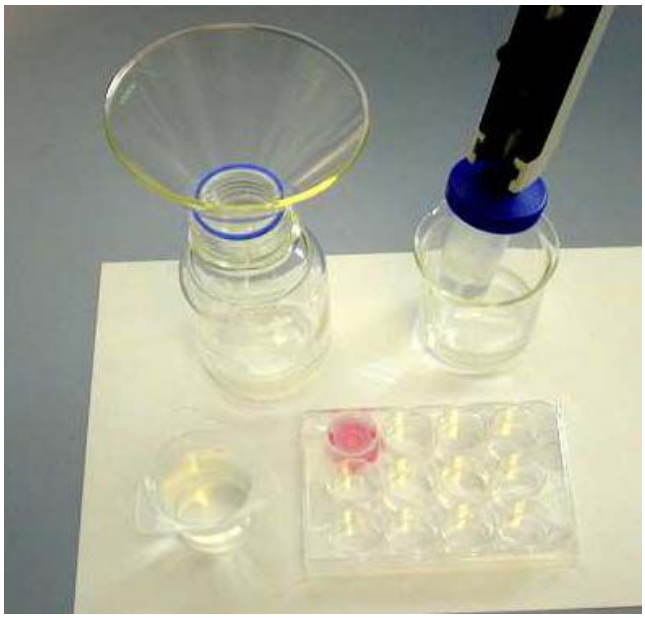
Positive displacement pipette deposit.

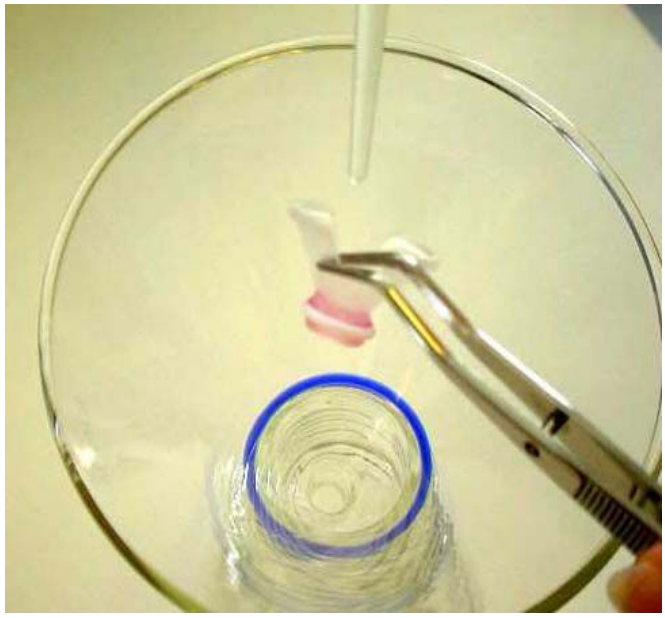
Spatula spreading (viscous, waxy …)

- Keep the plate (lids on) containing the treated *epidermis* **for 15 minutes** (± 0.5 minute) in the ventilated cabinet at room temperature (comprised between 19°C to 23°C).

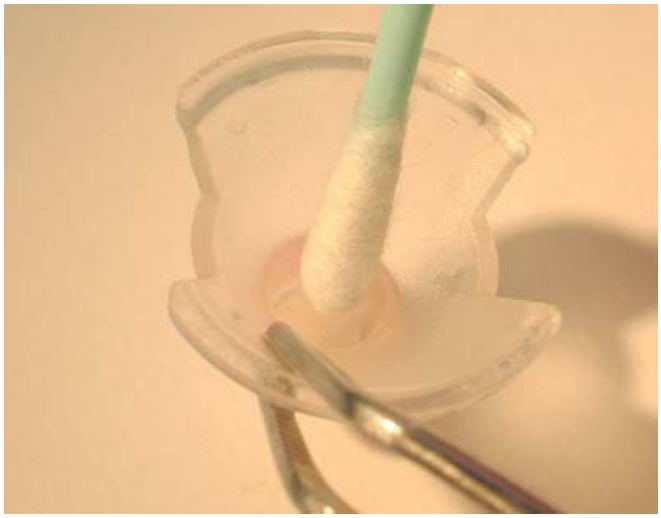
End of the treatment and removal of the test chemical
After 15 minutes exposure (+/- 0.5 min):

(a) Remove the treated units using forceps, and rinse thoroughly with 25 ml sterile PBS (ready to use) filling and emptying the tissue inserts, to remove all residual test chemical from the epidermal surface.

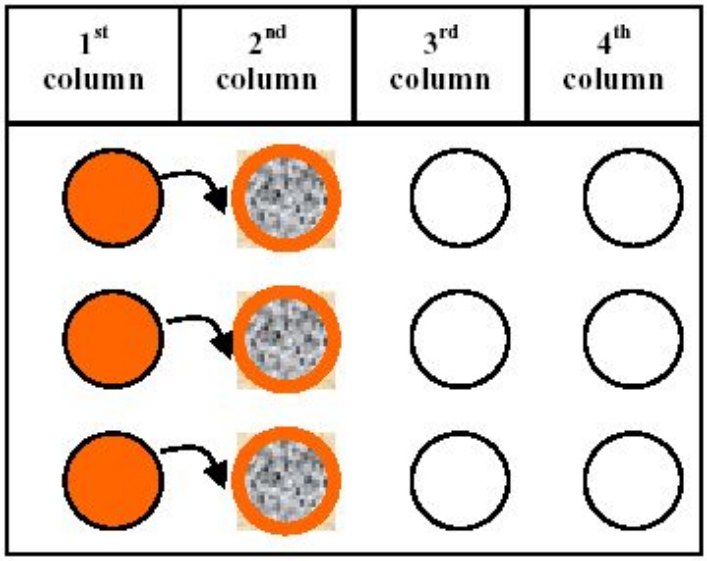




(b) Place the units on an absorbent paper, remove the remaining PBS from the epidermal surface by gently taping, and sweep the surface with a cotton-bud if necessary without damaging the *epidermis*.



(c) Transfer the blotted tissue units in the new maintenance medium pre-filled wells (2nd column).



Post treatment Incubation: 42 hours

- Incubate the treated and rinsed *epidermis* at 37°C, 5% CO_2, 95% humidified atmosphere for **42 hours ± 1 hour.** Record incubation start time in the ***MDS Annex 9.***

Recommended media sampling for mediators and enzyme release measurement (optional)

At the end of the 42 +/- 1 hour incubation period (Day 3):

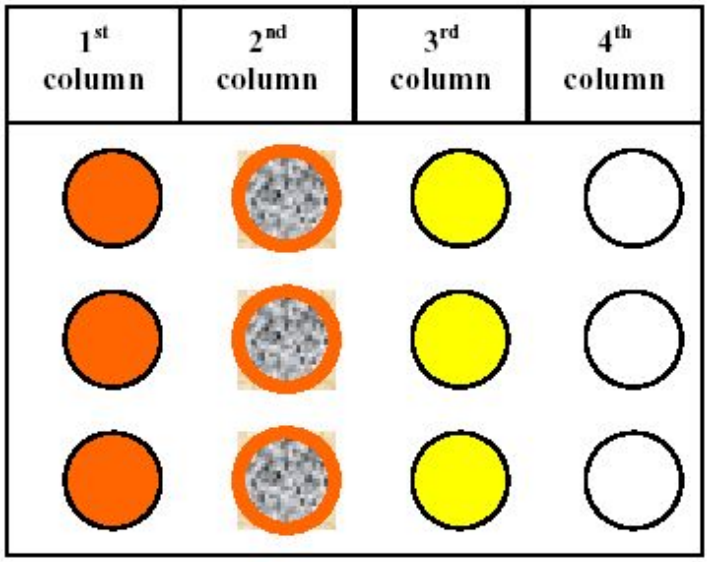
- Before starting, label an appropriate numbers of polypropylene tubes with caps (one per tissue unit): **batches number**, chemicals **code** number, **replicate** number and **date**.
- Out of the incubator, shake the plates containing the treated *epidermis* (lids on) on a plate shaker for 15 minutes +/- 2 min, medium speed (300 rpm/min). This step helps to homogenise the released mediators in the medium before sampling.
- Transfer 1.6 ml of incubation medium from each tissue to the pre-labeled tubes. Stock Frozen at -**20°C until analyses (for at least one year).**

Note: This optional step is not included in TG439. The procedure allows for the collection of additional information on the response of the test system to the chemical tested. Please refer to the contact persons of this protocol for more details of the relevant auxiliary measurements.

Endpoint Measurement

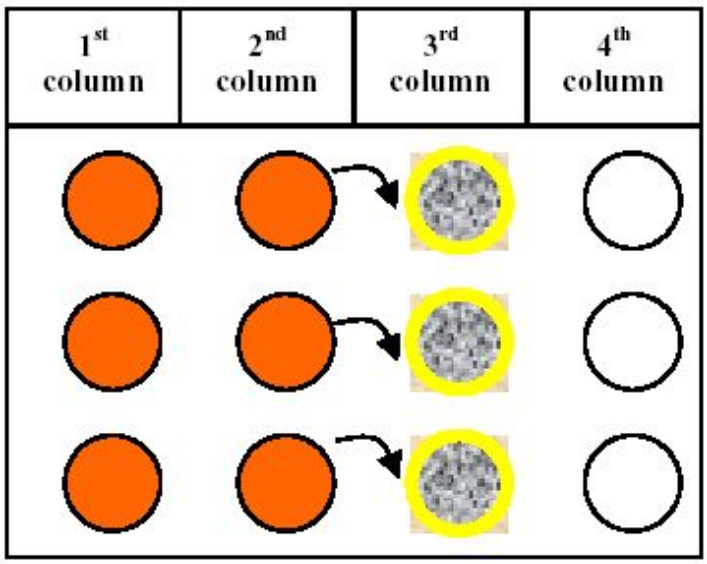
MTT test after the 42 hours incubation period (Day 3)

(a) Fill the appropriate number of wells of the assay plate (3 rd column) with 2 ml per well of 0.3 mg/ml MTT in assay medium.

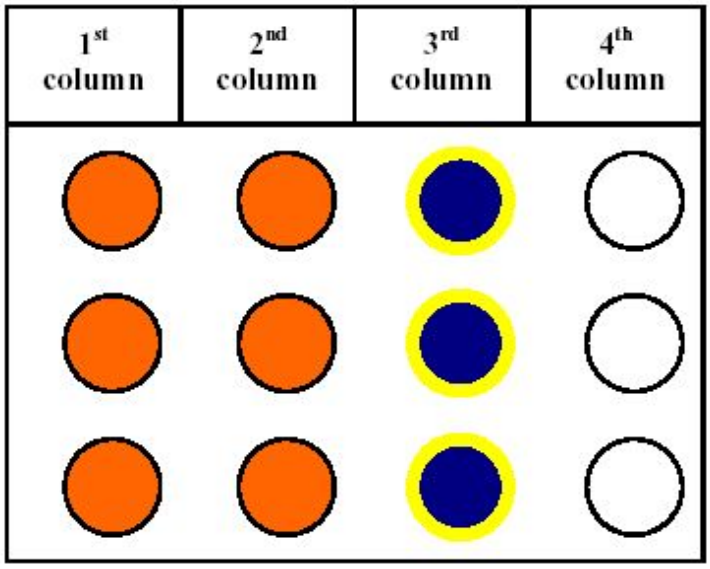


(b) Transfer the EpiSkin™ units to the MTT filled wells:

- Sweep excess medium on the unit bottom with absorbent paper before setting down on the well.
- Replace the lid on the plate.



(c)
Incubate for **3 hours** (± 5 minutes) at 37°C, 5% CO_2, 95% humidified atmosphere.
Record start time in the MDS Annex 9.
Note: Viable cells metabolize MTT. The blue color of the *epidermis* is due to the intracellular formation of formazan crystals.



(d) At the end of the incubation, record observations and comments in ***MDS Annex 10***.

Formazan extraction (Day 3)

(a) Prior to extraction, label 1.5 ml conic “safelock” type micro tubes with the chemicals code number and replicate N° (1 tube/epidermis).

Note: Steps **(b)** to **(d)** should be followed chemical by chemical.

(b) Place the 3 tissue units on absorbent paper to dry the tissues.

(c) After placing the *epidermis* units on the plate lid (as a support), make a total biopsy of the *epidermis* by using the special biopsy punch.

(d) Gently separate the *epidermis* from the collagen matrix with the aid of forceps, and place **both** parts (*epidermis* and collagen matrix) into the labeled microtubes.

> ***Important:***
> ***- Place the epidermis treated side onto the collagen matrix.***
> ***- If the collagen matrix is colored by the chemical, replace it during the extraction by a new collagen matrix (biopsy punched) taken from killed de-epidermised non treated epidermis.***

(e) When all the tissues of an application set are punched and ready in the micro tubes for, **add 500 µl of acidic isopropanol per tube.**

(f) Plug each tube to avoid evaporation and mix thoroughly using a vortex mixer.

(g) Ensure that all the biological material is correctly immersed in the solvent.
Record start time of extraction in *MDS Annex 9*.

(h) For readings on the same day as extraction:

- Store 4 hours at room temperature (recommended +18 to 23°C) protected from light.
- Vortex each tube at the middle of the incubation period to help extraction.

or

- For readings on Monday morning: store refrigerated at +4°C protected from light.

Record timings and necessary details *in MDS Annex 9.*

(i) Remove (using suction pump and toxic waste trap) the MTT solution from each well of the remaining plate. Discard the empty plate in the special waste container.

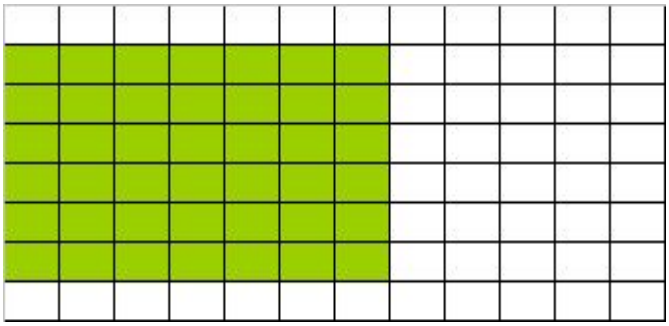
At the end of the formazan extraction period :

(j) Mix each tube using a vortex mixer, until solution color becomes homogeneous.

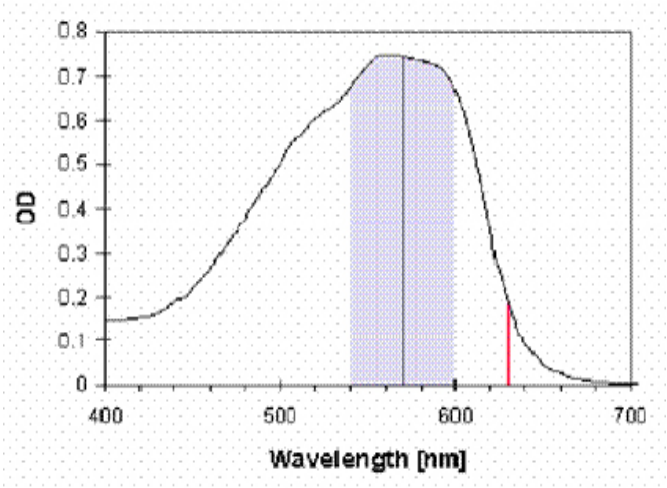
(k) If any visible cell/tissue fragments are in suspension, centrifuge (500 rpm) to eliminate the fragments and avoid further possible interference with the absorbance readings.

Absorbance / Optical Density measurements (Day 3 or 6)

(a) Transfer per tissue 2 x 200 µL sample/well (= 2 wells per tissue) from each tube into a 96 well flat bottom microtiter plate (labeled appropriately).
Note: Beware of isopropanol evaporation in 96 well plates. It is recommended to fill not more than 42 wells per plate and to make all readings in the same run (ex: maximum area to be used is marked in green).



(b) Read the Optical Densities (**OD**) in a 96-well plate spectrophotometer using wavelength filter centered at **570 nm** pass-band ± 30 nm and acidified isopropanol solution as blank without using a reference filter.
Note: Readings are performed without reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters have a tolerance their use can lead to reduction of the dynamics of the signal (OD).



Do not use the “empty” wells for readings (see example below).

	Blank	chem 1	chem 2	chem 3	chem 4	chem 5	chem 6					
A	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
B	blank	tissue 1	tissue 1	tissue 1	tissue 1	tissue 1	tissue 1	empty	empty	empty	empty	empty
C	blank	tissue 1	tissue 1	tissue 1	tissue 1	tissue 1	tissue 1	empty	empty	empty	empty	empty
D	blank	tissue 2	tissue 2	tissue 2	tissue 2	tissue 2	tissue 2	empty	empty	empty	empty	empty
E	blank	tissue 2	tissue 2	tissue 2	tissue 2	tissue 2	tissue 2	empty	empty	empty	empty	empty
F	blank	tissue 3	tissue 3	tissue 3	tissue 3	tissue 3	tissue 3	empty	empty	empty	empty	empty
G	blank	tissue 3	tissue 3	tissue 3	tissue 3	tissue 3	tissue 3	empty	empty	empty	empty	empty
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty
	1	2	3	4	5	6	7	8	9	10	11	12

(c) Record results on ***prepared templates***. Templates are normally linked to specific readers/programs used by the test laboratory and may slightly differ form the example above. A direct print of the readings should be made immediately and identified with date and signature and kept as raw data.

Acceptance Criteria

EpiSkin™-SM kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the *epidermis* and the kit culture medium have been tested for the absence of viruses, bacteria and mycoplasma. The quality of the final product is assessed by an MTT cytotoxicity test using sodium dodecyl sulphate (SDS) and by histological scoring. Additional criteria could be added in the future.

Method Documentation Sheets (MDS) allow for compliant Quality Control: correct set up, calibration, function of the equipment and quality of the preparations are an intergal part of the protcol. **See Annexes 1 to 13.**

Assay acceptance criteria for negative and positive controls

Negative control (NC) acceptance: OD of the negative control (PBS treated) reflects the viability of the tissues used in the test conditions (after shipping, storing in specific conditions). An absolute OD below the historical established lower boundary of the confidence interval indicates abnormal viability of tissues thus indicating possible difference in sensitivity to chemicals. These tissues should not be used for this application.

It can be considered that the NC meets the acceptance, if the mean OD value of the 3 tissues is > 0.6 and the Standard Deviation value (SD) of the % viability is 18.

Positive control (PC) acceptance: OD of the positive control (5% aq. SDS-treated) reflects the sensitivity of the tissues used in the test conditions (after shipping, storing in specific conditions). One positive control should be included in each run (maximum one PC per day).

It can be considered that the Positive Control meets the acceptance if the mean viability expressed as % of the NC, is 40% and the SD is≤ 18.

Batch acceptance: all chemical data from one batch are accepted if both the negative and the positive control fulfill the above requirements.

Chemical data acceptance: The inter-batch viability mean is calculated from the three independent assays using intra-batch tissue mean. The 3 intra-batch means must predict the same class of irritation.

For a given chemical, if the batch gives an SD > 18, the chemical is retested once (possible technical problem or error). If 2 or 3 batches give SDs > 18 the assay is not repeated (variability probably linked to the chemical itself).

Data Analysis

All data/calculations should be recorded on prepared ***Data Report Forms***. Excel prepared spreadsheets could be used (not provided by EpiSkin supplier). Blank data and chemicals data (OD) are copied and pasted in the Excel prepared tables named: “IMPORT sheet”.

Note: The figures below are snapshots of the actual calculation spreadsheets and are presented here for illustration only.

Correspondend Informations of MDS

Exp. no.:	xxxxxxx
Tissue-BATCH	xxxxxxx
Date:	xxxxxxx
Operator:	xxxxxxx

IMPORT

PLATE 1

	Blank	NC	PC	C1	C2	C3	C4						
A													
B	0,038	1,657	0,201	1,605	1,657	1,439	1,439						Tissue1
C	0,039	1,673	0,187	1,612	1,673	1,455	1,455						
D	0,041	1,541	0,162	1,654	1,541	1,368	1,368						Tissue2
E	0,039	1,543	0,156	1,755	1,543	1,407	1,407						
F	0,040	1,734	0,149	1,490	1,734	1,544	1,544						Tissue3
G	0,040	1,712	0,152	1,451	1,712	1,581	1,581						
H													
	1	2	3	4	5	6	7	8	9	10	11	12	

Test Chemical N°	Code N°
Negative control	NC
Positive control	PC
Test Chemical No. 1	C1
Test Chemical No. 2	C2
Test Chemical No. 3	C3
Test Chemical No. 4	C4

PLATE 2

	Blank	C5	C6	C7	C8	C9	C10						
A													
B	0,038	1,605	1,439	1,605	1,605	1,657	1,439						Tissue1
C	0,039	1,612	1,455	1,612	1,612	1,673	1,455						
D	0,041	1,654	1,368	1,654	1,654	1,541	1,368						Tissue2
E	0,039	1,755	1,407	1,755	1,755	1,543	1,407						
F	0,040	1,490	1,544	1,490	1,490	1,734	1,544						Tissue3
G	0,040	1,451	1,581	1,451	1,451	1,712	1,581						
H													
	1	2	3	4	5	6	7	8	9	10	11	12	

Test Chemical N°	Code N°
Test Chemical No. 5	C5
Test Chemical No. 6	C6
Test Chemical No. 7	C7
Test Chemical No. 8	C8
Test Chemical No. 9	C9
Test Chemical No. 10	C10

PLATE 3

	Blank	C11	C12	C13	C14	C15							
A													
B	0,038	1,608	1,492	0,252	0,153	0,329							Tissue1
C	0,070	1,688	1,491	0,254	0,147	0,329							
D	0,041	1,586	1,646	0,291	0,162	0,235							Tissue2
E	0,039	1,622	1,714	0,249	0,147	0,232							
F	0,040	1,528	1,251	0,250	0,163	0,272							Tissue3
G	0,040	1,548	1,391	0,255	0,165	0,267							
H													
	1	2	3	4	5	6	7	8	9	10	11	12	

Test Chemical N°	Code N°
Test Chemical No. 11	C11
Test Chemical No. 12	C12
Test Chemical No. 13	C13
Test Chemical No. 14	C14
Test Chemical No.15	C15

PLATE 4

	Blank	C16	C17	C18	C19	C20							
A													
B	0,038	0,153	1,492	0,252	0,252	0,153							Tissue1
C	0,070	0,147	1,491	0,254	0,254	0,147							
D	0,041	0,162	1,646	0,291	0,291	0,162							Tissue2
E	0,039	0,147	1,714	0,249	0,249	0,147							
F	0,040	0,163	1,251	0,250	0,250	0,163							Tissue3
G	0,040	0,165	1,391	0,255	0,255	0,165							
H													
	1	2	3	4	5	6	7	8	9	10	11	12	

Test Chemical N°	Code N°
Test Chemical No. 16	C16
Test Chemical No. 17	C17
Test Chemical No. 18	C18
Test Chemical No. 19	C19
Test Chemical No. 20	C20

date:	xxxxxx	performed by:	xxxxxxxx	signature:	xxxxxxx

Example of **IMPORT sheet** form tables

Exp. no.:	xxxxxxx
Tissue-BATCH no.:	xxxxxxx
Date:	xxxxxxx
Operator:	xxxxxxx

PLATE 1

Test Chemical N°	Code N°
Negative control	NC
Positive control	PC
Test Chemical No. 1	C1
Test Chemical No. 2	C2
Test Chemical No. 3	C3
Test Chemical No. 4	C4

Blank - plate	mean	sd
0,038	0,0395	0,001
0,039		
0,041		
0,039		
0,04		
0,04		

PLATE 3

Test Chemical N°	Code N°
Test Chemical No. 11	C11
Test Chemical No. 12	C12
Test Chemical No. 13	C13
Test Chemical No. 14	C14
Test Chemical No.1 5	C15

Blank - plate	mean	sd
0,038	0,0447	0,0125
0,07		
0,041		
0,039		
0,04		
0,04		

PLATE 2

Test Chemical N°	Code N°
Test Chemical No. 5	C5
Test Chemical No. 6	C6
Test Chemical No. 7	C7
Test Chemical No. 8	C8
Test Chemical No. 9	C9
Test Chemical No. 10	C10

Blank - plate	mean	sd
0,038	0,0395	0,001
0,039		
0,041		
0,039		
0,04		
0,04		

PLATE 4

Test Chemical N°	Code N°
Test Chemical No. 16	C16
Test Chemical No. 17	C17
Test Chemical No. 18	C18
Test Chemical No. 19	C19
Test Chemical No. 20	C20

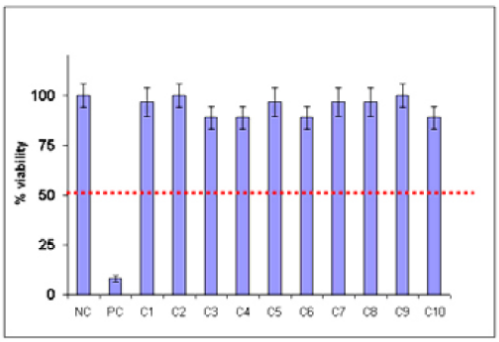
Blank - plate	mean	sd
0,038	0,0447	0,0125
0,07		
0,041		
0,039		
0,04		
0,04		

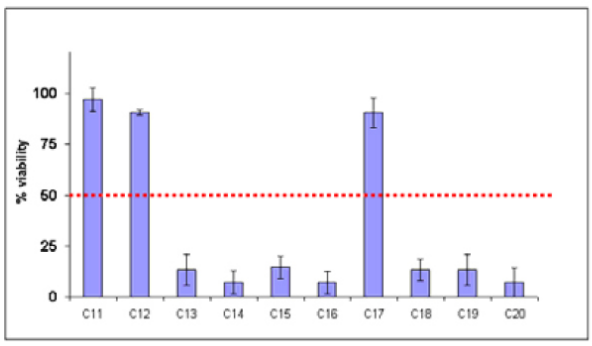
Code N°	Raw data aliquot 1	aliquot 2	Blank corrected data aliquot 1	aliquot 2	Mean of aliquotes	Viability	Tissue n
NC	1,657	1,673	1,618	1,634	1,626	101,4	1
	1,541	1,543	1,502	1,504	1,503	93,7	2
	1,734	1,712	1,695	1,673	1,684	105,0	3
PC	0,201	0,187	0,162	0,148	0,155	9,6	1
	0,162	0,156	0,123	0,117	0,120	7,5	2
	0,148	0,152	0,110	0,113	0,111	6,9	3
C1	1,605	1,612	1,566	1,573	1,569	97,8	1
	1,654	1,755	1,615	1,716	1,665	103,8	2
	1,490	1,451	1,451	1,412	1,431	89,2	3
C2	1,657	1,673	1,618	1,634	1,626	101,4	1
	1,541	1,543	1,502	1,504	1,503	93,7	2
	1,734	1,712	1,695	1,673	1,684	105,0	3
C3	1,439	1,455	1,400	1,416	1,408	87,8	1
	1,368	1,407	1,329	1,368	1,348	84,0	2
	1,544	1,581	1,505	1,542	1,523	95,0	3
C4	1,439	1,455	1,400	1,416	1,408	87,8	1
	1,368	1,407	1,329	1,368	1,348	84,0	2
	1,544	1,581	1,505	1,542	1,523	95,0	3
C5	1,605	1,612	1,566	1,573	1,569	97,8	1
	1,654	1,755	1,615	1,716	1,665	103,8	2
	1,490	1,451	1,451	1,412	1,431	89,2	3
C6	1,439	1,455	1,400	1,416	1,408	87,8	1
	1,368	1,407	1,329	1,368	1,348	84,0	2
	1,544	1,581	1,505	1,542	1,523	95,0	3
C7	1,605	1,612	1,566	1,573	1,569	97,8	1
	1,654	1,755	1,615	1,716	1,665	103,8	2
	1,490	1,451	1,451	1,412	1,431	89,2	3
C8	1,605	1,612	1,566	1,573	1,569	97,8	1
	1,654	1,755	1,615	1,716	1,665	103,8	2
	1,490	1,451	1,451	1,412	1,431	89,2	3
C9	1,657	1,673	1,618	1,634	1,626	101,4	1
	1,541	1,543	1,502	1,504	1,503	93,7	2
	1,734	1,712	1,695	1,673	1,684	105,0	3
C10	1,439	1,455	1,400	1,416	1,408	87,8	1
	1,368	1,407	1,329	1,368	1,348	84,0	2
	1,544	1,581	1,505	1,542	1,523	95,0	3

Code N°	Raw data aliquot 1	aliquot 2	Blankcorrected data aliquot 1	aliquot 2	Mean of aliquotes	Viability	Tissue n
C11	1,608	1,688	1,563	1,643	1,603	100,0	1
	1,586	1,622	1,541	1,577	1,559	97,2	2
	1,528	1,548	1,483	1,503	1,493	93,1	3
C12	1,492	1,491	1,447	1,446	1,447	90,2	1
	1,646	1,714	1,601	1,669	1,635	102,0	2
	1,251	1,391	1,206	1,346	1,276	79,6	3
C13	0,252	0,254	0,207	0,209	0,208	13,0	1
	0,291	0,249	0,246	0,204	0,225	14,0	2
	0,250	0,255	0,205	0,210	0,208	13,0	3
C14	0,153	0,147	0,108	0,102	0,105	6,6	1
	0,162	0,147	0,117	0,102	0,110	6,8	2
	0,163	0,165	0,118	0,120	0,119	7,4	3
C15	0,329	0,329	0,284	0,284	0,284	17,7	1
	0,235	0,232	0,190	0,187	0,189	11,8	2
	0,272	0,267	0,227	0,222	0,225	14,0	3
C16	0,153	0,147	0,108	0,102	0,105	6,6	1
	0,162	0,147	0,117	0,102	0,110	6,8	2
	0,163	0,165	0,118	0,120	0,119	7,4	3
C17	1,492	1,491	1,447	1,446	1,447	90,2	1
	1,646	1,714	1,601	1,669	1,635	102,0	2
	1,251	1,391	1,206	1,346	1,276	79,6	3
C18	0,252	0,254	0,207	0,209	0,208	13,0	1
	0,291	0,249	0,246	0,204	0,225	14,0	2
	0,250	0,255	0,205	0,210	0,208	13,0	3
C19	0,252	0,254	0,207	0,209	0,208	13,0	1
	0,291	0,249	0,246	0,204	0,225	14,0	2
	0,250	0,255	0,205	0,210	0,208	13,0	3
C20	0,153	0,147	0,108	0,102	0,105	6,6	1
	0,162	0,147	0,117	0,102	0,110	6,8	2
	0,163	0,165	0,118	0,120	0,119	7,4	3

Code Nr.	OD mean	Viability mean (%)	SD of OD	SD of viability	CV (%)
NC	1,604	100,0	0,09	5,76	5,76
PC	0,128	8,0	0,02	1,44	17,97
C1	1,555	97,0	0,12	7,33	7,56
C2	1,604	100,0	0,09	5,76	5,76
C3	1,426	88,9	0,09	5,55	6,24
C4	1,426	88,9	0,09	5,55	6,24
C5	1,555	97,0	0,12	7,33	7,56
C6	1,426	88,9	0,09	5,55	6,24
C7	1,555	97,0	0,12	7,33	7,56
C8	1,555	97,0	0,12	7,33	7,56
C9	1,604	100,0	0,09	5,76	5,76
C10	1,426	88,9	0,09	5,55	6,24

Code Nr.	OD mean	Viability mean (%)	SD of OD	SD of viability	CV (%)
C11	1,552	96,8	0,06	3,45	3,57
C12	1,453	90,6	0,18	11,20	12,36
C13	0,214	13,3	0,01	0,62	4,66
C14	0,112	7,0	0,01	0,45	6,41
C15	0,233	14,5	0,05	3,01	20,73
C16	0,112	7,0	0,01	0,45	6,41
C17	1,453	90,6	0,18	11,20	12,36
C18	0,214	13,3	0,01	0,62	4,66
C19	0,214	13,3	0,01	0,62	4,66
C20	0,112	7,0	0,01	0,45	6,41





date:	xxxxxx		**performed by:**	xxxxxxxx

signature:

Example of Spread sheet form tables

Data calculation steps
These calculation steps are applicable to the majority of test-substances characterized as follows: no interaction with the MTT reagent, non colored, with a low ability to stain the tissues and measured non specific color value 5% relative to negative control.

Main steps to be followed by the prepared template

(a) Blanks: calculate the OD mean from the 6 replicates for each plate.
(b) Negative PBS-treated controls: Subtract blanks mean value from individual tissues OD. Corrected OD mean for the 3 tissues corresponds to 100% viability.
(c) Positive control (SDS 5%): Subtract blanks mean value from individual tissues OD. Calculate the OD mean for each individual tissue.
(d) Tested compound: Subtract blanks mean value from individual tissues OD. Calculate the OD mean for each individual tissue.
(e) The viability % for each treated *epidermis*, are calculated relative to the mean of negative controls. (See formula below)
(f) Standard deviations are calculated on OD and % viabilities.
(g) CV are calculated on % viabilities.

- **The mean OD of the three Negative Controls (PBS treated) corresponds to 100% reference viability.**
- **For each individual treated tissue (TT) with a test substance or the positive control (PC), the relative viabilities are calculated as follows:**

OD Treated tissue OD Negative control (OD_{NC}) OD Positive control (OD_{PC})	= OD $_{TTraw}$ - OD $_{blank\ mean}$ = OD $_{NCraw}$ - OD $_{blank\ mean}$ = OD $_{PCraw}$ - OD $_{blank\ mean}$
Individual viabilities (%) %Positive Control1 = [OD_{PC1} /mean OD_{NC}] x 100 %Positive Control2 = [OD_{PC2} /mean OD_{NC}] x 100	

- **The mean relative viability is used for classification according to the prediction model.**

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

- Non specific MTT reduction calculation (NSMTT):
  OD_{ku}: untreated killed tissues
  OD_{kt}: chemical treated killed tissues
  $NSMTT = [(OD_{kt} - OD_{ku}) / OD_{NC}] \times 100$

  Note: NSMTT should be ≤ 50% relative to the negative control OD.
- True MTT metabolic conversion (TOD_{TT}):
  OD_{TV}: chemical treated viable tissues
  TOD_{TT}: true MTT metabolic conversion for treated tissue
  $TOD_{TT} = [OD_{TV} – (OD_{kt} - OD_{ku})]$

Relative viability% tissue1 = [TOD_{TT1} / OD_{NC}] x 100 *Relative viability*% tissue2 = [TOD_{TT2} / OD_{NC}] x 100 *Relative viability*% tissue3 = [TOD_{TT3} / OD_{NC}] x 100

Remark: **If non specific MTT reduction is > 50% relative to the negative control (living *epidermis*), additional steps must be undertaken if possible or the chemical must be considered as incompatible with the test.**

Data calculation for dyes and 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 color (unrelated to mitochondrial activity) and to subtract it before calculations of the “true” viability %.

- Non specific color % (NSC%):
  OD_{CT}: chemical treated tissue (not incubated with MTT)
  OD_{NC}: Control PBS treated tissue (incubated with MTT)
  $NSC\% = [OD_{CT} / OD_{NC}] \times 100$

  Note: NSC should be: 5% < NSC% ≤ 50%. If NSC% is < 5% then use normal calculation mode.
- True MTT metabolic conversion (TOD_{TT}):
  OD_{TV}: chemical treated viable tissues
  TOD_{TT}: true MTT metabolic conversion for treated tissue
  $TOD_{TT} = [OD_{TV} – OD_{CT}]$

Relative viability% tissue1 = $[TOD_{TT1} / OD_{NC}] \times 100$ *Relative viability*% tissue2 = $[TOD_{TT2} / OD_{NC}] \times 100$ *Relative viability*% tissue3 = $[TOD_{TT3} / OD_{NC}] \times 100$

Remark: If non specific OD due to coloration is > 50% relative to the negative control, additional steps must be undertaken if possible or the chemical must be considered as incompatible with the test.

Prediction Model

Irritation potential of test chemical is determined according to the UN GHS and EU CLP as "Category 2: Irritant" or “No Category” (UN, 2009; No. 1272/2008, EU, 2008). An irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.
The prediction model is defined as described below:

***In vitro* results**	***In vivo* classification**
Mean tissue cell viability ≤ 50%	Category 2: Irritant
Mean tissue cell viability > 50%	No Category

Annexes

Pages below contain snapshots from the validation study SOP. The actual MDS sheets are attached to the protocol and can be downloaded and printed separately, if needed. Go to DB-ALM webite (http://ecvam-dbalm.jrc.ec.europa.eu/), section related to protocol No. 131 and select Related information: **Downloads**.

Annex 1: METHODS DOCUMENTATION SHEET (MDS)

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

Main equipment verification

Identification

- Microbiological Safety Cabinet: .....
- Refrigerator: .....
- Spectrophotometer (96 well plate reader):.....

INCUBATOR VERIFICATION

Incubator n°	CO2 (%)	Temperature (°C)	Water bath level (OK)

Date: **ID and signature:**

BALANCE VERIFICATION

N° :	10 mg weight	1g weight
weighing	1 :.....mg 2 :.....mg 3 :.....mg	1 :.....g 2 :.....g 3 :.....g
Mean	.....mg	.....g
Tolerance	**9.9 mg to 10.1 mg**	**999. 5 mg to 1000. 5 mg**

Date: **ID and signature:**

PIPETTES VERIFICATION

Water temperature: .....

Balance N°:	pipette n° ..... 2 mL	pipette n° ..... 500 µL	pipette n°.....	pipette n° ..... 200 µL	pipette n° ..... 10 µL
mg mg mg	1 : 2 : 3 :	1 : 2 : 3 :	1 : 2 : 3 :	1 : 2 : 3 :	1 : 2 : 3 :
Mean					
SD					
CV (%)					
Tolerance	**5%**	**5%**	**5%**	**5%**	**5%**

Date: **ID and signature:**

Annex 2: METHODS DOCUMENTATION SHEET (MDS)

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

EPISKIN KIT RECEIPT

- Receipt date:.....
- Batch number: .....
- Check :
  - Sending date: .....
  - Shipment medium colour : ☐ Orange ☐ Yellow ☐ Violet
  - temperature indicator : ☐ Pale grey ☐ Dark grey
  - Expiration date: .....:
- kit compliance:
  - Control certificate N°: .....
  - Compliance: YES: ☐.....NO: .☐.....

Maintenance medium:

- Batch N°: .....
- Expiration date: .....
- Stocking temperature: 4°C.....
- Stocking place: Refrigerator N°.....

Essai medium :

- Batch N° : .....
- Expiration date: .....
- Stocking temperature: 4°C.....
- Stocking place: Refrigerator N° .....

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

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

Annex 4: METHODS DOCUMENTATION SHEET (MDS)

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

EVALUATION OF CHEMICAL-MTT DIRECT REDUCTION

Test-Chemical Name or code number	Amount Weight (gr) or volume (µl)	MTT solution Volume (ml)	Start Incubation Time:	End of incubation Time:	Interaction Blue Colour Yes / No

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

Annex 4: METHODS DOCUMENTATION SHEET (MDS)

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

EVALUATION OF CHEMICAL-MTT DIRECT REDUCTION

Test-Chemical Name or code number	Amount Weight (gr) or volume (µl)	MTT solution Volume (ml)	Start Incubation Time:	End of incubation Time:	Interaction Blue Colour Yes / No

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

Annex 5: METHODS DOCUMENTATION SHEET (MDS)

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

EVALUATION OF CHEMICAL-MTT REDUCTION ON WATER-KILLED EPIDERMIS

Test Chemical Name or code*	Solids Weight before application mg			Solids Weight after application mg:			Solids Applied Quantity mg			Liquids Volume µl	Start Incubation Time: First repl.	End Incubation Time: Third repl.
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3			

*: add an asterisk if grinding was necessary

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

Annex 6: METHODS DOCUMENTATION SHEET (MDS)

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

EVALUATION OF CHEMICAL TISSUE STAINING NON SPECIFIC COLOR

Test Chemical Name or code*	Solids Weight before application mg			Solids Weight after application mg:			Solids Applied Quantity mg			Liquids Volume µl	Start Incubation Time: First repl.	End Incubation Time: Third repl.
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3			

*: add an asterisk if grinding was necessary

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

Annex 7: METHODS DOCUMENTATION SHEET (MDS)

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

TEST-CHEMICALS APPLICATION ON THE EPIDERMIS

Test Chemical Name or code*	Solids Weight before application mg			Solids Weight after application mg:			Solids Applied Quantity mg			Liquids Volume µl	Start Incubation Time: First repl.	End Incubation Time: Third repl.
	Rep 1	Rep 2	Rep	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3			

*: add an asterisk if grinding was necessary

Date: ..... ID and signature:.....

Annex 8: METHODS DOCUMENTATION SHEET (MDS)

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

SOLUTIONS PREPARATION

- SDS 5% solution in distlilled stérile water(w/v) :
  - SDS, reference,batch N°.....
  - weight: .....
  - Distilled water, reference, batch N°: .....
  - Distilled water, volume added:.....
  - Preparation date: .....
  - Expiration date : .....
  - Stocking place , Refrigerator N°.....
  - Solution N°.....
- MTT stock solution preparation: 3 mg/ml :
  - MTT batch N° : .....
  - Weight : .....
  - PBS batch N°: .....
  - PBS Volume added: .....
  - Preparation date: .....
  - Expiration date : .....
  - Stocking place : Refrigerator N°.....
  - Solution N°.....
- Acidified isopropanol (v/v) :
  - HCl batch N° : .....
  - Volume of HCl 12N used: .....
  - Isopropanol batch N°: .....
  - Volume of isopropanol used: .....
  - Preparation date: .....
  - Expiration date : .....
  - Stocking place , Refrigerator N°.....
  - Solution N°.....

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

Annex 9: METHODS DOCUMENTATION SHEET (MDS)

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

MAIN INCUBATION TIMINGS

Test- chemicals Name or Code	42h post-traitement		3h MTT incubation		Formazan extraction	
	Start time date	End time date	Start time date	End time date	Start time date	End time date

Date: …… ID and signature:……

Annex 10: METHODS DOCUMENTATION SHEET (MDS)

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

OBSERVATIONS AND COMMENTS AFTER 3 h. MTT INCUBATION

Test-chemical	tissue 1: a	tissue 2: b	tissue 3: c

Date: …………………… ID and signature:……………………..

Annex 11: METHODS DOCUMENTATION SHEET (MDS)

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

PLATE CONFIGURATION FOR READINGS (Spread Sheets)

Plate 1

1	Blank
2	Negative control
3	Positive control
4	
5	
6	
7	
8	
9	
10	
11	
12	

Plate 2

1	Blank
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

Plate 3

1	Blank
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

Plate 4

1	Blank
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	

Annex 12: METHODS DOCUMENTATION SHEET (MDS)

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

REMARKS AND COMMENTS PROTOCOLE MODIFICATIONS IF NECESSARY	Date and Signature

Annex 13: METHODS DOCUMENTATION SHEET (MDS)

Laboratory: Study N°: Assay N° :

TEST-CHEMICALS APPLICATION: weight prints

Paste hereafter the weight prints of powder chemicals.

Bibliography

- Botham, P.A., Earl, L.K., Fentem, J.H., Roguet R., and van de Sandt, J.M. (1998)
  Alternative methods for skin irritation testing: the current status.
  Alternatives to Laboratory Animals (ATLA) 26, 195-211
- Cotovio, J., Grandidier, M. H., Portes, P., Roguet, R., Rubinstenn, G. (2005)
  The in vitro skin irritation of chemicals: optimisation of the EPISKIN prediction model within the framework of the ECVAM validation process.
  Alternatives to Laboratory Animals (ATLA) 33, 329-349
- Draize J.H., Woodard G.K. and Calvery H.O. (1944)
  Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes.
  Journal of Pharmacology and Experimental Therapeutics 82, 377-390
- ESAC (2009)
  ESAC Statement on the Performance Standards (PS) for *in vitro* skin irritation testing using reconstructed human epidermis, 31st meeting of ECVAM Scientific Advisory Committee (ESAC), 7-8 July, 2009.
  ECVAM Scientific Advisory Committee.
- ESAC (2009)
  Statement on the performance under UN GHS of three *in vitro* assays for skin irritation testing and the adaptation of the reference chemicals and defined accuracy values of the ECVAM Skin Irritation Performance Standards, 30th meeting of ECVAM Scientific Advisory Committee (ESAC), 9-10 March, 2009.
  ECVAM Scientific Advisory Committee.
- ESAC (2007)
  Statement on the validity of *in vitro* tests for skin irritation, 26th meeting of ECVAM Scientific Advisory Committee (ESAC), 26-27 April, 2007.
  ECVAM Scientific Advisory Committee.
- EU (2009)
  Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). **Link to document** (last access 27.05.2013)
  Official Journal of the European Communities L 220, 1-94
- EU (2008)
  Council Regulation (EC) No 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Annex, part B. Methods for the determination of toxicity and other health effects.
  Official Journal of the European Communities L 142, 141-443
- EU (2008)
  Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP). Entered into force on the 20 January 2009 which will stepwise replace the Council Directive 67/548/EEC (substances), Directive 1999/45/EC (preparations), amending Regulation (EC) No 1907/2006 and subsequent amendments and adaptations. **Link to document** (last access 27.03.2013)
  Official Journal of the European Communities L 353, 1-1355
- EU (2001)
  Commission Directive 2001/59/EC of 6 August 2001 adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances.
  Official Journal of the European Communities L 225, 1-333
- Mosmann, T. (1983)
  Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays.
  Journal of Immunological Methods 65, 55-63
- OECD (2015)
  OECD Test Guideline No. 404: Acute Dermal Irritation/Corrosion. First adopted in 1981. Latest update

adopted on the 28th of July 2015. **Link to document** (last access: 16.06.2016)
OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects

- OECD (2010)
  OECD Test Guideline No. 439: *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method, adopted on the 22nd of July 2010. **Link to document** (last access 16.08.2012)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2009)
  Explanatory Background Document to the OECD Draft Test Guideline on *in vitro* Skin Irritation Testing. *Prepared by Griesinger, C., Barroso, J., Zuang, V., Cole, T., & Manfred, E. G.* **Link to document** (last access 19.06.2013)
  OECD Environmental Health and Safety Publications
- OECD (2004)
  OECD Test Guideline No. 431: *In Vitro* Skin Corrosion: Human Skin Model Test, adopted on the 13th of April 2004. **Link to document** (last access 16.08.2012)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- Portes, P., Grandidier, M.H., Cohen, C., Roquet, R. (2002)
  Refinement of the EPISKIN protocol for the assessment of acute skin irritation of chemicals: follow-up to the ECVAM pravalidation study.
  Toxicology In Vitro 16, 765-770
- Tinois, E., Gaetini, Q., Gayraud, B., Dupont, D., Rougier, A., and Pouradier Duteil, X. (1994)
  The Episkin model: successful reconstruction of human epidermis in vitro.
  Alternative Methods in Toxicology 10, 133-140
- United Nations (2013)
  Globally Harmonised System of Classification and Labelling of Chemicals (GHS); New York and Geneva. Fifth revised edition. **Link to document** (last access 15.01.2014)
- Zuang, V., Balls, M., Botham, P.A., Coquette, A., Corsini, E., Curren, R.D., Elliott, G.R., Fentem, J.H., Heylings, J.R., Liebsch, M., Medina, J., Roguet, R., van de Sandt, J.M., Wiemann, C. and Worth, A.P. (2002)
  Follow-up to the ECVAM Prevalidations Study on *In Vitro* Tests for Acute Skin Irritation.
  Alternatives to Laboratory Animals (ATLA) 30, 109-129
- van de Sandt, J., Roguet, R., Cohen, C., Esdaile, D., Ponec, M., Corsini, E., Barker, C., Fusening, N., Liebsch, M., Benford, D., de Brugerolle de Fraissinette, A., and Fartasch, M. (1999)
  The use of human keratinocytes and human skin models for predicting skin irritation.
  Alternatives to Laboratory Animals (ATLA) 27, 723-743