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Protocol

# In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT) For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm™ (EPI-200-SIT)

*Note 1: This protocol is based on Zebet's SOP version 7.0 drafted by Manfred Liebsch and Dieter Traue (ZEBET at the BfR) which was used in the follow-up validation study of the Modified EpiDerm™ Skin Irritation Test (SIT).*

*The Modified EpiDerm™ Skin Irritation Test (SIT) was validated in 2007 in an international validation trial involving BASF SE (Ludwigshafen, Germany), IIVS Inc. (Gaithersburg, USA), ZEBET at the BfR (Berlin, Germany), and Zet-LSL (Linz, Austria). The study was conducted in line with requirements of OECD GD 34 and ECVAM Performance Standard document for applying human skin models to in vitro skin irritation.*

*Performing the EpiDerm SIT as outlined fulfills criteria set forth in OECD TG439.*

*The ECVAM Scientific Advisory Committee (ESAC) formally endorsed the scientific validity of the Modified EpiDerm™ Skin Irritation Test (SIT) at its November, 2008 meeting. ESAC concluded that the Modified EpiDerm™ SIT has sufficient accuracy and reliability for the prediction of skin irritating and non-irritating test substances and that it should be considered a validated, stand-alone in vitro replacement for animal skin irritation testing.*

*Note 2: A detailed video demonstrating use of this protocol is available via the Journal of Visualized Experiments (JoVE):*

*https://www.jove.com/video/1366/an-vitro-skin-irritation-test-sit-using-epiderm-reconstructed-human*

# Table of Contents

## 1.) Rationale and Background

The potential of chemicals to induce skin irritation (hazard) is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. Skin irritation refers to the production of reversible damage to the skin following the application of a test substance for up to 4 hours [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS)] (**1**). Skin irritation in vivo is determined by modification of the Draize rabbit skin irritation test as described in the OECD TG 404 (**2, 3**). Because systemic reactions play a minor role in modulating local skin toxicity potential of chemicals, skin irritation potential may be predicted by *in vitro* systems, provided they are sufficiently complex to mimic skin barrier in vivo and cell reactivity.

The method described in this SOP is based on method initially developed and refined by L'Oreal for EPISKIN model (**4, 5**). The SOP was applied on EpiDerm™ model, with the aim to develop for both systems a common protocol able to predict skin irritation potential according to the EU classification system and replace the in vivo acute skin irritation test in rabbits (**6, 7**). Upon review of existing information by the ECVAM Skin Irritation Task Force and an ECVAM Workshop both EPISKIN and EpiDerm™ skin irritation tests (SIT) were regarded as sufficiently promising predictors for skin irritancy potential and ready to enter the formal validation study. Due to the under-prediction of several chemicals in the second Phase of the ECVAM validation study (**8**), ESAC recommended to increase sensitivity of the EpiDerm™ SIT to better match in vivo rabbit data (**9**).

Following the recommendation of ESAC (**9**), the EpiDerm skin irritation test was further optimized by MatTek Corporation during 2006 and 2007. The extended exposure time (60 min) and minor modification of exposure conditions improved the sensitivity of the assay. The applicability domain, prediction model (50% viability border for identification of irritants) as well as the endpoint (MTT cytotoxicity assay) did not change; thus the concept of common protocol was maintained (**10**).

The predictive capacity of the modified EpiDerm™ SIT was initially assessed by MatTek Corporation, USA in an intra-laboratory study (**10**). Transferability of the method was evaluated in 2007 in an external international validation study between 4 laboratories: ZEBET at the BfR, Berlin, Germany; BASF, Ludwigshafen, Germany; IIVS, Gaithersburg, MD and Zet-LSL, Linz, Austria (**11, 12**). The validation trial was in accordance with the principles and criteria documented in OECD Guidance Document No. 34 on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (**13**) and ECVAM (2007) Performance Standards for applying human skin models to *in vitro* skin irritation (**14**).

In 2008, ESAC concluded that the Modified EpiDerm SIT has sufficient accuracy and reliability for prediction of R38 skin irritating and no-label (non-skin irritating) test substances (**15**). The Modified EpiDerm™ SIT is an *in vitro* procedure that, depending on information requirements, allows determining the skin irritancy of chemicals as a stand-alone replacement test, as a screen, or within a testing strategy in combination with, if appropriate, a weight of evidence approach (**16**).

## 2.) Specific Purpose of the Method

The EpiDerm™ SIT was developed and designed to predict skin irritation potential of neat test substances in the context of identification and classification of skin irritation hazard according to the EU classification system (R38 or no label). Since the EU and GHS systems were harmonized in 2008, the procedure described in this SOP also allows for hazard identification of irritant substances in accordance with UN GHS. The Modified EpiDerm™ SIT allows discrimination between irritants of category 2 and non-irritants. The test does not discriminate between non-mandatory subcategories of the UN GHS, i.e. it does not distinguish between GHS category 2 and category 3 irritants.

## 3.) Basis of the Method

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 dehydrogenase conversion of MTT [*(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide*], present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues (**17**). The reduction of the viability of tissues exposed to chemicals in comparison to negative controls (treated with water) is used to predict the skin irritation potential. Recent comparative studies in RhE models employing various endpoints to predict skin irritancy of topical formulations have shown that the MTT endpoint had clear advantages, even over mechanistically based endpoints like the release of IL-1$\alpha$ (**18, 19**).

## 4.) Test System Description

The reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, MA, USA and Bratislava, Slovakia) consists of normal human-derived epidermal keratinocytes, which have been cultured to form a multilayered highly differentiated model of the human epidermis. It consists of organized basal, spinous, and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. A generic description of general and functional conditions that reconstructed human skin models need to comply with can be found in the new OECD Test Guidelines 431 *In vitro* Skin Corrosion: Human Skin Model (**20**) and 439 *In vitro* Skin Irritation: Reconstructed Human Epidermis test Method (**21**).

The EpiDerm™ tissues (surface 0.6 cm²) are cultured on specially prepared cell culture inserts and shipped world-wide as kits, containing 24 tissues on shipping agarose together with the necessary amount of culture media, DPBS, 6-well plates, and 24-well plates. In addition, the MTT kit (containing MTT concentrate, diluent, and extractant) is provided by MatTek per request.

### 3.1.1 Quality Control of the Test System

The EpiDerm™ System is manufactured according to defined quality assurance procedures. All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (1%) for each EpiDerm lot. The ET-50 must fall within a range established based on a historical database of results. Histology is provided upon request.

### 3.1.2 Precautions

The epidermal cells are taken from healthy volunteers negative to HIV, and Hepatitis. Nevertheless, handling procedures for biological materials should be followed:
a) It is recommended to wear gloves during handling with the skin and kit components.
b) After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using 10% bleach, special containers or autoclaving).

*Note: Due to long post-incubation period, it is necessary to perform the test under aseptic conditions in the microbiological safety cabinet (laminar flow hood).*

## 3.2 Assay Quality Controls

### 3.2.1 Assay Acceptance Criterion 1: Negative Control

The **absolute OD** of the negative control (NC) tissues (treated with sterile DPBS) in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

**The assay meets the acceptance criterion if the mean $OD_{570}$ of the NC tissues is ≥ 1.0 and ≤ 2.8.**

### 3.2.2 Assay Acceptance Criterion 2: Positive Control

A **5% SDS** (in $H_2O$) solution (see **7.6.3**) is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95±1 % confidence interval of the historical data.

**The assay meets the acceptance criterion if the mean viability of PC tissues expressed as % of the negative control tissues is < 20%.**

### 3.2.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

Since in each test skin irritancy potential is predicted from the mean viability determined on 3 single tissues, the variability of tissue replicates should be acceptably low.

**The assay meets the acceptance criterion if the SD calculated from individual % tissue viabilities of the 3 identically treated replicates is ≤ 18%.**

***Note**: Chemicals that provide tissue viabilities in a range of 30 – 70 % may provide high SD. If the high SD (above acceptance limits) is typical for the chemical and the classification of the chemical is consistent in all independent runs, it is recommended to accept this result, although the Assay Acceptance Criterion 3 is not met.*

## 4. Limitations of the Method

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substances are a problem only if at the time of the MTT test (i.e. 42 hours after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified by a procedure described in Section **7.3**. and Annex D.

The method is not designed for testing of highly volatile test substances, gases, and aerosols.

# 5. Brief Basic Procedure

On the day of receipt, EpiDerm tissues are conditioned by incubation to release transport-stress related compounds and debris overnight. After pre-incubation, tissues are topically exposed to the test chemicals for 60 minutes. Preferably, three tissues are used per test chemical (TC) and for the positive control (PC) and negative control (NC). Tissues are then thoroughly rinsed, blotted to remove the test substances, and transferred to fresh medium.

After a 24 hr incubation period, the medium is collected for analysis of cytokines (*Note: This step is optional, since no improvement in assay performance was noted by using IL-1α or other cytokines as complementary endpoint*). Tissues are incubated for another 18 hours. Afterwards, the MTT assay is performed by transferring the tissues to 24-well plates containing MTT medium (1 mg/mL). After a 3 hr MTT incubation, the blue formazan salt formed by cellular mitochondria is extracted with 2.0 mL/tissue of isopropanol (extractant solution, part # MTT-100-EXT) and the optical density of the extracted formazan is determined using a spectrophotometer at 570 nm. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin irritation potential of the test material is predicted if the remaining relative cell viability is below 50% (Section 6).

*Note: Detailed video demonstrating the protocol is available via Journal of Visualized Experiments* *https://www.jove.com/video/1366/an-vitro-skin-irritation-test-sit-using-epiderm-reconstructed-human* ***(22)***

# 6. Data Interpretation Procedure (Prediction Model)

According to the EU and GHS classification (R38/ Category 2 or no label), 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.

| ***In vitro* result** | ***In vivo* prediction** | |
|---|---|---|
| | ***EPI-200-SIT classification*** | ***OECD/OCDE TG No. 439 (adopted and updated in 2019)*** |
| mean tissue viability ≤ 50% | Irritant (I) (R38 or GHS category 2) | Requiring classification and labeling (GHS Category 1 or 2) |
| mean tissue viability > 50% | Non-irritant (NI) | Non-irritant to skin (GHS No Category) |

# 7. Materials and Methods

## 7.1 Materials provided by MatTek

### 7.1.1 EPI-200-SIT Kit Components

EPI-200-SIT kits are shipped from Boston and Bratislava each Monday. Upon receipt of the EpiDerm tissues, transfer the tissue inserts into the assay medium and pre-incubate the cultures overnight at 37±1°C, 5±1 % $CO_2$ in air and 90% ± 10% relative humidity (RH) (for detailed procedure see section 7.7.1 Tissue conditioning). Place the rest of the assay medium into the refrigerator (5±3°C), MTT concentrate containing vial in the freezer (-20±5°C) and the MTT diluent in the refrigerator (5±3°C). Record lot numbers of all kit components into the Methods Documentation Sheet (MDS – see Annex B)

## Standard Assay Kit Components (order under the part # EPI-200-SIT)

| | | |
|---|---|---|
| 1 | Sealed 24-well EpiDerm™ (EPI-200) plate | Contains 24 tissues in cell culture inserts, packaged on agarose |
| 2 | 24-well plates (sterile) | Used for MTT viability assay |
| 8 | 6-well plates (sterile) | Used for maintaining tissues during assay protocol |
| 1 bottle, 100 mL | Assay Medium (EPI-100-NMM) | DMEM based medium |
| 1 vial, 1 mL | 5% SDS Solution (TC-SDS-5%) | Skin irritant reference chemical – Positive Control |
| 1 bottle, 100 mL | DPBS Rinse Solution (TC-PBS) | Used for rinsing the inserts |
| 25 pieces | Nylon mesh circles, 8 mm diameter, 200 µm pore (EPI-MESH) | Used for spreading test chemicals |
| 1 | MK-24-007-023 | Complete EpiDerm™ Skin Irritation Test (SIT) protocol is sent electronically |

## MTT-100 Assay Kit Components (MTT-100 must be ordered separately)

| | | |
|---|---|---|
| 1 vial, 2 mL | MTT concentrate (MTT-100-CON) | Frozen MTT concentrate |
| 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 crystals |

### 7.1.2 Expiration and Component Storage Conditions

| Part # | Description | Conditions | Shelf life |
|---|---|---|---|
| EPI-200-SIT | EpiDerm tisssues | refrigerator (5±3°C) | 96 hours |
| EPI-100-NMM | Assay medium | refrigerator (5±3°C) | 7 days |
| MTT-100-DIL | MTT diluent | refrigerator (5±3°C) | 2 months |
| MTT-100-CON | MTT concentrate | freezer (-20±5°C) | 2 months |

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

## 7.2 Other Materials (not provided with the EPI-200-SIT kit)

| | |
|---|---|
| • Laminar flow hood | For safe work under sterile conditions |
| • Humidified incubator (37±1°C, 5±1% $CO_2$, 90% ± 10% relative humidity (RH)) | For incubating tissues prior to and during assays |
| • Vacuum source/trap (optional) | For aspirating media and solutions |
| • Laboratory balance | For pipette verification and checking spoonful weight |
| • 96-well plate photometer | For reading OD |
| • Plate shaker | For extraction of formazan |
| • Stop-watch | For timing of application of test materials and other timed steps in protocol |
| • Sterile, blunt-edged forceps | For handling tissue inserts |
| • 500 mL plastic wash bottle | For rinsing tissue with DPBS |
| • 200 mL beaker | For collecting DPBS rinses |
| • 37±1°C water bath | For warming Media and MTT solution |
| • Mortar and pestle | For grinding granular solids |
| • Adjustable pipette / multi-step pipette | For pipetting 0.9 mL assay medium,<br>For pipetting 300 µL MTT medium<br>For pipetting 2 mL MTT extraction solution<br>For pipetting 200 µL formazan extract from 24-well plate into 96-well plate for the plate photometer<br>For application of 30 µL liquid test materials and 25 µL for application of DPBS when wetting the tissue surface before application of solid substances |
| • Positive displacement pipet, 30 µL | For application of gels, creams, viscous materials, semi-solid test materials and suspensions |
| • Sharp spoon (NaCl weight: 25 mg), e.g. Aesculap, Part #: FK 623R | For application of solids |
| • Bulb headed glass Pasteur pipette | To aid leveling the spoonful of solid test articles and for spreading on the tissue surface |
| • Dulbecco's Phosphate Buffered Saline (DPBS) (TC-PBS) | For rinsing tissues. |
| • Freeze-killed EpiDerm™ tissue (EPI-200-FRZN-EA) | Needed for colored test materials or for materials that directly reduce MTT. |
| • Extra 6-well plates - sterile (FALCON recommended) | For transferring tissue inserts to fresh media (instead of replacing the media using the same plate). |
| • Cryovials - polypropylene (NeoLab # 7-4581) | Collecting and freezing of media samples for each tissue |
| Adhesive tape (NeoLab # 7-2220 or # 2-5082) or Parafilm M | Covering plates during formazan extraction |
| • Cotton tip swabs (sterile) | For drying the tissue surface |
| • MTT-100 assay kit | Contains MTT - Thiazolyl Blue Tetrazolium Bromide reagent (Sigma, # M-5655) and isopropanol extractant. Note: The MTT-100 kit must be ordered separately. |

## 7.3 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 D). 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 **30 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 % $CO_2$, 90% ± 10% 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 **30 µL** of liquid test substance 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 7.7 except incubate the tissue for 3-h incubation in culture media without MTT (37±1°C, 5±1% $CO_2$, 90% ± 10% 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**
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 **30 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 $CO_2$, 90% ± 10% 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.

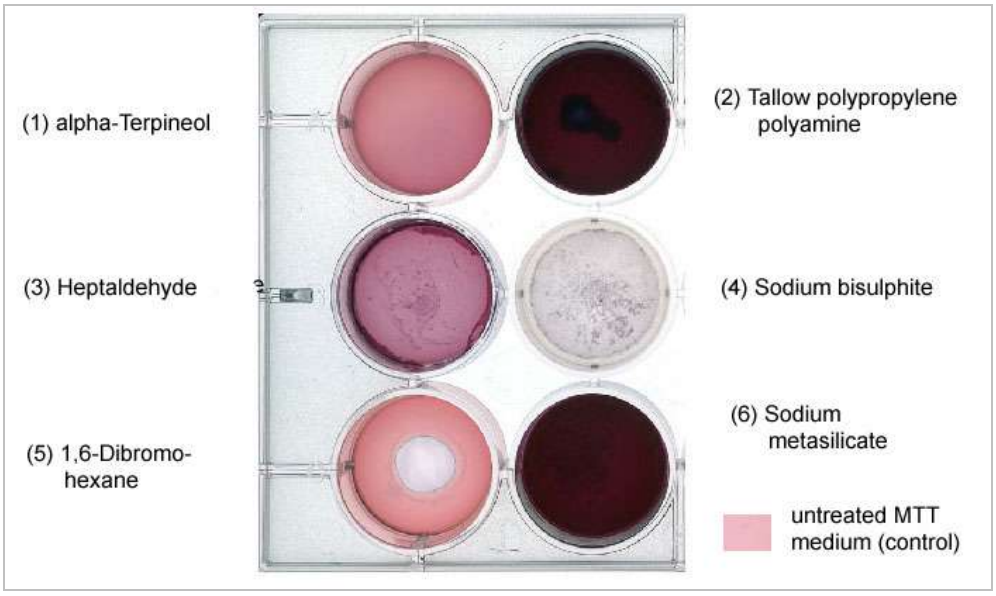



**Figure 1:** Example of test for direct MTT reduction ability (Step 3). Test substances (2) (3) and (6) have directly reduced MTT. In these cases, Step 4 (below) must be performed.

**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 can be ordered separately from MatTek Corporation (part # EPI-200-FRZN-EA). 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**

**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 by the SIT (tissue viability <50 %), the correction procedures are not necessary.*

*Note 2: Frozen tissues (EPI-200-FRZN-EA) can be ordered separately from MatTek Corporation.*

## 7.4 Test for Mesh Compatibility (liquid test substances only)

Capillary effects (surface tension effects) were observed if low volumes of lipophilic liquid test chemicals were applied on EpiDerm™ surface. Therefore, a nylon mesh (provided by MatTek) is used as a spreading support (see **7.7**). Twenty-five (25) nylon mesh samples are provided with the EPI-200-SIT kit. The mesh can be used for a wide range of liquid substances. However, some chemicals may react with the mesh and therefore the compatibility of each liquid chemical with nylon mesh has to be checked.

To test if a test chemical interacts with the mesh, place the mesh on a slide and apply 30 µL test substance. After 60 minutes exposure, check using a microscope (**Figure 3**), if an interaction between test substance and the mesh is noticed (**B**). In that case, the test substance has to be applied without using a mesh as a spreading aid.

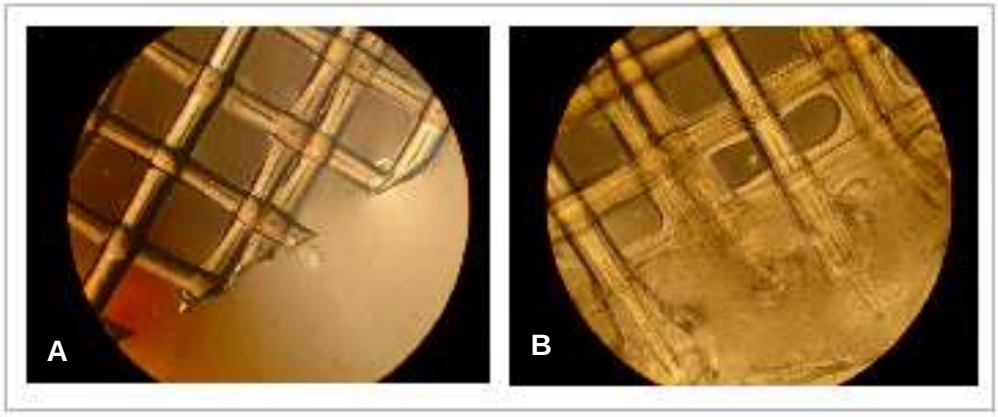



**Figure 3:** The mesh compatibility test showing: **A)** Normal mesh (no reaction with test material) and **B)** Damaged mesh (reaction with test materials).

## 7.5 Preparations

### 7.5.1 MTT solution (prepare freshly on the day of testing)

Thaw the MTT concentrate (MTT-100-CON) and dilute the concentrate with the MTT diluent (MTT-100-DIL). Store the remaining MTT solution in the dark at 5±3°C for later use on the same day (do not store overnight 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 (**see 7.2**) 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 and its solutions!

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

### 7.5.2 Dulbecco's PBS

Sterile ready-to-use DPBS should be used. About two liters are sufficient for all rinsing performed with one kit. If DPBS is prepared from 10x concentrates or powder, the pH needs to be adjusted to 7.0 and solution must be sterilized. Record the preparation in the MDS.

## 7.6 Test substances

**Safety Instruction**

1. For handling of known test substances, follow instructions given in the Material Safety Data Sheet.
2. If coded materials or unknown samples 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 irritating and toxic. Work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).
3. Store all test substances according to recommendations. Follow all the storage conditions provided (temperature, protection from light, protection from oxidization by nitrogen, etc.).

**Liquids**: Dispense **30 µL** directly atop the tissue and gently spread the chemical using bulb headed Pasteur pipette. Try to avoid contact of the pipette with the tissue surface. Afterwards, carefully place the nylon on the tissues surface. If necessary, gently position the mesh using the bulb headed glass Pasteur pipette. Record the use of mesh as a spreading tool in the MDS.

**Figure 4:** Application of liquids

**Semisolids**: Dispense **30 µL** using a positive displacement pipette directly atop the tissue. If necessary spread the chemical with Pasteur pipette to match size of tissue. Record the use of spreading in the MDS.

**Figure 5:** Application of semisolids (positive displacement pipette - detail) and subsequent spreading using bulb headed Pasteur pipette.

**Solids**: Crush and grind test material using a mortar with pestle. Shortly before application of the solid substance, moisten the tissue surface with **25 µL of sterile DPBS** to improve contact of the tissue surface with the test chemical. Fill **25 mg** sharp application spoon (see **Figure 6**) 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. A bulb headed Pasteur pipette can be used to empty the spoon completely.

Gently shake the inserts to improve the spreading of the solid on the surface. If necessary, gently spread the chemical to match size using bulb headed sound (or bulb headed Pasteur pipette). Do not press on the tissue surface. Record observations of the solubility of the material into the MDS. Record in the MDS if grinding was not necessary.

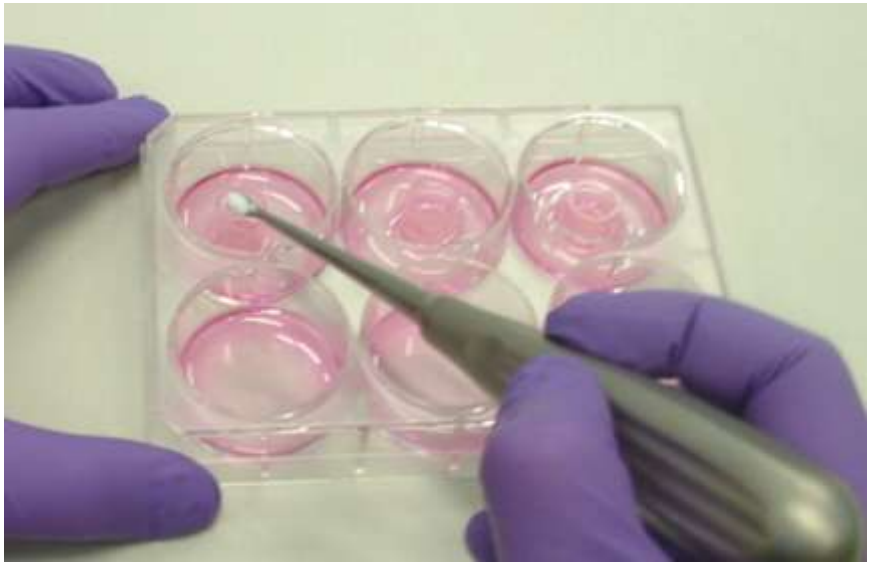


**Figure 6**: Application of solids

**Waxes:** For test substances with **waxy consistency,** the spoon application will not work. In these cases try to form a flat "cookie like" piece of about 8 mm diameter and place it atop the tissue, wetted with sterile **DPBS.** To improve the contact between the test substance and the tissue weigh down the "cookie" with a stainless steel aid like that shown in **Figure 7**.

**Figure 7**: The stainless steel aid used application of waxy materials

*Note: Highly volatile toxic test substances may affect neighbouring tissues within the same 6-well treatment plate. In these cases, plates should be covered with an adhesive plate cover, or other measures should be taken, like testing the volatile substances on separate plates.*

## 7.7 Experimental Procedure (see also ANNEX A)

### 7.7.1 DAY 0 – Day prior to dosing

1. Upon receipt of the shipment, examine all kit components for integrity. If there is a concern call MatTek Corporation or MatTek IVLSL immediately.

Contact persons:

| | | |
|---|---|---|
| Yulia Kaluzhny (US)<br>Phone: +1-508-881-6771<br>Email: *ykaluzhny@mattek.com* | Paul Kearney (US)<br>Phone: +1-508-881-6771<br>Email: *pkearney@mattek.com* | Silvia Letasiova (EU)<br>Phone: +421-2-3260-7401<br>Email: *sletasiova@mattek.com* |

2. Record all information about supplied material into the MDS.
3. Store the DPBS at room temperature and the vial containing the MTT concentrate in the freezer (-20±5°C).

**Tissue conditioning:**

1. Let the assay medium reach room temperature (20-25°C). Do not pre-heat to 37°C.
2. Pipette 0.9 mL of the assay medium into each well of sterile 6-well plates (For 24 inserts prepare eight 6-well plates. Use one 6-well plate for pre-incubation of three inserts).
3. Under sterile conditions, open the plastic bag containing the 24-well plate with epidermal tissues. Under a sterile airflow, remove the sterile gauze and carefully (using sterile forceps) take out each insert containing the epidermal tissue. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper or gauze, and place the tissues in the empty, sterile 24-well plate.

**Figure 8:** Visual inspection of the epidermal tissues.

4. Perform visual inspection of the inserts within the next 5 min. Record any tissue defects and excess moisture on the surface. Do not use tissues with defects or tissues with excessive moisture on the surface.
5. Dry the surface of the tissues with a sterile cotton tip swab and transfer tissues to a 6-well plate pre-filled with 0.9 mL medium. Place the plates for 60 ± 5 min into the incubator (37±1°C, 5±1% $CO_2$, 90% ± 10% RH).
6. At the end of the first (60 minute) pre-incubation period, transfer the inserts from upper wells into the lower wells of the 6-well plate. Further, pre-Incubate the tissues (37±1°C, 5±1% $CO_2$, 90% ± 10% RH) overnight for 18 ± 3 hr.

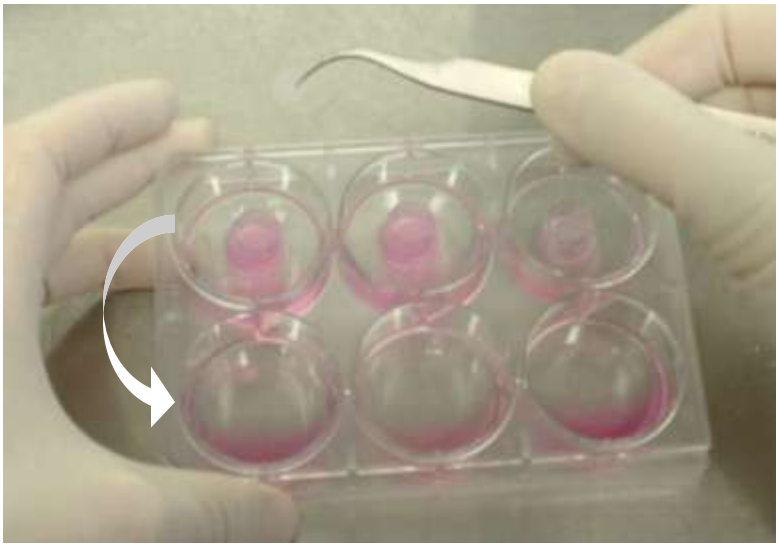


**Figure 9:** Pre-incubation plate design

7. Place the plates back into incubator for overnight pre-incubation.
8. Place the rest of the assay medium into the refrigerator (5±3°C) and the vial containing the MTT concentrate in the freezer (-20 ±5°C).

*Note 1: Any air bubbles trapped underneath the insert should be released.*
*Note 2: The visual quality check of the tissues has to be done quickly.*
*Note 3: Removal of the moisture collected on the tissues surface is important.*

***Do not use in the assay tissues which are extensively covered with liquid (>40% of surface).***

9. If necessary, prepare sufficient amount of rinsing DPBS for the next day (approximately 1 L per 24 inserts).
10. Prepare and sterilize all devices which will be used in the assay:
    - 150 mL vials (autoclave or heat sterilization),
    - cotton tips (autoclave),
    - mesh (UV irradiation; do not autoclave),
    - bulb headed Pasteur pipettes (70% Ethanol or heat sterilization),
    - washing bottles (UV/ gamma irradiation or 70% Ethanol),
    - blotting paper (autoclave),
    - spatula or spoon for application of solids (70% Ethanol),

## 7.7.2 DAY 1 – Chemical Exposure

1. Place all devices, solution and chemicals necessary for the test into the sterile hood.
   Checklist:
   - sterile 150 mL vials pre-filled (for 1 chemical (=3 inserts) one vial is required)
   - sterile cotton tips
   - sterile mesh
   - sterile bulb headed Pasteur pipettes
   - sterile washing bottles pre-filled with sterile DPBS
   - sterile blotting paper
   - sterile tips and pipettes
   - all vials with chemicals heated at room temperature (~25°C), NC (sterile DPBS) and PC (5% SDS)
   - digital timer
   - sharp, pointed tweezers
   - sharp spoon
   - 1 big vial for waste

*Note: Wipe all non-sterile material bottles with 70% EtOH.*
*Note: If you are not using the spoon for the application of solids, weight out 3x 25 mg of the test materials into the appropriate vials and prepare spatula for the application.*

2. Prepare a sufficient number of 6-well plates pre-filled with 0.9 mL of assay medium in the upper row (1 plate = 1 chemical).
3. Remove the pre-equilibrated, 6-well plates from the incubator approximately 5 min before exposure to chemicals will begin.
4. Evaluate the surface of tissues and exclude completely wet tissues or tissues with any visible defects.
5. Remove any moisture using sterile cotton tip.
6. Before test chemical exposure, label all 6-well plate lids with the test material codes or names.

*Note: The assay is designed to test maximum of 6 chemicals (using n=3 tissues/test chemical) in one testing SET by ONE technician using application and washing interval of 1 minute.* ***Deviations from this SOP may cause different outcomes.***

## Test Substance Exposure

*Note: Do not dose more than 18 tissues (= 6 test articles including PC and NC in a block (SET), in order to be able to perform all steps as required by this protocol.*

1. Apply **30 µL** (liquid) or **25 mg** (solid) of the undiluted test substance, NC or PC to three single tissues each according to **7.7.** Dose tissues at the time intervals needed later for rising off the test substances (optimal and highly recommended is 1 minute interval).
2. Keep the plates with dosed tissues in the laminar flow hood, until the last tissue is dosed.
3. After dosing the last tissue, transfer **all plates for 35 ± 1 minutes** to the humidified incubator (37±1°C, 5±1% $CO_2$, 90% ± 10% RH).
4. After 35 min, remove **all plates** from the incubator, place them into the sterile hood and wait until the period of 60 min is completed for the first dosed tissue.
5. After the **60 ± 1 minute** test substance exposure, rinse the tissues with sterile DPBS, filling and emptying the tissue insert **15 times** to remove any residual test material (**Figure 10)**. Use constant stream of DPBS applied from 1.5 cm distance from the tissue surface. (The stream of DPBS should not be too soft, otherwise, the test article will not be removed. Optimal wash bottle, with pointed endings is shown in **Figure 10**)

*Note: Washing procedure – tissues with mesh: wash the tissues 5 times from the wash bottle to remove excess of the test chemical. Then remove the mesh carefully with pointed, sharp forceps (see Figure 9 and 10) and only then continue with the washing procedure (10 times). If the test substance reacts with the insert, the mesh may stick to the edge of insert. This may complicate its removal.*

6. After the 15th rinse from washing bottle, completely submerge the insert 3 times in 150 mL DPBS (shake to remove all rests of test material).

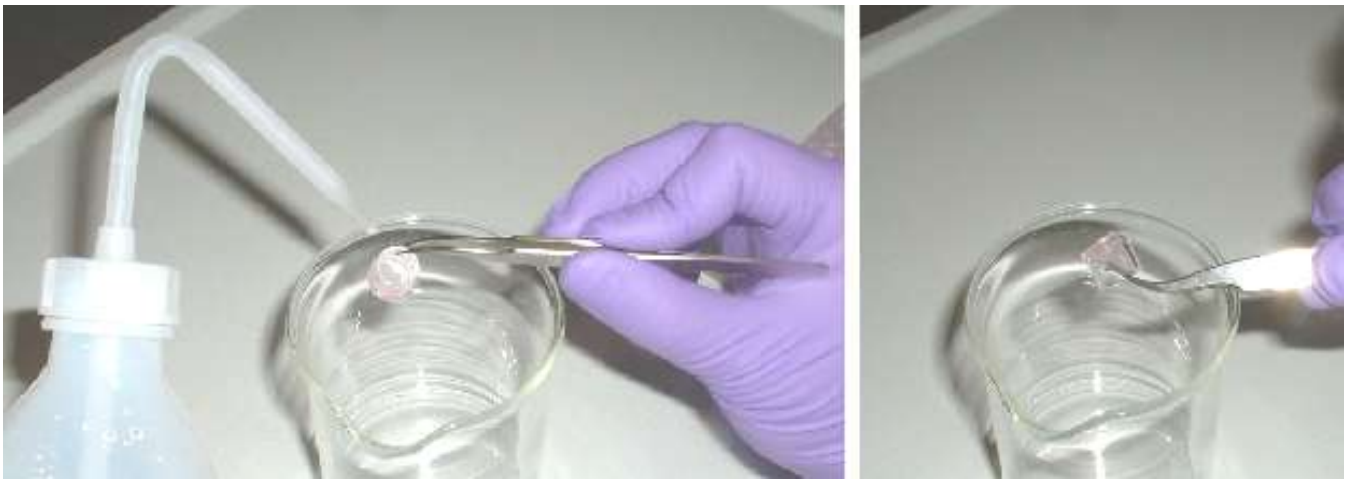


**Figure 10:** Removal of test articles - washing procedure

7. Finally, rinse the tissue once from inside and once from outside with sterile DPBS. Remove excess of DPBS by gentle shaking the insert, blot insert on sterile blotting paper (**Figure 11**).
8. Transfer the blotted tissue inserts to new 6-well plates pre-filled with 0.9 mL of fresh assay medium.
9. After all inserts are washed**, DO NOT FORGET to carefully dry** the surface of each tissue with a sterile cotton tipped swab (**Figure 11**). In case that traces of the chemical are still present on the surface, try to remove it with the sterile wetted cotton swab. Record this procedure in the MDS. You may evaluate visually tissue surface under a dissecting stereoscope.
10. Incubate tissues in the incubator for next 24±2 hours. Record start time of incubation in the MDS.

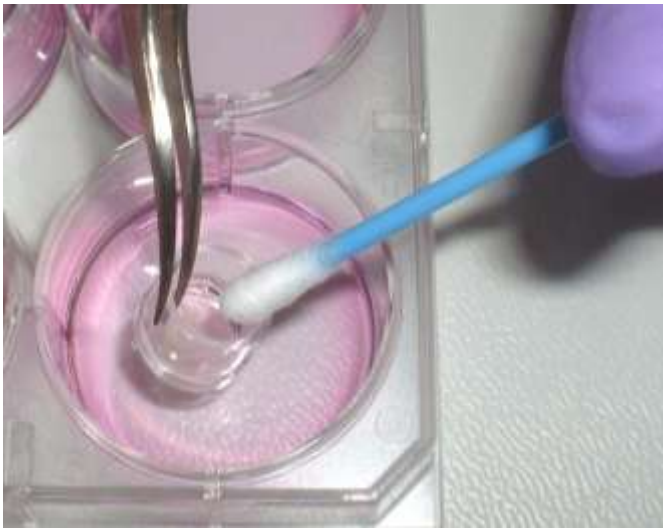



**Figure 11:** Completion of tissue washing - blotting and drying the tissue surface

### 7.7.3 DAY 2 – Change medium (mandatory – steps 1-2)

### Collect media for cytokine analysis (optional – steps 3-7)

1. At the end of the 24 ± 2 hr incubation period, pre-fill the lower row of the 6-well plates with 0.9 mL of fresh assay medium.
2. Transfer the inserts from the upper row of the 6-well plates into the lower row and place the 6-well plates back into the incubator for an additional 18 ± 2 hr post-incubation.
3. If the medium from the 24 hour incubation will be analyzed for cytokine or chemokine release, prepare a sufficient number of sterile vials (e.g. cryotubes, volume 1.5 mL). Alternatively, the media can be stored in a labeled 24-well plate (design shown below).

| NCa | NCb | NCc | PCa | PCb | PCc |
|---|---|---|---|---|---|
| 1a | 1b | 1c | 2a | 2b | 2c |
| 3a | 3b | 3c | 4a | 4b | 4c |
| 5a | 5b | 5c | 6a | 6b | 6c |

4. Mark the cryotubes or a 24-well plate with names or codes of the test substances and replicate code (e.g. a, b, c). Include the tissue lot number and date of the experiment. Use a water resistant marker.
5. Place the 6-well plates containing inserts on a plate shaker (500 rpm/min) for 5 minutes.
6. Transfer the medium (approximately 0.9 mL) from the 24-hour incubation plates into the cryotubes or 24-well plate. Use fresh pipette tips between samples.
7. Close the vials properly. If used, the 24-well plate should be sealed with parafilm. Store the samples at -20 ± 5°C (for up to 12 months) until analysis.

## 7.7.4 DAY 3 – MTT Viability Test

**MTT Test**

1. Prior to the MTT assay, label a sufficient number of 24-well plates.
2. Prepare MTT medium from frozen concentrate according to **7.5.1** and pipette 300 µL of MTT medium in each well.
3. Remove inserts from the 6-well plates, blot the bottom of the inserts, and transfer them into the 24-well plates, pre-filled with 0.3 mL of MTT (1 mg/mL). Place the plates in the incubator (37±1°C, 5± 1% $CO_2$, 90% ±10% RH), record the start time of MTT incubation in the MDS and incubate for **3 hours ± 5 min**.

*Note: The 3 hours +/- 5 min MTT incubation time must be strictly adhered to. Deviations from the 3 hour time for MTT incubation will result in different MTT readings.*

4. After MTT incubation is complete, gently aspirate the MTT medium from all the wells (e.g. using a suction pump plus toxic waste trap). Refill the wells with DPBS and aspirate again. Repeat this rinsing twice and make sure that tissues are dry after the last aspiration. Alternatively, after aspirating the MTT medium, the blot the bottom of the insert on an absorbent material. Transfer inserts to new 24-well plates.
5. Immerse the inserts by gently pipetting 2.0 mL of isopropanol (extractant solution MTT-100-EXT) into each insert. The level will rise above the upper edges of the insert, thus completely covering the tissues from both sides.
6. Seal the 24-well plates (e.g. with Parafilm or place into a sealable plastic bag) to inhibit extractant evaporation. Record start time of extraction in the MDS and extract formazan for at least 2 hours at room temperature with gentle shaking on a plate shaker (~ 120 rpm).
7. As an alternative, overnight extraction is also possible. Seal plates as described above and extract at room temperature in the dark, without shaking. Before using the extracts, shake for at least 15 min on plate shaker. After the extraction period is complete, pierce the inserts with an injection needle (~gauge 20, ~ 0.9 mm diameter) and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Before transferring the extract to 96 well plates pipette up and down 3x until the extractant solution is homogenous.
8. For each tissue, transfer 2 × 200µL aliquots of the blue formazan solution into a 96-well flat bottom microtiter plate according to the fixed plate design given in spreadsheet (example is given in **Figure 12**). Use isopropanol (MTT-100-EXT) as blanks. Read OD in a 96-well plate spectrophotometer using a wavelength between 540 and 595 nm, preferably at **570 nm**, **without using a reference filter.**

**PLATE 1**

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

NC - Negative control ; PC – Positive control; C1, C2... - Chemical No. 1, Chemical No.2..; BL - Blank

**Figure 12:** Fixed 96 well-plate design (for OD reading in plate photometer, 2 aliquots per tissue)

In contrast to other photometers, in plate readers pipetting errors influence the OD. Therefore, 2 formazan extract aliquots shall be taken from each tissue extract. In the Excel data sheet, these 2 aliquots will be automatically reduced to one value by calculating the mean of the two aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.

The plate design is dictated by the plate design used in the Spreadsheet, which was used in the validation study for data collection and preliminary calculations. It is necessary to strictly keep the plate design given here. Otherwise, the calculation of results will be incorrect.

*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 30%.*

## 7.8 Documentation

### 7.8.1 Method Documentation Sheet, MDS (see ANNEX B)

The Method Documentation Sheet allows a GLP compliant QC of the correct set up, calibration and function of the equipment, as well as all preparations.

If the test is performed as a GLP compliant procedure in a non-GLP environment filling in all information is mandatory. However, in a full GLP certified laboratory many records (in particular equipment calibrations, temperatures etc.) may be recorded centrally by other means. In this case, reference to the relevant laboratory notebook or other documentation is sufficient.

**Per each experiment, make a hardcopy of the MDS, fill in and sign the requested information, starting on 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. If adjustable pipettes are used, the correct adjustment shall be checked and recorded in the MDS on each test day.*
*Note (2): It is recommended at the beginning of the study to check the weight of a levelled application spoon of each solid test substance and record this weight in Annex C.*

### 7.8.2 Test Data

A blank, password protected MS EXCEL workbook **EpiDerm-SIT-SPREAD.XLS** can be provided by ZEBET or MatTek Corporation. A copy should be made before the first data entry. The workbook consists of two single spreadsheets named: IMPORT and SPREAD.

Data files of optical densities (ODs) generated by the microplate reader (without blank subtraction) are copied from the reader software to the Windows Clipboard and then pasted into the first spreadsheet of the EXCEL workbook (IMPORT – see **Figure 13**). The blank corrections, calculation of results and statistical parameters are done automatically in the second part of the workbook (SPREAD – see **Figure 14**).

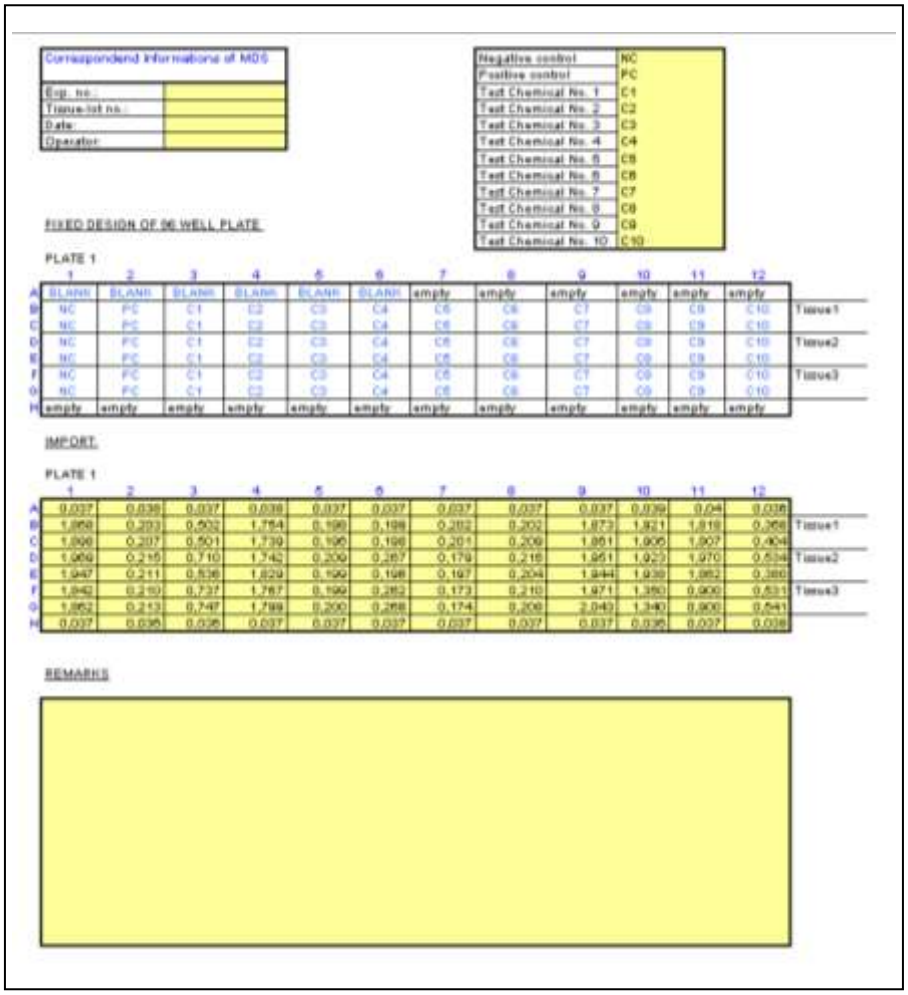



**Figure 13:** First part of the excel workbook EpiDerm-SIT-SPREAD.XLS.

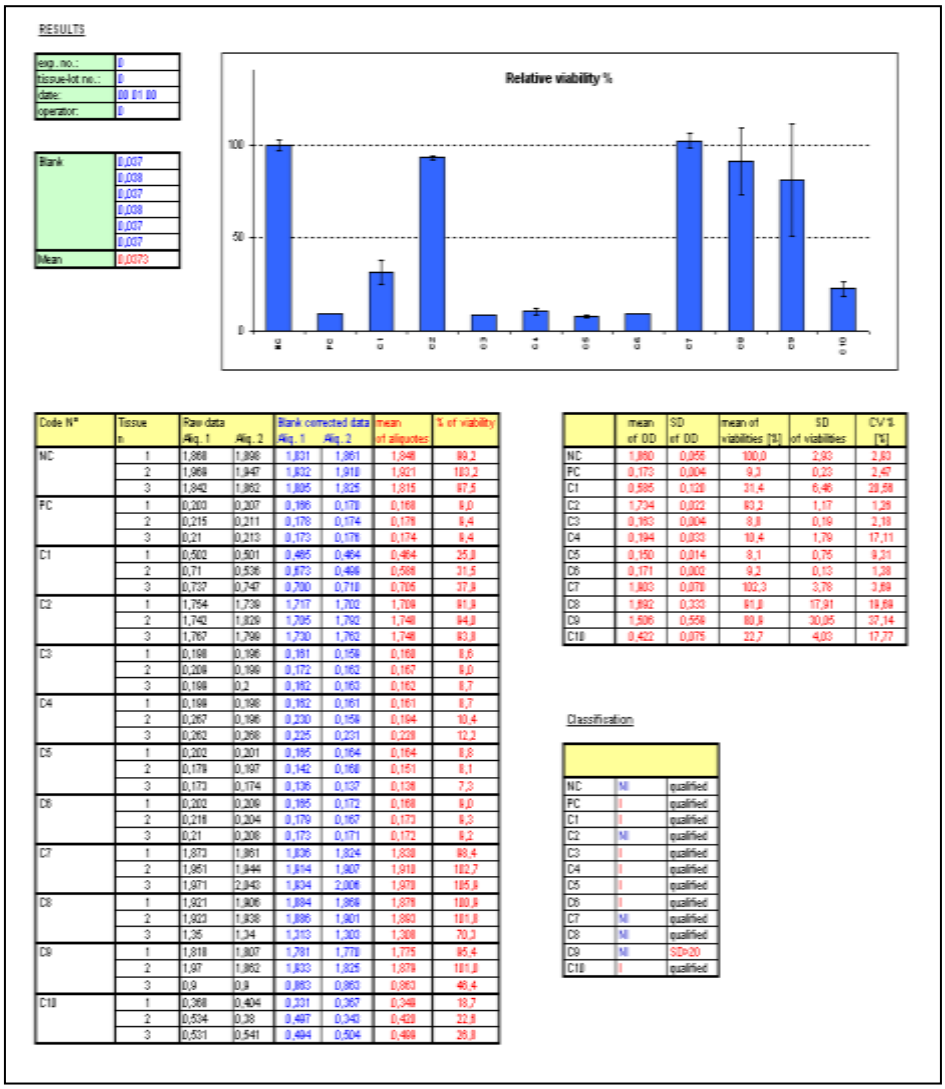
Use the fixed 96-well plate design as specified in the SOP. In addition to the entry of the reader raw data, some requested information has to be filled in the first map of the spreadsheet: (tissue lot numbers, test material codes, date, lab personnel).

**After data entry, the spreadsheet performs the following calculations**

1. Blank correction
2. For each individual tissue treated with a test substance (TS), the positive control (PC) and the negative control (NC) the individual relative tissue viability is calculated according to the following formulas

   Relative viability TS (%) = [$OD_{TS}$ / Mean of $OD_{NC}$] x 100
   Relative viability NC (%) = [$OD_{NC}$ / mean of $OD_{NC}$] x 100
   Relative viability PC (%) = [$OD_{PC}$ / mean of $OD_{NC}$] x 100

3. For each test substance, negative control, and the positive control, the mean relative viability of the three individual tissues is calculated and used for classification according to the Prediction Model (see section 6).
4. The spreadsheet shows a graph of the results (% of relative viability ± SD) see **Figure 14**.



RESULTS

| | |
|---|---|
| exp. no.: | 0 |
| tissue-lot no.: | 0 |
| date: | 00.01.00 |
| operator: | 0 |

| | |
|---|---|
| Blank | 0,037 |
| | 0,038 |
| | 0,037 |
| | 0,038 |
| | 0,037 |
| | 0,037 |
| Mean | 0,0373 |



| Code N° | Tissue n | Raw data Aliq. 1 | Raw data Aliq. 2 | Blank corrected data Aliq. 1 | Blank corrected data Aliq. 2 | mean of aliquotes | % of viability |
|---|---|---|---|---|---|---|---|
| NC | 1 | 1,868 | 1,898 | 1,831 | 1,861 | 1,846 | 99,2 |
| | 2 | 1,969 | 1,947 | 1,932 | 1,910 | 1,921 | 103,2 |
| | 3 | 1,842 | 1,862 | 1,805 | 1,825 | 1,815 | 97,5 |
| PC | 1 | 0,203 | 0,207 | 0,166 | 0,170 | 0,168 | 9,0 |
| | 2 | 0,215 | 0,211 | 0,178 | 0,174 | 0,176 | 9,4 |
| | 3 | 0,21 | 0,213 | 0,173 | 0,176 | 0,174 | 9,4 |
| C1 | 1 | 0,502 | 0,501 | 0,465 | 0,464 | 0,464 | 25,0 |
| | 2 | 0,71 | 0,536 | 0,673 | 0,499 | 0,586 | 31,5 |
| | 3 | 0,737 | 0,747 | 0,700 | 0,710 | 0,705 | 37,9 |
| C2 | 1 | 1,754 | 1,739 | 1,717 | 1,702 | 1,709 | 91,8 |
| | 2 | 1,742 | 1,829 | 1,705 | 1,792 | 1,748 | 94,0 |
| | 3 | 1,767 | 1,798 | 1,730 | 1,762 | 1,746 | 93,8 |
| C3 | 1 | 0,168 | 0,196 | 0,131 | 0,159 | 0,160 | 8,6 |
| | 2 | 0,204 | 0,199 | 0,172 | 0,162 | 0,167 | 9,0 |
| | 3 | 0,199 | 0,2 | 0,162 | 0,163 | 0,162 | 8,7 |
| C4 | 1 | 0,199 | 0,198 | 0,162 | 0,161 | 0,161 | 8,7 |
| | 2 | 0,257 | 0,196 | 0,220 | 0,159 | 0,194 | 10,4 |
| | 3 | 0,262 | 0,268 | 0,225 | 0,231 | 0,228 | 12,3 |
| C5 | 1 | 0,202 | 0,201 | 0,165 | 0,164 | 0,164 | 8,8 |
| | 2 | 0,179 | 0,197 | 0,142 | 0,160 | 0,151 | 8,1 |
| | 3 | 0,173 | 0,174 | 0,136 | 0,137 | 0,136 | 7,3 |
| C6 | 1 | 0,202 | 0,209 | 0,165 | 0,172 | 0,168 | 9,0 |
| | 2 | 0,216 | 0,204 | 0,179 | 0,167 | 0,173 | 9,3 |
| | 3 | 0,21 | 0,206 | 0,173 | 0,171 | 0,172 | 9,2 |
| C7 | 1 | 1,873 | 1,861 | 1,836 | 1,824 | 1,830 | 98,4 |
| | 2 | 1,851 | 1,944 | 1,814 | 1,907 | 1,910 | 102,7 |
| | 3 | 1,971 | 2,943 | 1,934 | 2,886 | 1,979 | 185,8 |
| C8 | 1 | 1,921 | 1,906 | 1,884 | 1,868 | 1,876 | 100,8 |
| | 2 | 1,923 | 1,838 | 1,886 | 1,801 | 1,893 | 101,8 |
| | 3 | 1,35 | 1,34 | 1,313 | 1,303 | 1,308 | 70,3 |
| C9 | 1 | 1,818 | 1,807 | 1,781 | 1,770 | 1,775 | 95,4 |
| | 2 | 1,97 | 1,962 | 1,933 | 1,925 | 1,879 | 101,0 |
| | 3 | 0,9 | 0,9 | 0,863 | 0,863 | 0,863 | 46,4 |
| C10 | 1 | 0,368 | 0,404 | 0,331 | 0,367 | 0,349 | 18,7 |
| | 2 | 0,534 | 0,38 | 0,497 | 0,343 | 0,420 | 22,6 |
| | 3 | 0,531 | 0,541 | 0,494 | 0,504 | 0,499 | 26,8 |

| | mean of OD | SD of OD | mean of viabilities [%] | SD of viabilities | CV% [%] |
|---|---|---|---|---|---|
| NC | 1,890 | 0,056 | 100,0 | 2,93 | 2,93 |
| PC | 0,173 | 0,004 | 9,3 | 0,23 | 2,47 |
| C1 | 0,585 | 0,128 | 31,4 | 6,46 | 20,58 |
| C2 | 1,734 | 0,022 | 93,2 | 1,17 | 1,26 |
| C3 | 0,163 | 0,004 | 8,8 | 0,19 | 2,19 |
| C4 | 0,194 | 0,033 | 10,4 | 1,79 | 17,11 |
| C5 | 0,150 | 0,014 | 8,1 | 0,75 | 9,21 |
| C6 | 0,171 | 0,002 | 9,2 | 0,13 | 1,38 |
| C7 | 1,903 | 0,078 | 102,3 | 3,78 | 3,69 |
| C8 | 1,892 | 0,333 | 91,8 | 17,91 | 19,69 |
| C9 | 1,936 | 0,556 | 80,8 | 30,05 | 37,14 |
| C10 | 0,422 | 0,075 | 22,7 | 4,03 | 17,77 |

Classification

| | | |
|---|---|---|
| NC | NI | qualified |
| PC | I | qualified |
| C1 | I | qualified |
| C2 | NI | qualified |
| C3 | I | qualified |
| C4 | I | qualified |
| C5 | I | qualified |
| C6 | I | qualified |
| C7 | NI | qualified |
| C8 | NI | qualified |
| C9 | NI | SD>20 |
| C10 | I | qualified |

**Figure 14:** Second spreadsheet of the excel workbook EpiDerm-SIT-SPREAD.XLS.

- **For each experiment, make a hardcopy of the raw data (i.e. plate reader data).**
- **For each experiment, save your secondary data in one copy "EpiDerm-SIT-SPREAD.XLS".**
- **Fill in the requested information in "EpiDerm-SIT-SPREAD.XLS".**
- **In addition, per each experiment, keep signed hardcopies of "EpiDerm-SIT-SPREAD.XLS" together with the signed hardcopy of the MDS.**

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# ANNEX A: EpiDerm™ Skin Irritation Test: Flowchart

**TRANSFER TISSUES FROM AGAROSE TO ASSAY MEDIUM**

⬇

**INCUBATE (37±1°C, 5±1 % $CO_2$, 90% ± 10% RH) FOR 60 ± 5 MIN**

⬇

**TRANSFER TISSUES TO FRESH ASSAY MEDIUM**

⬇

**INCUBATE (37±1°C, 5±1 % $CO_2$, 90% ± 10% RH) OVERNIGHT (18±3 H)**

⬇

**DOSE 3 TISSUES EACH WITH 30 µL / 25 MG TS, OR PC, OR NC**

⬇

**EXPOSE 60 MIN (37±1°C, 5±1 % $CO_2$, 90% ± 10% RH)**

⬇

**STOP EXPOSURE AFTER 60 MINUTES BY RINSING WITH DPBS**

⬇

**TRANSFER TISSUES TO FRESH ASSAY MEDIUM**

⬇

**INCUBATE (37±1°C, 5±1 % $CO_2$, 90% ± 10% RH) FOR 24 ±2 HOURS**

⬇

**COLLECT MEDIUM FOR IL-1A ANALYSIS (*OPTIONAL STEP*)**

⬇

**INCUBATE (37±1°C, 5±1 % $CO_2$, 90% ± 10% RH) FOR NEXT 18±2 HOURS**

⬇

**BLOT TISSUES AND PERFORM MTT ASSAY**

⬇

**READ OD IN A PLATE SPECTROPHOTOMETER AT 550-570 NM**

## ANNEX B: Methods Documentation Sheet (MDS)

**Assay**:……

**Date:** ……

**Corresponding XLS Data File Name:** ……

**PERFORMED BY: .....SIGNATURES: .....**

**Time Protocol**

**Receipt of EpiDerm™ tissues** (date, day, hour):

……

**ID:**

**Experimental schedule**

| **Procedure** | **Date (dd-mm-yy)** | **Set 1** | | **Set 2** | | **Remark** |
|---|---|---|---|---|---|---|
| | | start | stop | start | stop | |
| **Pre-incubation 1** (60 ± 5 min) | | | | | | |
| **Pre-incubation 2** (18 ± 3h) | | | | | | |
| **Exposure** (60 ±1 min) | | | | | | |
| **Washing** | | | | | | |
| **Post-incubation 1 - start** (24 ± 2h ) | | | | | | |
| **Medium change** (24h after exposure ± 2h) | | | | | | |
| **Post-incubation 2 – start** (18 ± 2h) | | | | | | |
| **MTT test** (3h ± 5 min) | | | | | | |
| **Extraction** (minimum 2h) | | | | | | |
| **Measurement** | | | | | | |

## Devices Verification

**Incubator verification**

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

ID/ Date:

**Refrigerator verification** **Water bath verification**

| Refrigerator # | Temperature < 5°C ± 3°C > | | Water bath # | Temperature < 37 ± 1°C > |
|---|---|---|---|---|
| | | | | |

ID/ Date: 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** |
|---|---|---|
| | | |
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| | | |

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 | 25 µL | 30 µL |
|---|---|---|---|---|---|---|
| | .....$H_2O$ weight in g..... | | | | | |
| 1. | | | | | | |
| 2. | | | | | | |
| 3. | | | | | | |
| Mean | | | | | | |
| SD | | | | | | |

ID/ Date:

## Kit Components *(**MTT-100 Kit Must Order Separately)*

| | |
|---|---|
| EpiDerm™ (EPI-200-SIT)<br>Lot no.: | Production date: |
| Assay medium (EPI-100-NMM)<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: |
| DPBS (TC-PBS); 100 mL<br>Lot no: | Expiration date: |
| 5% SDS – Positive Control (TC-SDS-5%) | Expiration date: |
| Position of Ice-packs:<br>(direct contact of the ice-packs with the skin must be avoided) | |
| Other remarks | |

ID/ Date:

**Visual Quality Control Of The Skin**

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

| APPERANCE | **KIT 1** | **KIT 2** |
|---|---|---|
| MACRO. | | |
| No of excluded tissues with:<br>- edge defects<br>- air bubbles<br>- extensive moisture on the surface | | |

Specific observations:

## Solutions

**Positive Control**

*Note: In case you are preparing your own MTT stock solution and/or DPBS fill in the following* forms:

**MTT Stock Solution Preparation**: 5 mg/mL:

- MTT batch N° : ……..
- Weight : ……..
- DPBS batch N°: ……...
- DPBS Volume added: …….
- Preparation date: ……...
- Expiration date : ……..
- Stocking place : Refrigerator N°…….....

**DPBS Solution Preparation**:

- Preparation date: …….
  - pH adjustment (to 7.0)……..…….....
  - Type of sterilisation.....
- Preparation date: ……..…….....
- Expiration date : ……...

## Dosing Procedure

Tick (✓) in the columns the type of application (pipette, patch, spoon...).
(**P**:pasty; **V:** viscous; **W**:waxy; **ST**:sticky; **C**:creamy)

| Code | Liquid/ Solid (L/S) | Application (25 mg / 30 µL) | | | | | Remarks |
|---|---|---|---|---|---|---|---|
| | | Pipette (✓) | Mesh (✓) | Spoon (✓) | Pin (✓) | Wetting (✓) | |
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ID/ Date:

## Remarks to Single Tissues

If during the assay you observe any abnormality, have to substitute tissues, or deal with any technical problems, fill in following table.

Record the tissue number, substance code and your observation or remark.

| Substance code | Tissue No. | Remark |
|---|---|---|
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ID/ Date:

## MTT Plate Configuration

PLATE 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 |

PLATE 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 |

ID/ Date:

## Spectrophotometrical Measurement

**Plate Configuration for Reading (for transfer to Spreadsheet EpiDerm SIT.xls):**
Record the positions of substances on 96-well plate.
Strictly adhere to the fixed plate design of the SOP (version January 14, 2009).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | |
| B | **NC** | **PC** | | | | | | | | | | | Tissue1 |
| C | **NC** | **PC** | | | | | | | | | | | |
| D | **NC** | **PC** | | | | | | | | | | | Tissue2 |
| E | **NC** | **PC** | | | | | | | | | | | |
| F | **NC** | **PC** | | | | | | | | | | | Tissue3 |
| G | **NC** | **PC** | | | | | | | | | | | |
| H | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | **Blank** | |

***Note:*** **Turn on the reader 10 min before reading plate.**

**Check plate photometer filter**
Tick correct (✓) filter setting

| | |
|---|---|
| Reading Filter:<br>570 (550-570) nm | |
| No Reference Filter | |

ID/ Date:

**Archival**

Raw data saved in/as:

Spreadsheet saved in/as:

MDS saved in/as:

## ANNEX C: Characterization of Test Substances

| Code | Physical state (S/L) | Color | Change of the color in aqueous environment (+ / -) | MTT reduction (+ / -) | Reaction with mesh (+ / - ) | Remarks | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Known R-phrases | Known S-phrases | Special Storage Requirements | Other |
| | | | | | | | | | |
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## Solid Substances Only

| Code | Weight of substance (when applied by spoon) in mg | | | Grinding (yes / no) | Remarks |
|---|---|---|---|---|---|
| | 1. | 2. | 3. | | |
| | | | | | |
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# ANNEX D: 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 experimenter 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.