MatTek LIFE SCIENCES

Protocol

# In Vitro EpiDerm™ Skin Corrosion Test (EPI-200-SCT)

For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm™

*Note: This protocol is based on ZEBET's SOP dated May 7, 2006 (drafted by Manfred Liebsch and Dieter Traue of ZEBET at the BfR and approved by Helena Kandarova).*

*Performing the EpiDerm SCT as outlined fulfills criteria set forth in OECD TG 431.*

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## 1. Rationale and Background

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material, as defined by the Globally Harmonized System (GHS) for Classification and Labeling of Chemical Substances and Mixtures (**1**).

The potential for chemical induced skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. Various systems for classification of corrosive potential are included in international regulatory requirements.

The present test is based on the experience that corrosive chemicals are cytotoxic after a short-term exposure to the stratum corneum of the epidermis, if cytotoxicity is immediately observed after chemical exposure. It is designed to predict and classify skin corrosivity potential of a chemical by using a three-dimensional human epidermis model.

In the year 1998 the EPISKIN and TER *in vitro* corrosivity test were successfully validated and met the acceptance criteria previously defined by the Management Team of the ECVAM International Validation Study (**2**). Because EPISKIN was not available after the Study, a Catch-up (validation study was performed with the epidermis model EpiDerm (**3**).

In 2002 National Coordinators of OECD Test Guideline Programme (WNT) endorsed New Draft Test Guidelines TG430 (TER) and TG431 (Human Skin Model) for *In Vitro* Skin Corrosion Testing which were finally adopted in 2004. In Guideline TG 431 (paragraphs 9 – 11) general functional and performance criteria were defined if other (or new) skin or epidermis models are used in the context of this guideline (**4, 6**).

## 2. Specific Purpose of the Method

This test can reliably discriminate chemicals that are corrosive to skin from non-corrosive chemicals and can therefore be used for the classification of skin corrosion hazard according to the GHS System adopted by the OECD (**1**). This test allows sub-categorisation of corrosive substances and mixtures into optional Sub-category 1A, in accordance with the UN GHS (1), as well as a combination of Sub-categories 1B and 1C (**7**) (**8**) (**9**). This test does not distinguish between Sub-categories 1B and 1C and it is not designed to predict skin irritation potential.

## 3. Basic Procedure

EpiDerm tissues are conditioned by pre-incubation (1 hour or overnight) for release of transport stress related compounds and debris. After pre-incubation tissues are transferred to fresh Maintenance Medium and topically exposed with the test chemicals for 3 min and 1 hr, respectively. Two (alternatively three) tissues each are used per treatment, negative control (NC) and positive control (PC). After exposure tissues are rinsed and blotted and assay medium is replaced by MTT-medium. After 3 hr incubation, tissues are washed with PBS, blotted, and the blue formazan salt is extracted with MatTek extractant solution. The optical density (OD) of the formazan extract is determined spectrophotometrically at 540 -570 nm, and cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin corrosivity potential of the test materials is classified according to the remaining cell viability obtained after 3 minutes or 1 hour exposure with the test chemical.

# 4. Limitations of the Method

One limitation is possible interference of the test substance with the endpoint MTT: A test substance may directly reduce MTT, thus mimicking dehydrogenase activity of the cellular mitochondria. This property of the test substance is only a problem, if at the time of the MTT test (after the chemical has been rinsed off) there is still sufficient amounts of the test substance present on (or in) the tissues. In this case the (true) metabolic MTT reduction and the (false) direct MTT reduction can be differentiated and quantified by a procedure described in section **6.2**.

The method is not designed to be compatible with highly volatile test substances. However, possible toxic interference across plate wells can be avoided by sealing the wells with an adhesive cover sheet or testing volatile chemicals on separate plates.

# 5. Materials

## 5.1 Materials Required for the Experiments

| | |
|---|---|
| Sterile, blunt-edged forceps | For transferring tissues from agarose |
| 500 mL wash bottle | For rinsing tissue after test material exposure |
| 200 mL beaker | For collecting PBS washes |
| Sterile disposable pipettes, pipette tips and pipetters | For diluting, adding, and removing media and test materials. For topically applying test materials to tissues |
| 37°C incubator 5% $CO_2$ | For incubating tissues prior to and during assays |
| Vacuum source/trap (optional) | For aspirating solutions |
| Laminar flow hood (optional) | For transferring tissues under sterile conditions |
| Cotton swabs | For drying the tissue surface |
| Mortar and Pestle | For grinding solids |
| Adjustable Pipette 1 mL | For pipetting assay medium under inserts (1 mL) |
| Pipette 300 µL | For pipetting MTT medium into 24-well plates |
| Pipette 2 mL | For pipetting MTT extraction solution into 24-well plate |
| Pipette 200 µL | For pipetting extracted formazan from 24-well plate into 96 well plate to be used in a plate photometer |
| Pipette 50 µL | For application of liquid test materials |
| Positive displacement pipette 50 µL | For application of semi-solid test materials |
| Sharp spoon (NaCl weight: 25 ± 1 mg) | For application of solids<br>Aesculap, Purchase No.: FK 623<br>MEDKA KG<br>Arzt- und Krankenhausbedarf<br>Bismarrckstr. 101<br>D –10625 Berlin |
| bulb headed Pasteur pipette | To aid leveling the spoon and spreading the chemical |
| Laboratory balance | For pipette verification and checking spoonful weight |
| 96-well plate photometer 570 or 540 nm | For reading OD |
| Shaker for microtiter / MILLICELL® plates | For extraction of formazan |
| Stop-watches | To be used during application of test materials |
| Potassium Hydroxyde, 8 N (Sigma P4494) | To be used as positive control with each kit |
| Dulbecco's Phosphate Buffered Saline (TC-PBS) | Used for rinsing tissues |
| MTT (MTT-100-CON) | For preparing MTT-medium |
| HCl | For pH adjustment of PBS (if applicable) |

| | |
|---|---|
| NaOH | For pH adjustment of PBS (if applicable) |
| Nylon Mesh 200 µm (EPI-MESH) | Use as a spreading aid for liquid test materials, provided a pre-test shows the compatibility of test material and nylon mesh and only for chemicals which are not mechanically spread. |
| $H_2O$, pure (distilled or aqua-pure) | To be used as negative control with each kit |
| Extractant solution (MTT-100-EXT) | For MTT extraction |

## 5.2 EpiDerm™ Skin Corrosion Test Kit Components

EPI-200-SCT kits are shipped from Ashland, MA, USA or from Bratislava, Slovakia on Monday (an alternative shipping procedure – over weekend - is possible). Upon receipt of the EpiDerm tissues, place the sealed 24-well plates and the assay medium into the refrigerator (2-8°C). Place the MTT concentrate containing vial in the freezer (-20°C) and the MTT diluent in the refrigerator (2-8°C).

Record lot numbers of all kit components in the Methods Documentation Sheet (MDS) (see ANNEX A).

### *EPI-200-SCT Kit Components*

| Amount | Item | Contains/Used for: |
|---|---|---|
| 1 | Sealed 24-well plate (EPI-200-SCT) | Contains 24 tissues on agarose |
| 2 | 24-well plates (sterile) | For MTT viability assay |
| 4 | 6-well plates (sterile) | For corrosivity assay |
| 1 bottle, 100 mL | Assay Medium (EPI-100-ASY) | For corrosivity assay |
| 2 bottle, 100 mL | PBS Rinse Solution (TC-PBS) | For rinsing tissues |
| 25 pieces | Nylon Mesh circles 8 mm diameter, 200 µm pore (EPI-MESH) | For spreading test chemicals |
| 1 | MK-24-007-0024 | Skin corrosivity test (SCT) protocol |
| 1 vial, 10 mL | 1% Triton X-100 Solution (TC-TRI) | Skin irritant reference chemical<br>Do not use in present method |

### *MTT-100 Assay Kit Components (ready-to-use kit)*

| | | |
|---|---|---|
| 1 vial, 2 mL | MTT concentrate (MTT-100-CON) | Frozen MTT concentrate (5 mg/mL) |
| 1 vial, 8 mL | MTT diluent (MTT-100-DIL) | For diluting MTT concentrate prior to use in the MTT assay |
| 1 bottle, 60 mL | Extractant Solution (MTT-100-EXT) | For extraction of formazan |

### *Expiration and Kit Storage*

| Part # | Description | Conditions | Shelf life* |
|---|---|---|---|
| EPI-200-SCT* | EpiDerm cultures | refrigerator (2-8°C) | 96 hours |
| EPI-100-ASY | Assay medium | refrigerator (2-8°C) | 14 days |
| MTT-100-DIL** | MTT diluent | refrigerator (2-8°C) | 2 months |
| MTT-100-CON** | MTT concentrate | freezer (-20 ± 5°C) | 2 months |
| *Refers to storage time @ 2-8°C in unopened package.<br>** MTT-100 kits must be ordered separately. | | | |

*Note: Examine all kit components for integrity. If there is a concern call MatTek immediately.*

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|---|---|---|

# 6. Methods

## 6.1 Tissue and Medium Storage

For the EpiDerm reconstructed epidermal model and Medium refer to the relevant Technical Data and Safety Sheet, located in the plastic file inside the shipping box.

If the test is not performed on the day of receipt, store the EpiDerm™ tissues in the refrigerator at 4°C until next day. If you plan to determine any additional endpoints to MTT viability measurements, place the tissues immediately upon arrival into the EpiDerm Maintenance Medium and pre-incubate 1 hour (37°C, 5%. $CO_2$, humidified atmosphere). Afterwards replace the medium and continue with overnight pre-incubation.

Store Maintenance Medium at 4°C in the dark. The shelf life is limited (see Technical Data file in the shipping box). Use EpiDerm Maintenance Medium at room temperature, do not pre-heat.
Record lot numbers of all components in the Methods Documentation Sheet (see **ANNEX A**)

## 6.2 Test for Interference of Chemicals with MTT Endpoint and Correction Procedures

As specified in Section 4, a test substance may interfere with the MTT endpoint if: a) it is colored and/or b) able to directly reduce MTT (for possible combination of interactions, see **Annex B**). The MTT assay is affected only if the test material is present in the tissues when the MTT viability test is performed.

Some non-colored test materials may change into colored materials in wet or aqueous conditions and thus stain tissues during the 60 min exposure. Therefore, before exposure, a functional check for this possibility should be performed (**Step 1**).

**Step 1**
Add **50 µL** (liquid) or **25 mg** (solid - using a sharp spoon as per Section **7.2**) of the test substance into 0.3 mL of deionized water. Perform the test in a transparent, preferably glass test-tube since plastic test tubes may react with the test articles during the incubation time. Incubate the mixture in the incubator (37±1°C, 5±1 % $CO_2$, 95% RH) for 60 min. At the end of the exposure time, shake the mixture and evaluate the presence and intensity of the staining (if any). If the solution changes color significantly, the test substance is presumed to have the potential to stain the tissue. A functional check on viable tissues should be performed (**Step 2**).

**Step 2**
To check the tissue-binding of a **colored test article** (or a chemical that changes into a colored substance), expose one viable tissue to **50 µL** of liquid or **25 mg** of solid test substance. In parallel, expose a tissue to DPBS (negative control). Follow all procedures as described in this SOP Section 6.7 except incubate the tissue for 3-h incubation in culture media without MTT (37±1°C, 5±1% $CO_2$, 95% RH) instead of incubating in media containing MTT. After the 3 hour incubation, rinse the tissues and extract the tissues using 2.0 mL of isopropanol and measure the optical density (OD) at 570 nm.

*Note: If the colored test substance does not completely rinse off, pipette 1.0 mL of the extracting agent into each well so that the MTT is extracted through the bottom of the tissue culture insert. After extraction is complete, remove the insert and add an additional 1.0 mL of extractant to bring the total volume to 2.0 mL.*

**Data correction procedure – colored substances**
If the extract from tissues treated by colored substance (or substance detected in step 1) has an OD between 5% and 30% of the negative control tissue (treated with PBS), the chemical should be further tested on more tissues using the procedure described above. The real MTT OD (unaffected by interference with the colored test materials) is calculated using following formula:

**OD = OD colored tissue (MTT assay) – OD colored tissue (no MTT assay)**

*Note: If the extract from tissues treated by colored substance (or substance detected in step 1) has an OD <5% of the PBS treated control tissue and the tissue viability (determined in MTT assay) is not close to the classification cut-off (50%), correction of the results is not necessary.*

If OD of extract from the tissue treated by colored substance (or substance detected in **Step** 1) is > 30% of the PBS treated control tissue, additional steps and expert judgment must be performed to determine if the test substance must be considered as incompatible with the test.

**Step 3**
All test materials (including those already evaluated in **Step 1** and **Step 2**) should be further evaluated for their **potential to interfere with MTT assay**. To test if a material directly reduces MTT, add **50 µL** (liquid) or **25 mg** (solid - using sharp spoon **7.2**) of the test substance to 1 mL of the MTT medium and incubate in the incubator (37±1°C, 5±1 $CO_2$, 95% RH) for 60 min. Untreated MTT medium is used as control. If the MTT solution turns blue/purple, the test substance reduces MTT and additional functional check (**Step 4**) must be performed.

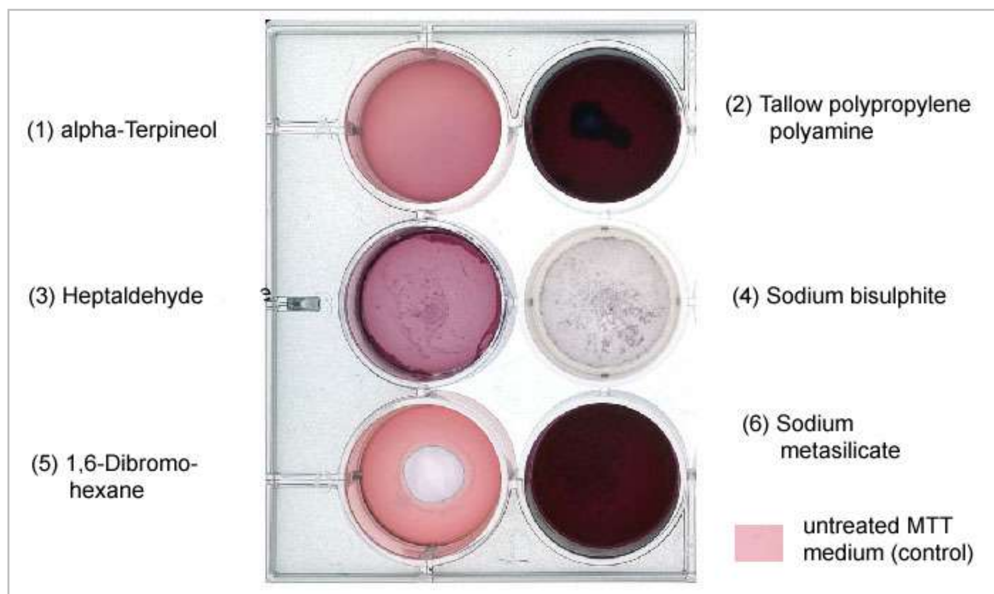


**Figure 1**: Example of test for direct MTT reduction ability (Step 3). Test substances (2) (3) and (6) have directly reduced MTT. In these cases, Step 4 (below) must be performed.

**Step 4**:
The procedure employs freeze-killed tissues that possess no metabolic activity but absorb and bind the test substance similar to viable tissues.

- Freeze-killed tissues (part # EPI-200-FRZN-EA) can be ordered separately from MatTek Corporation (US) or MatTek In Vitro Life Science Laboratories (Slovakia). The frozen tissues may be stored indefinitely in the freezer (-20 ± 5°C).
- Each MTT reducing chemical is applied to two freeze-killed tissues. In addition, two freeze killed tissues are left untreated (Note: The untreated killed controls will show a small amount of MTT reduction due to residual reducing enzymes within the killed tissue). The entire assay protocol is performed on the frozen tissues in parallel to the assay performed with the live EpiDerm tissues. Data are then corrected as follows:

**Data correction procedure – MTT reducers**

**True viability = Viability of treated tissue – Interference from test chemical = OD tvt – OD kt**
**where OD kt = (mean OD tkt – mean OD ukt)**

tvt = treated viable tissue — kt = killed tissues
tkt = treated killed tissue — ukt = untreated killed tissue (NC treated tissue)

If the interference by the test substance is greater than 30% of the negative control value, additional steps must be taken into account or the test substance may be considered incompatible with this test system (expert judgment).

If the interference by the test substance is < 30% of the negative control value, the net OD of the test substance treated killed control may be subtracted from the mean OD of the test substance treated viable tissues to obtain the true amount of MTT reduction that reflects metabolic conversion only.

*Note 1: If the colored test material or the MTT reducing chemical is classified as irritant (tissue viability <50 %) in the EpiDerm Skin Irritation test (MatTek protocol # MK-24-007-0023), the correction procedures are not necessary.*

*Note 2: Frozen tissues (EPI-200-FRZN-EA) can be ordered separately from MatTek Corporation and MatTek In Vitro Life Science Laboratories.*

## 6.3 Test for Mesh Compatibility (Liquid Test Substances Only)

Since the surface of the EpiDerm tissues is slightly hydrophilic, spreading of highly lipophilic substances might sometimes present a problem due to the surface tension effects. Therefore, a nylon mesh (see 5.1) can be used as a spreading support if mechanical re-spreading is not efficient. Some chemicals, however, may react with the mesh and therefore the compatibility of test chemical with nylon mesh must be performed. To test if a test chemical interacts with the mesh, place the mesh on a glass slide and apply 50 µL of test substance. After 60 minutes exposure, check using a microscope. If an interaction between test substance and the mesh is noticed (**Figure 2**), the test substance has to be applied without the mesh.

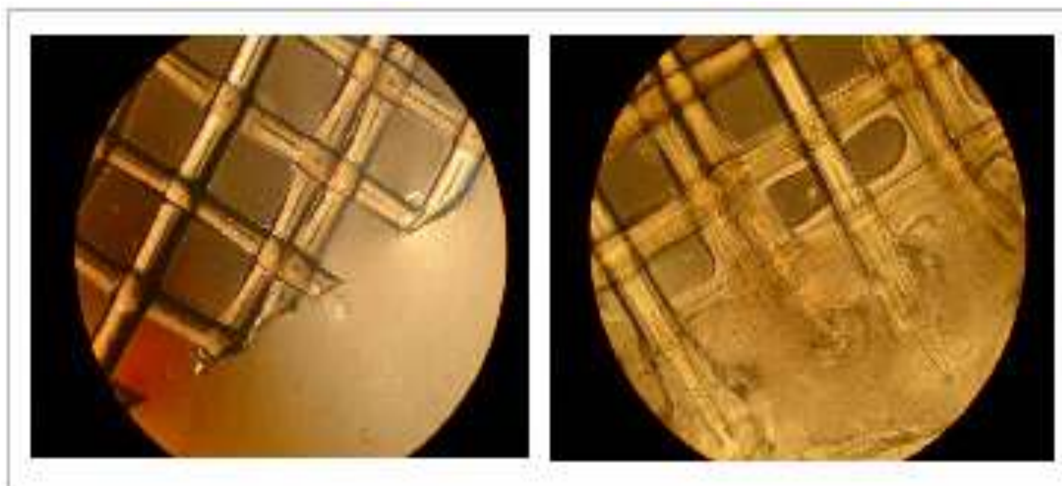

**Figure 2: The mesh compatibility test**

*Note: The mesh (25 pieces) is included in the EPI-200-SCT kit from MatTek Corporation and MatTek In Vitro Life Science Laboratories.*

## 6.4 Preparations

### 6.4.1 MTT Medium (Prepare Fresh on Day of Testing!)

If you buy MTT-100 kit (ready-to-use kit) from MatTek, thaw the MTT concentrate (MTT-100-CON) and dilute with the MTT diluent (MTT-100-DIL). Store the remaining MTT solution in the dark at 4°C for later use on the same day (do not store until next day since MTT will degrade with time).

If you are not using the MTT-100 kit provided by MatTek, prepare the stock solution (5 mg/mL) of MTT in DPBS. Stock solution can be stored frozen (-20 ±5°C) for up to 2 months. Before use, filter the stock solution and dilute the filtrate with the assay medium to final concentration (1 mg/mL). Record the preparation in the MDS. Do not store the diluted MTT solution overnight.

**Safety precaution**: MTT is toxic (Risk phrases: H315, H319, H335, H341). Wear protective gloves during manipulation with MTT solution!

*Note: MTT is light sensitive. Protect all solutions from light*

### 6.4.2 Dulbecco's PBS

Sterile ready-to-use DPBS should be used. About two liters are sufficient for all rinsing performed with one kit. If PBS is prepared from powder or 10x concentrated PBS is used, prepare according to supplier instructions and adjust to pH 7.0 with either NaOH or HCl. Record the pH adjustment in the MDS.

### 6.4.3 Test Substances

**Safety Instruction**

a) For handling of non-coded test substances follow instructions given in the Material Safety Data Sheet.

b) If coded chemicals are supplied, no (or possibly incomplete) information regarding the safe handling will be provided. Therefore, all test materials must be treated as if they were corrosive and toxic and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).

c) Store all test substances according to recommendations. Respect special store conditions (special temperature, protection from light, protection from oxidization by nitrogen, etc.)

**Liquids**: Dispense **50 µL** directly atop the EpiDerm tissue and spread with bulb headed Pasteur pipette. If necessary, gently place a nylon mesh (8 mm diameter) on the surface (see **Figure 3**, picture b). *Record the use of mesh as spreading tool in the MDS*.

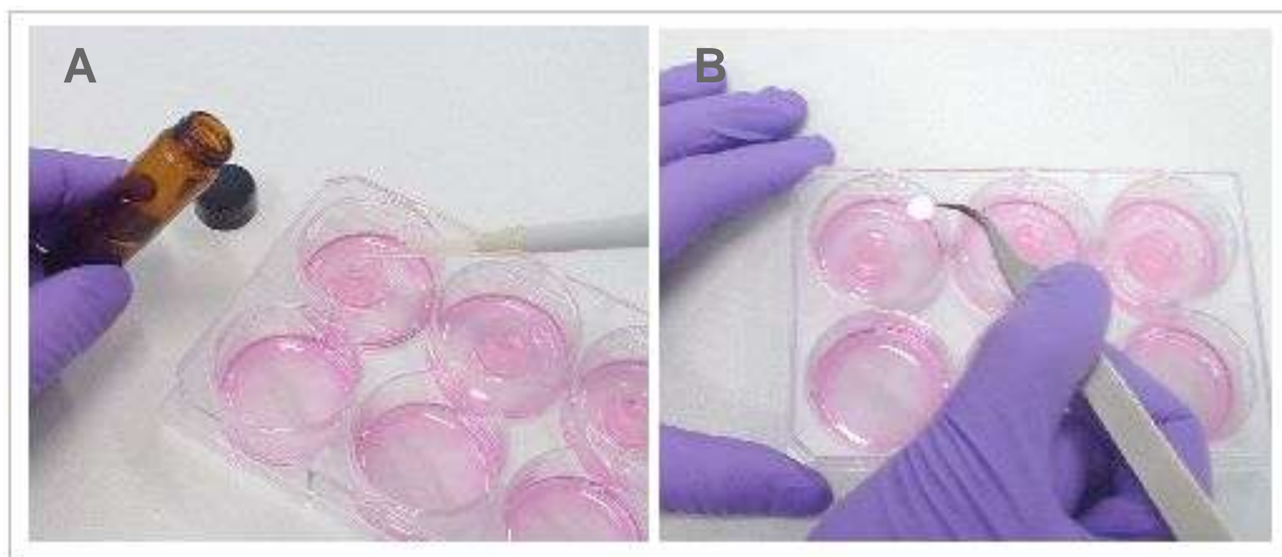


**Figure 3**: Application of liquids.

**Semisolids**: Dispense **50 µL** using a positive displacement pipette directly atop the tissue. If necessary, spread to match size of tissue. *Record the use of spreading in the MDS*.

**Figure 4:** Application of semisolids.

**Solids**: Crush and grind test material with a mortar with pestle wherever this improves the consistency. Fill **25 mg** sharp application spoon* (see **5.1** and **Figure 5**) with fine ground test material. Level the spoon by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material. "Packing" can be avoided by using a rod-shaped spoon instead of a flat spatula. If a bulb headed sound is used the bulb can be used to empty the spoon completely. Add the **25 µL of $H_20$** necessary for wetting of the test material to increase tissue surface contact. Increase the volume of $H_20$ in case of materials where this is not enough for wetting. If necessary, spread material to match the size of the tissue. Record in the MDS if grinding was not used and the $H_20$ volume necessary to wet the chemical.

**Figure 5:** Application of solids.

**Waxes:** For test substances with **waxy consistence** the spoon application does not work. In these cases, try to form a flat "disc like" piece of about 8 mm diameter and place it atop the tissue, wetted with **15 µL $H_2O$**. To improve the contact between test substance and tissue weigh down the "disc" with a stainless steel aid like that shown in **Figure 6**.

**Figure 6:** Stainless steel aid.

**Note: Since the surface of the solid covering the tissues is more important than the weight, the "leveled spoon technique" is an accepted dosing procedure. The spoon used here has been calibrated to equal 25 mg of fine grinded NaCl. The weight will be different if other materials are used.*

*****Note:** determine in a pre-test a volume of $H_2O$ necessary to wet test chemical.*

## 6.5 Prediction Model

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after a **3 min** treatment compared to the negative control tissues concurrently treated with $H_20$. A chemical is classified "**corrosive**" if the relative tissue viability after **3 min** treatment with a test material is decreased below 50%. In addition, materials are classified "**corrosive**" if the relative tissue viability after **1 hr** treatment with a test material is decreased below 15%.

| **Mean tissue viability** (expressed as % of negative control) | **Prediction** |
|---|---|
| 3 min < 50% | corrosive |
| 3 min ≥ 50% **and** 1 hour: < 15% | corrosive |
| 3 min ≥ 50% **and** 1 hour: ≥ 15% | non-corrosive |

For (optional) sub-categorisation of chemicals which are initially classified as "corrosive", a chemical is classified as Sub-category 1A if the tissue viability was < 25% after the 3 min treatment and is classified as a combination of sub-categories 1B/1C if the tissue viability was ≥ 25%.

| **Mean tissue viability** (expressed as % of negative control) | **Prediction** |
|---|---|
| 3 min < 25% | Sub-category 1A |
| 3 min ≥ 25% | A combination of Sub-categories 1B/1C |

## 6.6 Assay Quality Controls

### 6.6.1 Assay Acceptance Criterion 1: Negative Control (NC)

The **absolute OD** of the $H_2O$ treated NC tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping and storing procedure and under specific conditions of the assay. Tissue viability is meeting the acceptance criterion if the mean OD of the mean of NC is **OD** ≥ **0.8**.

### 6.6.2 Assay Acceptance Criterion 2: Positive Control (PC)

**8N KOH** (**Sigma** # P4494) is used as PC and must be tested once on each testing day. The mean tissue viability following 1 hour exposure of the PC must be ≤ 15%.

### 6.6.3 Assay Acceptance Criterion 3: Difference (Diff) and/or Coefficient of Variation (CV)

Based on the test design (explained in 6.7), the experiments are performed on two or three tissue replicates per exposure time. In the range 20 - 100% viability, the Difference (Diff) of viability between the two tissue replicates should not exceed 30%, or if using three tissue replicates per time point, the Coefficient of Variation (CV) between the tissue replicates must be ≤ 30%.

## 6.7 Experimental Procedure

***Introductory Note:***

*The experiment can be performed using either: a) two tissues per timepoint (as in the validation study), or b) three tissues per timepoint. Alternative b) is recommended mainly for laboratories establishing the test. Examples of both test design are given below.*

***ALTERNATIVE A**) **24 tissues** (1 EPI-200-SCT kit) are used for testing **4 test chemicals,** negative control and positive control, each of them applied both for **3 min** and **1 hr** to **two tissue replicates** per treatment.*

***ALTERNATIVE B**) **36 tissues** (1 EPI-200-SCT and 1 EPI-212-SCT kit) are used for testing **4 test chemicals,** negative control and positive control, each of them applied both for **3 min** and **1 hr** to **three tissue replicates** per treatment..*

**Day Prior To Testing**

- If the EpiDerm tissues are not used in the day of receipt, keep the originally sealed plates until the next day in the refrigerator at 4°C.
- If necessary, prepare sufficient amount of rinsing PBS for the next day according to **6.4.1**

*Note: Since the present test is a short-term test which makes use of the EpiDerm model over a period of only 5 hours, sterility is not as important as is in other applications of the EpiDerm model. Nevertheless, it is important to keep assay media sterile and to keep risk of contamination at a low level.*

## ALTERNATIVE A) – Example With 4 Chemicals, Two Tissues Per Timepoint

### Day of Testing

### Tissue Conditioning (Pre-incubation)

a) Prepare **two** 6-well plates for **four** chemicals and the negative (NC) and positive control (PC) for the **3 min application**. Pipette 0.9 mL Maintenance Medium in each well.

b) Prepare **two** 6-well plates for **four** chemicals and the negative (NC) and positive control (PC) for the **1 hr application**. Pipette 0.9 mL Maintenance Medium in each well.

c) Remove the shipped multiwell plate from the plastic bag. Open the 24-well plate under a sterile airflow and remove the sterile gauze. Carefully take out each insert containing the epidermal tissue, rapidly remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper, and immediately place it in a well of the prepared 6-well plate. Act quickly as the epidermal cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert!

d) Mark the 6-well plates as shown in **Figure 7**.

e) Place the 6-well plates containing the tissues into a humidified (37°C, 5% $CO_2$) incubator for 1 hour.

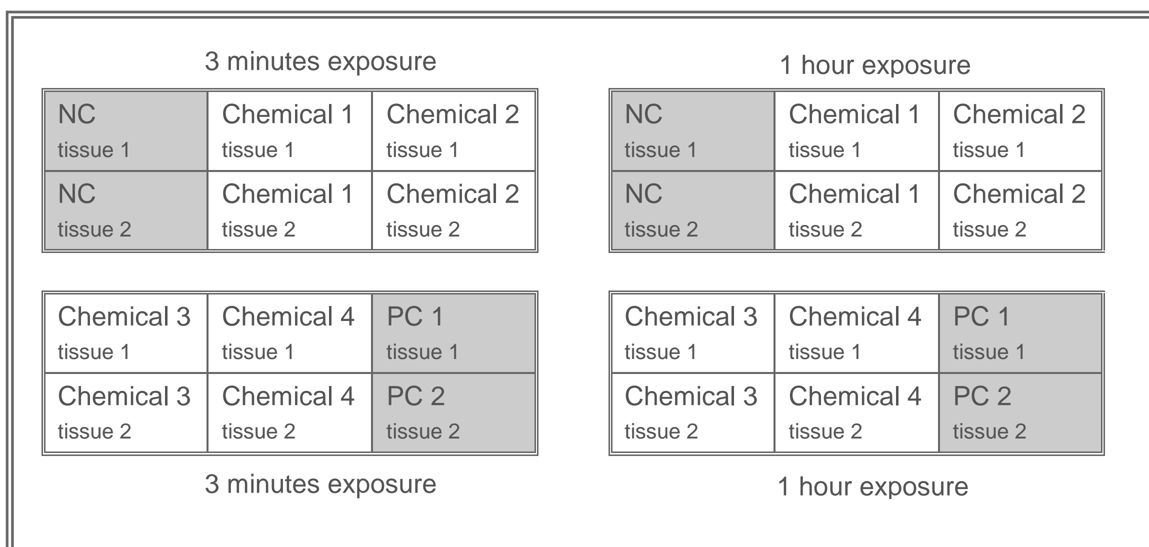


**Figure 7:** 6-well plate design

### Preparations for the Main Test (Make During the 1 Hr Pre-incubation Time)

a) Prepare the MTT medium according to 6.4.1.

b) Prepare two 24-well plates to be used as “holding and “MTT plates” one for the 3 min experiment, the other for the 1 hr experiment.

c) Use the plate design shown in **Figure 8**. Pipette 300 μL of either maintenance medium or MTT medium in each well.

d) Place the plates in the incubator.

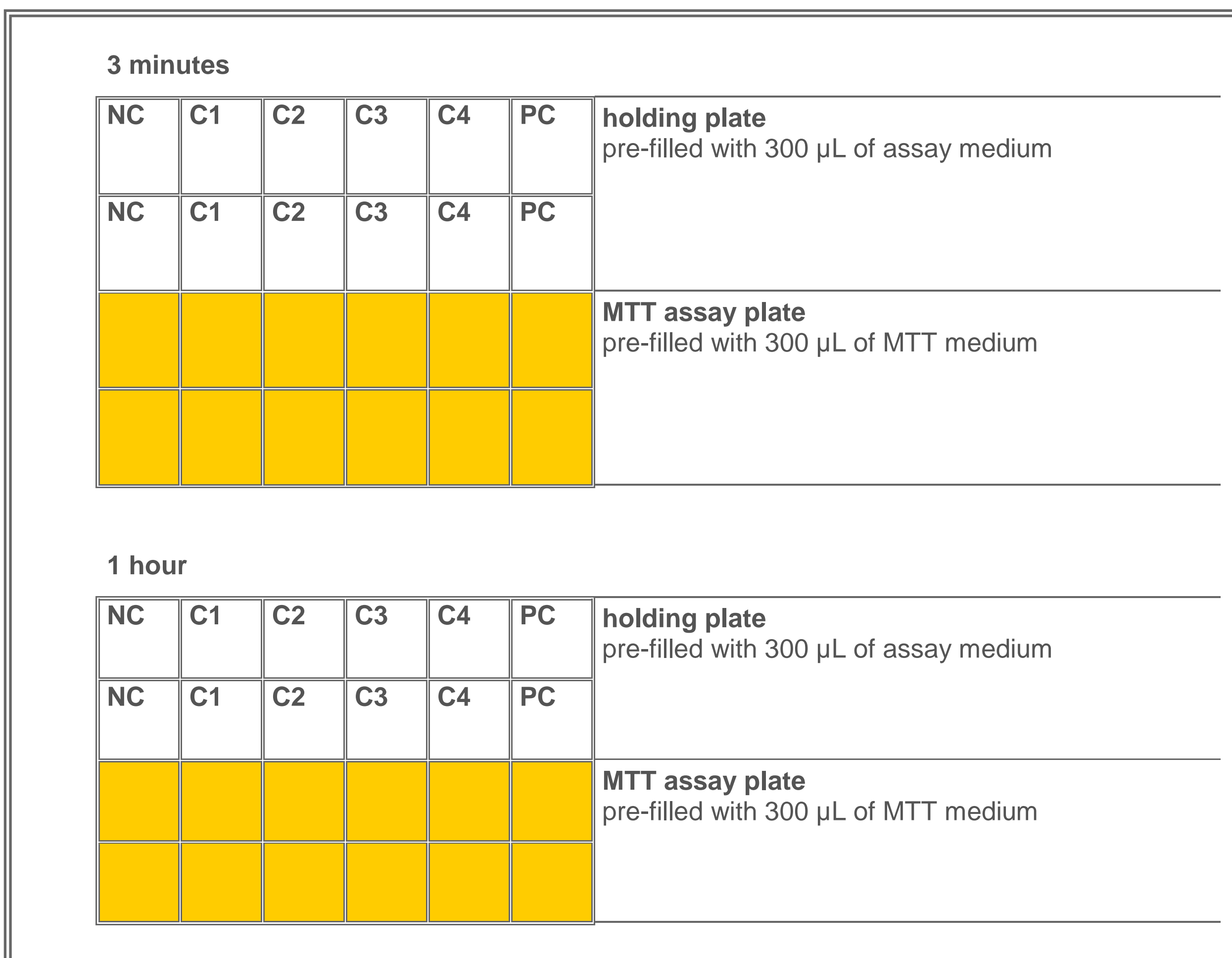


**Figure 8**: 24-well plate design. "Holding and MTT plate", both for the 1 hr experiment and the 3 min experiment. NC = negative control; C1 – C4 = test chemicals 1-4; PC = positive control.

### 1-Hour Application

***Note:*** *Dosing time interval is dictated by rinsing procedure. If the technician has performed the test already, 45 seconds intervals is sufficient for both application and washing procedures. However, if the test is performed for the first time, 1 minute dosing interval is recommended.*

a) After 1 hour of pre-incubation, transfer each insert to new 6-well plates with fresh medium (0.9 mL per well). Alternatively, aspirate the pre-incubation medium from the 6-well plates and pipet 0.9 mL of fresh medium into each well.

b) Set the timer to 1 hr and start it. Add 50 µL of $H_2O$ (negative control) into the first insert atop the tissue. After 45 sec repeat the procedure with the second tissue. Proceed with test material 1 - 4 (50 µL: liquids, 25 mg + 25 µL $H_2O$: solids) and the positive control in the same manner until all tissues are dosed.

c) Dosing interval scheme for the 1 hour experiment:

| | |
|---|---|
| 0.00-0.45 – tissue 1 (NC) | 4.30-5.15 – tissue 7 (C3) |
| 0.45-1.30 – tissue 2 (NC) | 5.15-6.00 – tissue 8 (C3) |
| 1.30-2.15 – tissue 3 (C1) | 6.00-6.45 – tissue 9 (C4) |
| 2.15-3.00 – tissue 4 (C1) | 6.45-7.30 – tissue 10 (C4) |
| 3.00-3.45 – tissue 5 (C2) | 7.30-8.15 – tissue 11 (PC) |
| 3.45-4.30 – tissue 6 (C2) | 8.15-9.00 – tissue 12 (PC) |

d) Place the 6-well plates into the incubator (37°C, 5% $CO_2$) for the rest of the exposure time until 1 hour exposure is reched for first tissue dosed. Record start time in the MDS.

e) After the 1 hour period of test material exposure is complete, with forceps remove the first insert from the 6-well plate. As shown in **Figure 9**, using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom on blotting paper. Place the insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all tissues are rinsed. Rinse all tissues in an interval of 45 sec.

**Figure 9:** Rinsing: fill and empty 20 x

f) Once all tissues have been rinsed and are in the holding plate, dry the surface with cotton swab, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay.

g) Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for 3 hours (37°C, 5% $CO_2$).

### 3 Min Application

**Note:** Dosing time interval is dictated by rinsing procedure. If the technician performs test already routinely, 45 seconds interval is sufficient for both application and washing procedures. However, if the test is performed for the first time, 1 minute dosing interval is recommended.

a) Start the timer for 3 min. Add 50 µL $H_2O$ (negative control) into the first insert atop the tissue. After 45 seconds repeat the procedure with the second tissue. Following 45 seconds intervals enable to dose 4 tissues. Afterwards, washing of the tissue 1 have to start.

b) After the 3 min period of exposure for the first four tissues is complete, start the timer for 3 min and with forceps remove the first insert from the 6-well plate. As shown in **Figure 9**, using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom on blotting paper. Place the insert in the prepared holding plate. After 45 seconds repeat the procedure with the second insert, after 1:30 minutes with the third tissue...etc.

**Dosing interval scheme for the 3 min experiment:**

SET 1 = NC and chemical 1 (6 minutes)

**Dosing:**
0.00-0.45 – tissue 1 (NC)
0.45-1.30 – tissue 2 (NC)
1.30-2.15 – tissue 3 (C1)
2.15-3.00 – tissue 4 (C1)

**Rinsing**
3.00-3.45 – tissue 1 (NC)
3.45-4.30 – tissue 2 (NC)
4.30-5.15 – tissue 3 (C1)
5.15-6.00 – tissue 4 (C1)

SET 2 = chemical 2, 3 (6 minutes)
SET 3 = chemical 4, PC (6 minutes)

c) Proceed with all test materials (50 µ: liquids, 25 mg + 25 µL $H_2O$: solid) and the positive control in the same manner until all tissues are dosed and rinsed.

d) Once all tissues have been rinsed and are in the holding plate, carefully dry the surface of the tissue with a cotton swab. Afterwards remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plates in the incubator, record start time of MTT incubation in the MDS and incubate for 3 hours (37°C, 5% $CO_2$).

## ALTERNATIVE B) – Example With 4 Chemicals, Three Tissues Per Timepoint

### Day of Testing

### Tissue Conditioning (Pre-Incubation)

a) Prepare three 6-well plates for four chemicals and the negative (NC) and positive control (PC) for the 3 min application. Pipette 0.9 mL Maintenance Medium in each well.

b) Prepare three 6-well plates for four chemicals and the negative (NC) and positive control (PC) for the 1 hr application. Pipette 0.9 mL Maintenance Medium in each well.

c) Remove the shipped multiwell plate from the plastic bag. Open the 24-well plate under a sterile airflow and remove the sterile gauze. Carefully take out each insert containing the epidermal tissue, rapidly remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper, and immediately place it in a well of the prepared 6-well plate. Act quickly as the epidermal cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert!

d) Mark the 6-well plates as shown in **Figure 10**.

e) Place the 6-well plates containing the tissues into a humidified (37°C, 5% $CO_2$) incubator for 1 hour.

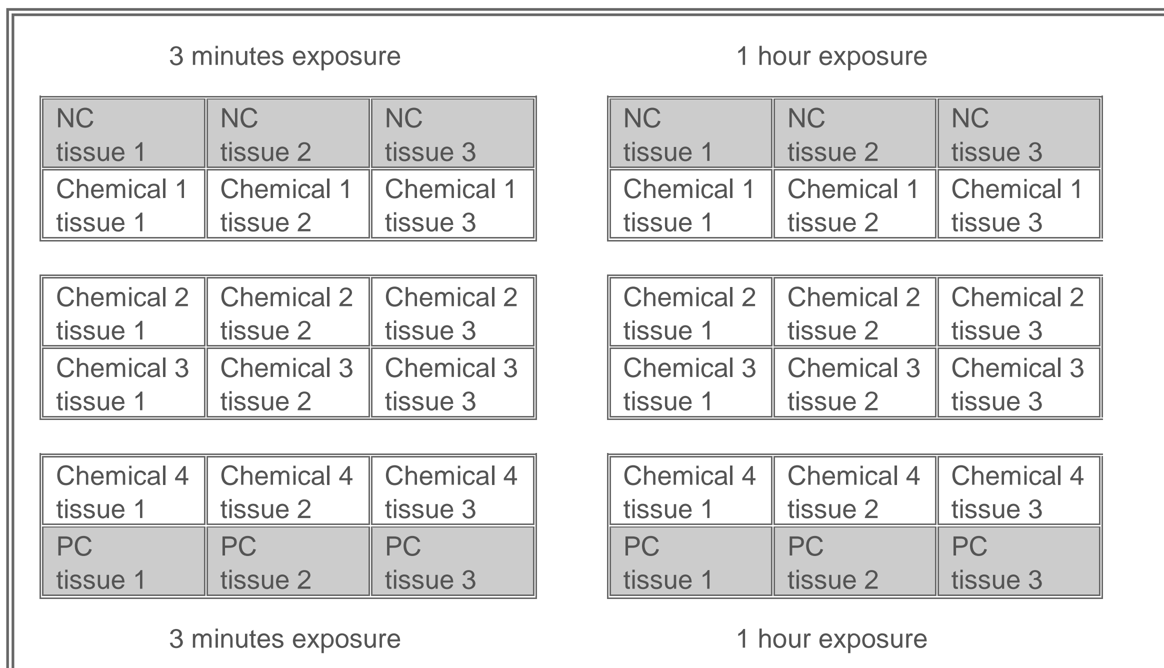


**Figure 10**: 6-well plate design

**Preparations for the main test (make during the 1 hr pre-incubation time)**

a) Prepare MTT medium according to 6.4.1.

b) Prepare two 24-well plates to be used as "holding plates." One for the 3 min experiment, the other for the 1 hr experiment. Pipette 300 µL of maintenance medium into each well of the holding plates. In addition, prepare two 24-well plates for the MTT assay. Pipette 300 µL of MTT medium in each well of the MTT assay plate.

c) Use the plate design shown in **Figure 11**.

d) Place the plates in the incubator.

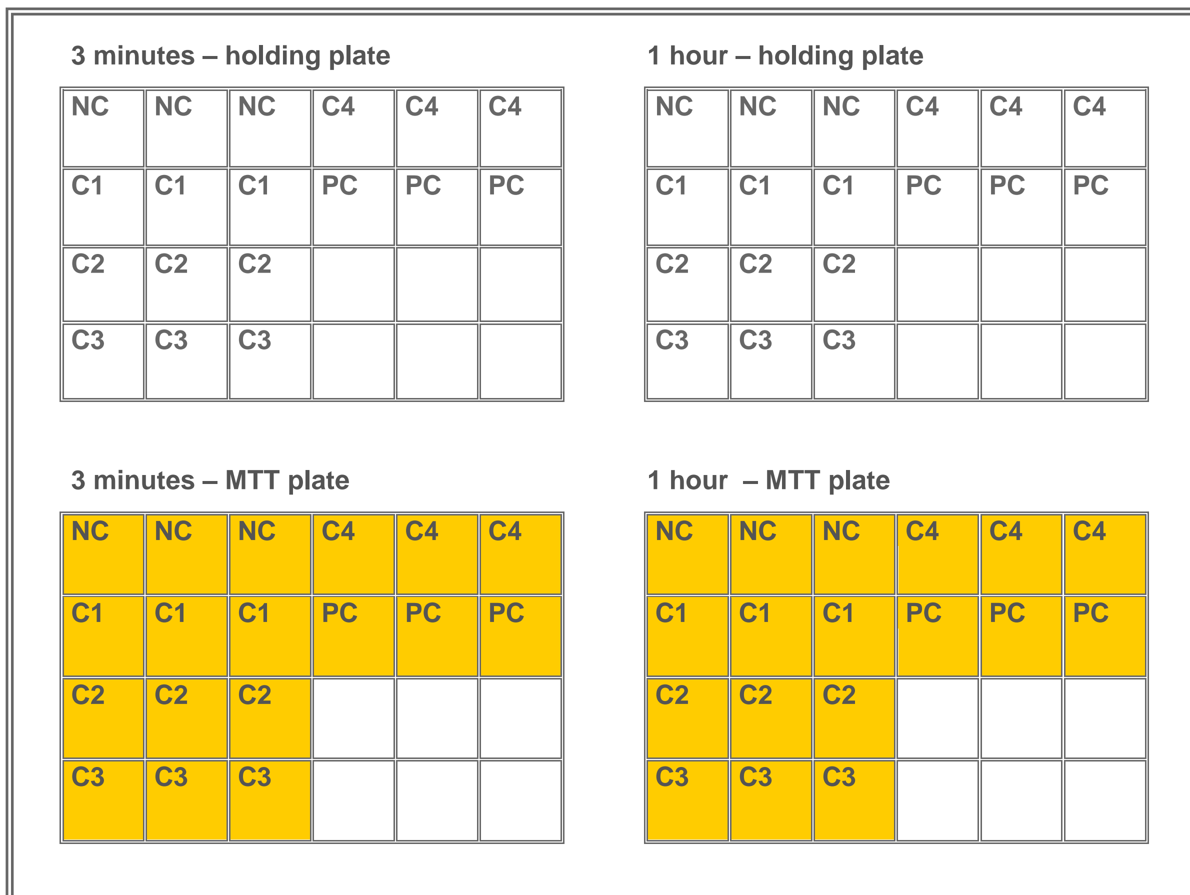


**Figure 11:** 24-well plate design. "Holding and MTT plates", both for the 3 min and 1 hour experiment. NC = negative control; C1 – C4 = test chemicals 1- 4; PC = positive control

### 1-hour application

*Note: Dosing time interval is dictated by rinsing procedure. In this test design, a 1 minute dosing interval is recommended.*

a) After 1 hour of pre-incubation, transfer each insert to new 6-well plates with fresh medium (0.9 mL per well). Alternatively, aspirate the pre-incubation medium from the 6-well plates and pipet 0.9 mL of fresh medium into each well.

b) Set a timer to 1 hr and start it. Add 50 µL of $H_2O$ (negative control) into the first insert atop the tissue. After 60 sec repeat the procedure with the second tissue. Proceed with test material 1 - 4 (50 µL: liquids, 25 mg + 25 µL $H_2O$: solids) and the positive control in the same manner until all tissues are dosed.

**Dosing interval scheme for the 1 hour experiment, alternative b):**

0.00-1.00 – tissue 1 (NC)
1.00-2.00 – tissue 2 (NC)
2.00-3.00 – tissue 3 (NC)
3.00-4.00 – tissue 4 (C1)
4.00-5.00 – tissue 5 (C1)
5.00-6.00 – tissue 6 (C1)
6.00-7.00 – tissue 7 (C2)
7.00-8.00 – tissue 8 (C2)
8.00-9.00 – tissue 9 (C2)
9.00-10.00 – tissue 10(C3)
10.00-11.00 – tissue 11 (C3)
11.00-12.00 – tissue 12 (C3)
12.00-13.00 – tissue 13 (C4)
13.00-14.00 – tissue 14 (C4)
14.00-15.00 – tissue 15 (C4)
15.00-16.00 – tissue 16 (PC)
16.00-17.00 – tissue 17 (PC)
17.00-18.00 – tissue 18 (PC)

c) Place the 6-well plates into the incubator (37°C, 5% $CO_2$) for the remaining exposure time until 1 hour exposure is reached for first tissue dosed. Record start time in the MDS.

d) After the 1 hour period of test material exposure is complete, use forceps to remove the first insert from the 6-well plate. As shown in **Figure 9**, using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot the bottom on blotting paper. Place the insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all tissues are rinsed. Rinse all tissues in an interval of 60 sec.

e) Once all the tissues have been rinsed and are in the holding plate, dry the tissue surface with a cotton swab. Remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay.

f) Place the plate in the incubator, record start time of MTT incubation in the MDS and incubate for 3 hours (37°C, 5% $CO_2$).

### 3 Min Application

***Note:*** *Dosing time interval is dictated by rinsing procedure. In this test design, 1 minute dosing interval is recommended.*

a) Start the timer for 3 min. Add 50 µL of $H_2O$ (negative control) into the first insert atop the tissue. After 60 seconds repeat the procedure with the second tissue. Following 60 seconds interval enable to dose 3 tissues (= 1 test substance).

b) After the 3 min period of exposure for the first three tissues is complete, remove the first insert from the 6-well plate. As shown in **Figure 10**, using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot the bottom on blotting paper. Place the insert into the prepared holding plate. After 60 seconds repeat the procedure with the second insert....etc.

**Dosing interval scheme for the 3 min experiment:**

NC (6 minutes)
**Dosing:**
0.00-1.00 – tissue 1 (NC)
1.00-2.00 – tissue 2 (NC)
2.00-3.00 – tissue 3 (NC)

**Rinsing**
3.00-4.00 – tissue 1 (NC)
4.00-5.00 – tissue 2 (NC)
5.00-6.00 – tissue 3 (NC)

chemical 1 = 6 minutes
chemical 2 = 6 minutes
chemical 3 = 6 minutes
chemical 4 = 6 minutes
PC = 6 min
Total duration of the test: 36 min

c) Proceed with all test materials (50 µL: liquids, 25 mg + 25 µL $H_2O$: solid) and the positive control in the same manner until all tissues are dosed and rinsed.

d) Once all the tissues have been rinsed and are in the holding plate carefully dry the tissue surface with a cotton swab. Afterwards remove inserts from the holding plate, blot the bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, and incubate for 3 hours (37°C, 5% $CO_2$). Record the start time of MTT incubation on the MDS.

### MTT Test and Reading – Both test designs

a) After the 3 hour MTT incubation period is complete, gently aspirate MTT from all wells (e.g. using a suction pump), refill wells with PBS and aspirate. Repeat the rinsing twice and make sure that tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.

b) Immerse the inserts by gently pipetting 2 mL extractant solution (MTT-100-EXT) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.

c) Seal the 24 well plate (e.g. with a zip bag) to inhibit the extractant solution evaporation. Record the start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.

d) After the extraction period is complete, pierce the inserts with an injection needle and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Place the 24-well plates on a shaker for 15 minutes until solution is homogeneous in colour.

e) Per each tissue transfer 200 µL of the blue formazan solution into a 96-well flat bottom microtiter plate, both from the 3 min exposure and from the 1 hr exposure. For the 96-well plate, use the exact plate design given in **Figure 12**, as this configuration is used in the data spreadsheets. Read the OD in a spectrophotometer at 570 nm, without reference filter. Alternatively, ODs can be read at 540 nm.

***Note**: Readings are performed without a 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 may have a ± tolerance in some cases the reference filter reduces the dynamics of the signal (OD) up to 40%.*

### Fixed 96 well-plate design for OD readings

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | NC-T1 | PC-T1 | C1-T1 | C2-T1 | C3-T1 | C4-T1 | C5-T1 | C6-T1 | C7-T1 | C8-T1 | C9-T1 | C10-T1 | 3 min |
| B | NC-T1 | PC-T1 | C1-T1 | C2-T1 | C3-T1 | C4-T1 | C5-T1 | C6-T1 | C7-T1 | C8-T1 | C9-T1 | C10-T1 | |
| C | NC-T2 | PC-T2 | C1-T2 | C2-T2 | C3-T2 | C4-T2 | C5-T2 | C6-T2 | C7-T2 | C8-T2 | C9-T2 | C10-T2 | |
| D | NC-T2 | PC-T2 | C1-T2 | C2-T2 | C3-T2 | C4-T2 | C5-T2 | C6-T2 | C7-T2 | C8-T2 | C9-T2 | C10-T2 | |
| E | NC-T1 | PC-T1 | C1-T1 | C2-T1 | C3-T1 | C4-T1 | C5-T1 | C6-T1 | C7-T1 | C8-T1 | C9-T1 | C10-T1 | 1 hour |
| F | NC-T1 | PC-T1 | C1-T1 | C2-T1 | C3-T1 | C4-T1 | C5-T1 | C6-T1 | C7-T1 | C8-T1 | C9-T1 | C10-T1 | |
| G | NC-T2 | PC-T2 | C1-T2 | C2-T2 | C3-T2 | C4-T2 | C5-T2 | C6-T2 | C7-T2 | C8-T2 | C9-T2 | C10-T2 | |
| H | NC-T2 | PC-T2 | C1-T2 | C2-T2 | C3-T2 | C4-T2 | C5-T2 | C6-T2 | C7-T2 | C8-T2 | C9-T2 | C10-T2 | |

**Figure 12.** Alternative A): Transfer 3 aliquots (200 µL) from each tissue into the 96-well plates using the plate design above. Use one 96-well plate for both time points. T1 and T2 refer to the duplicate (N=2) tissues used for each exposure time and test article.

## 6.8 Documentation

### 6.8.1 Method Documentation Sheet, MDS (see ANNEX A)

The MDS allows to check the correct set up, calibration and function of the equipment as well as correct weights, applications etc. The MDS is designed as a paper document "in the spirit of GLP". Per each test, make a hardcopy of the MDS, fill in and sign the requested information, starting the day prior to testing and ending after the test has been conducted.

*Note (1): If several tests are performed per week, pipette verification (weighing $H_20$ on a balance) is only necessary once at the beginning of each week. Nevertheless, if adjustable pipettes are used the correct adjustment shall be checked and recorded in the MDS before each test.*

*Note (2): If solids cannot be sufficiently ground to a fine powder, it is recommended to check the weight of the levelled application spoon and record this weight in the MDS.*

### 6.8.2 MS EXCEL Data Spreadsheets

The MS EXCEL workbook can be obtained from MatTek Corporation (USA) or MatTek In Vitro Life Science Laboratories (Slovakia). Data of optical densities (ODs) generated by the microplate reader **and already corrected by substraction of the blanks mean from all OD values** are copied from the Reader software or manually imputted to the Windows Clipboard and then pasted into the first spreadsheet (**Import**) of the EXCEL workbook in the 96-well format given above in **Figure 12**.

The workbook consists of three spreadsheets, named **Import**, **Results** and **Classification.** The first sheet (**Import**) is used for pasting or manually transposing the OD values. In addition, fill in required information on test substances, tissue lots, etc. in the first sheet (**Import**). The second sheet (**Results**) makes calculations and provides a column graph of the results. Entries on test materials made in the first sheet will be copied from there automatically to the correct positions in the **Results** sheet. A summary of the classification for each test article and whether the test was qualified (Q) or not (NQ) is presented on the **Classification** sheet. Add any special comment(s) related to the experiment or measurement in the last (Remarks) column on the third sheet.

## 7. REFERENCES


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9. Desprez B., Barroso J., Griesinger C., Kandárová H., Alépée N., and Fuchs, H. (2015). Two Novel Prediction Models Improve Predictions of Skin Corrosive Subcategories by Test Methods of OECD Test Guideline No. 431. Toxicol. In Vitro 29, 2055 2080.

## ANNEX A: Methods Documentation Sheet (MDS)

**Assay No:**......................................................................

**Date:**......................................................................................

**Corresponding XLS data file name:**.......................................................................

**Performed by:**..............................................................................................

### Time Protocol

**Receipt of EpiDerm tissues**:
Date:...........................................ID:.......................................

**Experimental schedule:**
Date:……………………………….ID:…………………………..

| procedure | preincubation (1 hour) | | exposure (1hour) | | exposure (3 min) | | MTT assay (3h) | | formazan extraction (2h or overnight) | |
|---|---|---|---|---|---|---|---|---|---|---|
| SET No. | start | stop | Start | stop | start | stop | start | stop | start | stop |
| 1 | | | | | | | | | | |
| 2 | | | | | | | | | | |
| 3 | | | | | | | | | | |
| 4 | | | | | | | | | | |
| 5 | | | | | | | | | | |
| 6 | | | | | | | | | | |
| 7 | | | | | | | | | | |
| 8 | | | | | | | | | | |
| 9 | | | | | | | | | | |
| 10 | | | | | | | | | | |
| 11 | | | | | | | | | | |
| 12 | | | | | | | | | | |

**Measurement** (date, hour):
Date:.............................................ID ..........................

## Devices Verification

**Incubator Verification**

| Incubator # | $CO_2$ < 5% ± 0,5 % > | Temperature < 37°C ± 1°C > | Check water in reservoir (✓) |
|---|---|---|---|
| | | | |

ID/ Date:

**Refrigerator Verification**

| Refrigerator # | Temperature < 5°C ± 2°C > |
|---|---|
| | |

ID/ Date:

**In case that your devices are controlled by central computer, fill in the following table instead of fields above:**

| **Name of the Device** | **Device #** | **Reference** |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |

ID/ Date:

**Pipette verification (triplicate weightings)**

Pipette 3 x $H_2O$ into a small baker on a laboratory scale and record readings in g. Perform pipette verification only once per week and refer to it in all assays of this week. If adjustable pipettes are used, check adjustment daily.

| | 0.9 mL | 2 mL | 300 µL | 200 µL | 50 µL | 25 µL |
|---|---|---|---|---|---|---|
| | ........$H_2O$ weight in g........ | | | | | |
| 1. | | | | | | |
| 2. | | | | | | |
| 3. | | | | | | |
| Mean | | | | | | |
| SD | | | | | | |

ID/ Date:

## Kit Receipt

| EpiDerm kit received (day/date/hour): | Day used: |
|---|---|
| EpiDerm (EPI-200-SCT)<br>Lot no.:<br>No. of inserts: | Production date: |
| Assay medium (EPI-100-ASY)<br>Lot no.: | Expiration date: |
| MTT concentrate (MTT-100-CON); 2 mL<br>Lot no.: | Expiration date: |
| MTT diluent (MTT-100-DIL); 8 mL<br>Lot no.: | Expiration date: |
| MTT extractant (MTT-100-EXT), 60 mL<br>Lot no.: | Expiration date: |
| PBS (TC-PBS); 100 mL<br>Lot no: | Expiration date: |
| No. of 6 well plates: | |
| No. of 24 well plates | |
| MTT protocol + packaging diagram | |
| Position of ice-packs:<br>(direct contact of the ice-packs with the skin must be avoided) | |
| Other remarks | |

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

## Quality Control of the Skin

**Use scores: 1**- very good, 2-good, 3- acceptable, 4- not acceptable

| APPERANCE | KIT 1 | KIT 2 | KIT 3 | KIT 4 |
|---|---|---|---|---|
| MACRO. | | | | |
| MICRO | | | | |
| OTHER as.<br>-edge defects<br>-air bubbles<br>-wet tissues<br>-- | | | | |

**If you have any special observations (listed in OTHER), make reference to related tissue(s)**

Kit No.1:......................

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 7 | 8 | 9 | 10 | 11 | 12 |
| 13 | 14 | 15 | 16 | 17 | 18 |
| 19 | 20 | 21 | 22 | 23 | 24 |

Kit No.2:......................

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 7 | 8 | 9 | 10 | 11 | 12 |
| 13 | 14 | 15 | 16 | 17 | 18 |
| 19 | 20 | 21 | 22 | 23 | 24 |

Kit No.3:......................

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 7 | 8 | 9 | 10 | 11 | 12 |
| 13 | 14 | 15 | 16 | 17 | 18 |
| 19 | 20 | 21 | 22 | 23 | 24 |

Kit No.4:......................

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 7 | 8 | 9 | 10 | 11 | 12 |
| 13 | 14 | 15 | 16 | 17 | 18 |
| 19 | 20 | 21 | 22 | 23 | 24 |

ID/ Date:

## Dosing Procedure

Please tick (✓) in the first three columns the type of application (pipette, patch, spoon)

**REMARKS:** record, if spreading was necessary, or if crushing and grinding was **not** used (because it did not improve consistence of test material)

| TEST CODE | Liquid/ Solid (50 µL)/ (25 mg) | Application | | | Material characterization § | Remarks |
|---|---|---|---|---|---|---|
| | | Pipette (✓) | Spoon (✓) | Mesh (✓) | | |
| | | | | | | |
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| | | | | | | |

§ use your own wording, like: "highly viscous", “waxy”, “sticky” etc.

ID/ Date:

## MTT Assay

**Remarks before and after incubation with MTT**

**Before MTT incubation**

| 3 min exposure | | 1 hour exposure | |
|---|---|---|---|
| Chemical tested | Observation (tissue #) | Chemical tested | Observation (tissue #) |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

**After MTT incubation**

| 3 min exposure | | 1 hour exposure | |
|---|---|---|---|
| Chemical tested | Observation (tissue #) | Chemical tested | Observation (tissue #) |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

*record observation as separation of the tissue from the insert during the washing procedure, reaction between insert and tested chemical, MTT reduction by chemical itself etc.

Date:……………………………ID………………………….

## Measurement

**Check plate photometer filter**

Tick correct (✓) filter setting

| reading filter: 570 nm | |
|---|---|
| no reference filter | |

Date:…………….ID……………………

## Archivation

**Raw data saved in/as:**

……………………………………………………………………………………………………..

**Spreadsheet saved in/as:**

……………………………………………………………………………………………………..

**MDS saved in/as:**

……………………………………………………………………………………………………...

Date:…………….ID……………………

# ANNEX B: Test for Interference of Chemicals with MTT Endpoint and Correction Procedures

**Possible interactions between test chemicals and test system**

| | Water coloration (Step 1, Section 7.3) | Tissue Staining (Step 2, Section 7.3) | MTT Interaction (Step 3, Section 7.3) | Test Conditions |
|---|---|---|---|---|
| Case 1 | - | - | - | A |
| Case 2 | + | - | - | A |
| Case 3 | - | + | - | B |
| Case 4 | + | + | - | B |
| Case 5 | - | - | + | C |
| Case 6 | + | - | + | C |
| Case 7 | - | + | + | B+C |
| Case 8 | + | + | + | B+C |

Test Conditions:
A – Perform all steps according to the basic SOP. Correction of results using additional controls is not needed,
B – Perform Step 2, Section 7.3 in addition to the basic SOP. Correction of results with viable tissue is needed.
C – Perform Step 4, Section 7.3 in addition to the basic SOP. Correction of results with frozen tissue is needed.

**B+C:**
A colored chemical (or a chemical that may turn colored after interaction with water) may cause both tissue staining and false MTT reduction. If the experimentator is interested only in the correction of final results, combination of test condition C is sufficient for this purpose, since the frozen tissues absorb approximately the same amount of chemicals (and color) as the viable tissue. However, since the frozen tissues are more hydrated than viable tissues, tissue staining by water soluble colorants (or non-colored chemicals that will turn in aqueous conditions into colored) can be overestimated. In addition, conditions may arise when information on the amount of non-specific coloration is required. For increased precision of the above mentioned case, Steps 2 & 4 (Section 7.3) need to be performed.