**DB-ALM Protocol n° 138 : In vitro EpiDerm™ Skin Irritation Test (EpiDerm SIT)**

**Skin Irritation and Corrosivity**

EpiDerm™ Skin Irritation Test (SIT) is designed for the prediction of skin irritation potential of test substances through their cytotoxic effect on the in vitro reconstructed human 3D epidermis equivalent EpiDerm™(EPI-200). Cytotoxicity is assessed in the MTT cell viability assay. The EpiDerm SIT is granted regulatory approval as a full replacement method for the in vivo rabbit skin irritation test under the EC Test Method Regulation (Method B.46, EU 2009) and under the conditions laid down in the OECD Test Guideline No. 439 – In Vitro Skin Irritation: Reconstructed Human Epidermis Model.

**Objective & Application**

- **TYPE OF TESTING**: Part of a test battery, Part of an integrated testing strategy, Replacement (partial), Replacement (full)
- **LEVEL OF ASSESSMENT**: Toxic potential, Hazard identification
- **PURPOSE OF TESTING**: Classification/labelling, Safety, Product development

**Context of Use:**
The EpiDerm™ SIT consists of a topical exposure of the neat test chemical to a reconstructed human epidermis (RhE, Epi-200) followed by a cell viability test. Cell viability is measured through MTT [2-(4,5-dimethyl thiazole-2-yl) 2,5-diphenyltetrazoliumbromide] reduction by a mitochondrial dehydrogenase into a blue formazan salt, which is quantified after extraction from tissues (Mosmann, 1983). The reduction of the viability of tissues exposed to highly coloured chemicals and/or MTT reducers may interfere with the cel viability measurement, if the test substance is still present in the test system at the time of the MTT test. The protocol includes a list of control steps to detect, quantify and compensate such interference. In a very unlikely case, highly interfering test substance can be judged incompatible with the test.

**Résumé**

The United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) defines skin irritation as production of reversible damage to the skin following the application of a test substance for up to 4 hours (United Nations, 2009). The classification has been determined so far by a modification of the in vivo Draize rabbit skin irritation test (Draize et al., 1944), as described in the OECD TG 404 (OECD, 2002). However, the Draize test had been widely criticised for inadequate predictivity for human skin irritants and ethical issues regarding pain and discomfort caused to the animals during the test. An alternative in-vitro method was developed during the last decennium, based on the biological process behind the skin irritation in situ (Welss et al., 2004). Since systemic reactions play a minor role in modulating local skin toxicity of chemicals, the skin irritation potential can be predicted reliably by in vitro models, derived from the human epidermis. The only prerequisite is that the test system is sufficiently complex to mimic in vivo skin barrier function and cell reactivity. Specific acceptance criteria are described in the OECD TG 431 “In Vitro Skin corrosion: Human Skin Model Test” (OECD, 2004) and OECD TG 439 “In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method” (OECD, 2010).

Irritants penetrate the barrier function of the stratum corneum and disrupt cell integrity. The damage may be caused directly by the physico-chemical properties of the irritant (e.g. surfactants destabilise the lipid bi-layers, create pores and denature membrane proteins) or indirectly, e.g. when irritant induces a release of reactive oxygen species which in turn damage membranes and proteins. Both scenarios lead eventually to cell death and an inflammatory response in the exposed tissue (Welss et al., 2004). Cell viability was judged as the most robust biomarker and selected as the main endpoint in the ECVAM-coordinated validation study. IL-1alpha measurement is considered only as a complementary endpoint, and it is not used for classification and labelling of skin irritation hazard (ESAC, 2007 and 2009).

The EpiDerm™ SIT consists of a topical exposure of the neat test chemical to a reconstructed human epidermis (RhE, model EPI-200) followed by a cell viability test. Cell viability is measured through MTT [3-(4,5-dimethyl thiazole-2-yl) 2,5-diphenyltetrazoliumbromide] reduction by a mitochondrial dehydrogenase into a blue formazan salt, which is quantified after extraction from tissues (Mosmann, 1983). 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. The protocol also describes optional sampling points for inflammatory cytokine measurements. However, note that IL-1 alpha measurement is not required by OECD TG439 and that comparative studies in RhE models employing various endpoints to predict skin irritancy of topical formulations have shown that the additional measurement of IL-1 alpha release did not increase the predictive capacity of the test for the classification of skin irritation hazard (Faller and Bracher 2002, Faller et al. 2002, Kandarova et al., 2009).

**Experimental Description**

**Endpoint and Endpoint Measurement:**

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

**CYTOKINE DETERMINATION:** the release of cytokines (e.g. IL-1 alpha) is determined in the culture medium by ELISA (Enzyme Linked Immunosorbent Assay) and expressed in pg/ml (an optional endpoint in the skin irritation test)

**Endpoint Value:**

**Experimental System(s):**

RHE EpiDerm™: The reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, USA and MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia) grown from normal human-derived epidermal keratinocytes, which have been cultured to form a multilayered highly differentiated model of the human epidermis. It consists of organised basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. The EpiDerm™ tissues (surface 0.63 cm²) are cultured on specially prepared cell culture inserts and shipped world-wide as kits, containing 12, 18 or 24 tissues on shipping agarose together with the necessary amount of culture media and handling plates.

**Basic Procedure**

On the day of receipt, EpiDerm tissues are conditioned overnight by incubation to release transport-stress-related compounds and debris. Three tissues are used for each test chemical (TC) and for the positive (PC) and negative (NC) controls. After a pre-incubation step, tissues are topically exposed to the test chemicals for 60 minutes. Tissues are then thoroughly rinsed and blotted to remove the test substances, and transferred to fresh medium. After a 24 hr incubation period, the medium can be collected for analysis of cytokines (optional step). 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 incubation, the blue formazan salt formed by cellular mitochondria is extracted with isopropanol (2.0 ml/tissue). The optical density of the extracted formazan is determined 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 positive if the remaining relative cell viability is below 50%.


**Data Analysis/Prediction Model**

Under the UN GHS and EU CLP systems (Category 2: Irritant or “No Category”; United Nations, 2009; EC No.1272/2008, EU, 2008), the test substance is classified as irritant if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls. The prediction model is defined as described below:

<table>
<thead>
<tr>
<th>In vitro results</th>
<th>In vivo classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean tissue cell viability ≤50%</td>
<td>Category 2: Irritant</td>
</tr>
<tr>
<td>Mean tissue cell viability &gt;50%</td>
<td>No Category</td>
</tr>
</tbody>
</table>

**Test Compounds and Results Summary**

The accuracy of the EpiDerm™ SIT was evaluated in a follow-up validation study with 55 well-characterised chemicals(Kandarova et al., 2009). The same chemicals were earlier used in the EpiDerm and EpiSkin optimisation studies(Kandarova et al., 2005, Cotovio et al. 2005) and in phase II ECVAM validation study (ECVAM, 2007; Spielmann et al. 2007). The selection focused on those which:

- were under-predicted in phase II ECVAM validation study, where a protocol with a shorter exposure time was used

cause slight to moderate erythema, which is a group most prone to prediction errors.

Table II: Accuracy values for the MTT endpoint of the in vitro EpiDermTM SIT in an evaluation study with 55 selected chemicals (Kandarova et al., 2009).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EpiDermTM SIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>76.3 %</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>86.1 %</td>
</tr>
<tr>
<td>Accuracy</td>
<td>80.6 %</td>
</tr>
</tbody>
</table>

The reproducibility of the EpiDerm SIT protocol was further tested in 3 independent laboratories using 20 reference chemicals of the 2007 Performance Standards document. EpiDermTM SIT met all pre-defined performance standards with regard to the method’s predictive capacity (ESAC, 2009).

Table III: Accuracy values for the 20 reference chemicals and MTT endpoint of the in vitro EpiDermTM SIT in comparison with the validated reference method (ESAC, 2009).

<table>
<thead>
<tr>
<th>Performance standard (validated reference method)</th>
<th>EpiSkinTM (validated reference method)</th>
<th>EpiDermTM SIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>≥70 %</td>
<td>76.9 %</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>≥80 %</td>
<td>85.7 %</td>
</tr>
<tr>
<td>Accuracy</td>
<td>≥75 %</td>
<td>75 %</td>
</tr>
</tbody>
</table>

Modifications of the Method

This protocol is based on a method initially developed and refined by L’Oreal for the EpiSkin model (Portes et al., 2002, Cotovio et al., 2005). The generic SOP was applied to the EpiDerm model, with the aim to develop a common protocol for both systems, able to predict skin irritation potential according to the EU classification system and replace the in vivo acute skin irritation test in rabbits (Kandarova et al., 2005; Spielman et al., 2007).

The first ECVAM-coordinated validation study was followed by the recommendation of ESAC to improve the sensitivity of the test (ESAC, 2007). The EpiDerm TM SIT was further optimised 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; so that the concept of common protocol was maintained (Kandarova et al., 2009).

The current version of the protocol is based on ZEBET’s SOP version 7.0 drafted by Manfred Liebsch and Dieter Traue (ZEBET at the BfR), approved by Helena Kandárová (MatTek Corporation) and was used in the follow-up validation study of the modified EpiDerm™ Skin Irritation Test (ESAC, 2009, Kandarova et al., 2009).

Acceptance Criteria and Proficiency Testing

OECD Test Guidelines 431 and 439 (OECD 2004 and 2010) contain a generic description of general and functional conditions that reconstructed human skin models need to comply with when the test is used for regulatory purpose.

OECD TG 439 (OECD, 2010), recommends that prior to the routine use of the test system, the laboratory should demonstrate its technical proficiency using the ten Proficiency Chemicals listed in the Table IV, which are a subset used in the validation study:

Table IV: Proficiency chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS NR</th>
<th>In vivo score in accordance with OECD TG 404</th>
<th>Physical state</th>
<th>UN GHS Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>naphthalene acetic acid</td>
<td>86-87-3</td>
<td>0</td>
<td>Solid</td>
<td>No Cat.</td>
</tr>
<tr>
<td>isopropanol</td>
<td>67-63-0</td>
<td>0.3</td>
<td>Liquid</td>
<td>No Cat.</td>
</tr>
<tr>
<td>methyl stearate</td>
<td>112-61-8</td>
<td>1</td>
<td>Solid</td>
<td>No Cat.</td>
</tr>
<tr>
<td>heptyl butyrate</td>
<td>5870-93-9</td>
<td>1.7</td>
<td>Liquid</td>
<td>No Cat. (Optional Cat.3)*</td>
</tr>
<tr>
<td>hexyl salicylate</td>
<td>6259-76-3</td>
<td>2</td>
<td>Liquid</td>
<td>No Cat. (Optional Cat.3)*</td>
</tr>
<tr>
<td>cyclamen aldehyde</td>
<td>103-95-7</td>
<td>2.3</td>
<td>Liquid</td>
<td>Cat. 2</td>
</tr>
<tr>
<td>1-bromohexane</td>
<td>111-25-1</td>
<td>2.7</td>
<td>Liquid</td>
<td>Cat. 2</td>
</tr>
</tbody>
</table>
**Discussion**

EpiDerm™ Skin Irritation Test is one of the three assays that have been validated by ECVAM for the assessment of the skin irritation potential of chemicals (together with SkinEthic™ RHE 42bis and Episkin™ SIT 15min-42h) (ESAC, 2007 and 2009). The test is based on the Reconstructed Human Epidermis model which is accepted by OECD and EU as a full in vitro replacement of the in vivo Draize skin irritation test (TG439, OECD, 2010; TMR B.46, EU, 2009).

Under OECD TG 439 and Test method B.46, new testing laboratories are advised to demonstrate a technical proficiency using the ten proficiency chemicals, listed in the Table IV, before commencing to a routine assessment. The SOP, available in the Downloads section of this protocol, includes a list of data reporting forms (Method Documentation Sheets) to facilitate a GLP-compliant quality control of the correct set up, calibration and function of the equipment, as well as all preparations in a non-GLP environment.

The test can be performed in a standard cell culture laboratory under aseptic conditions. No specific equipment is required.

Test system provider has prepared a detailed video demonstration of this protocol, which is publically available via the Journal of Visualized Experiments (JoVE):

http://www.jove.com/video/1366/

The test takes 3 days to complete and one experienced laboratory technician can assess up to 18-20 chemicals in a single test run.

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A coloured test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the viability measurement. In case of such event, the (true) metabolic MTT reduction and the contribution by a coloured test material or (false) direct MTT reduction by the test material can still be quantified. The SOP provided in the Downloads section of the protocol lists a number of control steps designed to detect and correct the calculation, so that either the true metabolic MTT reduction can be assessed or, based on the good scientific judgment, the test substance deemed incompatible with the test method.

**Status**

**Known Laboratory Use:**

ZEBET at the BfR (Germany) - Participation in Validation Study

BioMed-zet Life Science GmbH (Austria) - Participation in Validation Study

IIVS (United States) - Participation in Validation Study

BASF (Germany)- Participation in Validation Study

List of laboratories and CRO's trained and qualified to perform the validated EpiDerm SIT assay can be provided per request by MatTek Corporation.

**Participation in Validation Studies:**

ECVAM Skin Irritation Task Force regarded skin irritation tests EpiSkin and EpiDerm as promising predictors for skin irritancy potential and both tests entered a formal validation study (SIVS), coordinated by ECVAM (2003-2007). Due to the under-prediction of several chemicals (Spielman et al., 2007), ESAC recommended to increase sensitivity of the EpiDerm SIT to better match in vivo Draize skin irritation test (Kandarova et al., 2009). The validation method 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 (OECD, 2005) and ECVAM Performance Standards for applying human skin models to in vitro skin irritation (ESAC, 2009).
In 2008, ESAC concluded that the Modified EpiDerm™ SIT had sufficient accuracy and reliability for prediction of R38 skin irritating and no-label (non-skin irritating) test substances (ESAC, 2009).

**Regulatory Acceptance:**

In July 2009, the reconstructed human epidermis test methods for skin irritation testing which meet a defined set of performance criteria (such as EpiSkin™, EpiDerm™ SIT and SkinEthic™ RHE) have been included in Commission Regulation No 761/2009/EC, Method B.46 (EU, 2009). It was a 1st adaptation to the technical progress of the EU Test Methods Regulation 440/2008/EC (EU, 2008), which serves as a technical Annex to the Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH, EU, 2006).

In 2010, "In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method" was adopted as the OECD Test Guideline No 439, which is applicable to the assays for skin irritation employing reconstructed human epidermis models (EpiSkin™, EpiDerm™ SIT and SkinEthic™ RHE) (OECD, 2010).

**Ongoing development:**

The same reconstructed human epidermis models are also used for the skin corrosivity classification (OECD TG 431). The OECD is developing a new test guideline for Integrated Testing Strategy for skin irritation and corrosion for an unequivocal classification of a test compound.

**Abbreviations and Definitions**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CLP</td>
<td>Classification, Labelling and Packaging</td>
</tr>
<tr>
<td>DSD</td>
<td>Dangerous Substance Directive</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco's Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>EC</td>
<td>European Comission</td>
</tr>
<tr>
<td>ECVAM</td>
<td>European Centre for the Validation of Alternative Methods. As from 2011, ECVAM has been established as the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), hosted by the Joint Research Centre, Institute for Health and Consumer Protection (IHCP).</td>
</tr>
<tr>
<td>ESAC</td>
<td>ECVAM Scientific Advisory Committee</td>
</tr>
<tr>
<td>ET-50</td>
<td>Exposure time that induce 50% cell viability</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>IC50</td>
<td>Concentration that induce 50% decrease in cell viability</td>
</tr>
<tr>
<td>I</td>
<td>Irritant</td>
</tr>
<tr>
<td>IL-1alpha</td>
<td>Interleukin-1alpha</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4, 5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MDS</td>
<td>Methods Documentation Sheet</td>
</tr>
<tr>
<td>NC</td>
<td>Negative Control</td>
</tr>
<tr>
<td>NI</td>
<td>Non Irritant</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Positive Control</td>
</tr>
<tr>
<td>RHE</td>
<td>Reconstructed Human Epidermis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SIT</td>
<td>Skin Irritation Test</td>
</tr>
<tr>
<td>SIVS</td>
<td>ECVAM-coordinated Skin Irritation Validation study which took place in 2003-2007.</td>
</tr>
<tr>
<td>TG</td>
<td>Test Guideline</td>
</tr>
<tr>
<td>TMR</td>
<td>Test Method Regulation</td>
</tr>
<tr>
<td>UN GHS</td>
<td>United Nations Globally Harmonized System of Classification and Labelling of Chemicals</td>
</tr>
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</table>

_Last update: 29 January 2013_
PROCEDURE DETAILS, 4 February 2013

In vitro EpiDerm™ Skin Irritation Test (EpiDerm SIT)
DB-ALM Protocol n° 138

This protocol is compliant with the OECD TG 439 In Vitro Skin Irritation: Reconstructed Human EpiDermis Test Method (Adopted on 22 July 2010).

On request of the Test Method’s authors, the Technical description is presented in the form of the Standard Operating Procedure (SOP), provided and maintained by MatTek Corporation.

Access to the full SOP document is provided by DB-ALM (ecvam-dbalm.jrc.ec.europa.eu). It can be found in the section related to DB-ALM Protocol No. 138, under Related information: Downloads box.

A detailed video demonstrating this protocol step by step is also available via the Journal of Visualized Experiments (JoVE): http://www.jove.com/video/1366/

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EU (2006)


EU (2006)


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Mosmann T. (1983)
Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays.

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OECD (2004)


Refinement of the EPISKIN protocol for the assessment of acute skin irritation of chemicals: follow-up to the ECVAM pravalidation study.

The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test.

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