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™ Skin Irritation Test (EpiDerm™ SIT) is an *in vitro* procedure that can be used for the hazard identification of irritant chemical substances and mixtures in accordance with the **United Nations Globally Harmonized System of Classification and Labelling of Chemicals** (UN GHS, United Nations, 2009) and **European Commission Regulation on the classification, labelling and packaging of substances and mixtures** (CLP, EC No.1272/2008, EU, 2008). Positive results obtained with the test method enable the classification of test substances as "Category 2: Irritant". If the regulatory authorities do not require the optional UN GHS classification of "Category 3: Mild irritant", the remaining test substances are labelled as “No Category” without further *in vivo* testing (Method B.46, EU 2009; TG 439, OECD, 2010).The EpiDerm™ SIT should be used within the sequential dermal irritation/corrosion testing strategy for the full characterisation of the skin irritation/corrosion potential (TG 404, OECD, 2002; Method B.4, EC No. 440/2008, EU 2008).

Applicability Domain:
This method is applicable to a wide range of chemicals and chemical classess with different physicochemical properties including:

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

So far the method was not evaluated for testing of gases, aerosols and highly volatile test compounds.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 predicitvity 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 at 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 denaturate 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-1*alpha* 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%.

A detailed video demonstrating this protocol step by step is available via the Journal of Visualized Experiments (JoVE):

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

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 I: Prediction model for EpiDerm™ SIT of the in vivo skin irritation hazard

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

Test Compounds and Results Summary

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 EpiDerm™ SIT in an evalulation study with 55 selected chemicals (Kandarova et al., 2009).

	EpiDerm™ SIT
Specificity	**76.3 %**
Sensitivity	**86.1 %**
Accuracy	**80.6 %**

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 EpiDerm™ SIT in comparison with the validated reference method (ESAC, 2009).

	Performance standard	EpiSkin™ *(validated reference method)*	**EpiDerm™ SIT**
Specificity	≥70%	76.9 %	**69.2 %**
Sensitivity	≥80%	85.7 %	**85.7 %**
Accuracy	≥75%	80 %	**75 %**

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™ 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

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

potassium hydroxide (5% aq.)	1310-58-3	3	Liquid	Cat. 2
1- methyl-3-phenyl-1-piperazine	5271-27-2	3.3	Solid	Cat. 2
heptanal	111-71-7	3.4	Liquid	Cat. 2

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

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 (Austira) - 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 Coproation.

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 rabbit data, before the test could be endorsed as a full replacement (ESAC, 2007).

The EpiDerm™ SIT was further optimised by MatTek Corporation during 2006 and 2007. The predictive capacity of the modified EpiDerm™ SIT was initially assessed by MatTek Corporation, USA in an intra-laboratory study (Kandarova *et al*., 2009). Transferability of the method was evaluated in 2007 in an external international validation study between 4 laboratories: ZEBET (BfR, Berlin, Germany); BASF (Ludwigshafen, Germany); IIVS (Gaithersburg, MD, USA) and Zet-LSL (Linz, Austria). 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 (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 unequivolcal classification of a test compound.

Abbreviations and Definitions

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

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/

Contact Details

Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET)
Federal Institute for Risk Assessment (BfR)
Diedersdorfer Weg 1
Berlin D-12277
email: zebet@bfr.bund.de

Dr. Patrick Hayden
US Headquarters
MatTek Corporation
200 Homer Ave.
Ashland, MA 01721
United States
email: PHayden@mattek.com

Dr Helena Kandárová
In Vitro Life Science Laboratories
MatTek Corporation
Mlynske Nivy 73
Bratislava 821 05
Slovakia
email: kandarova@centrum.sk

Bibliography

- Cotovio, J., Grandidier, M. H., Portes, P., Roguet, R., Rubinstenn, G. (2005)
  The in vitro skin irritation of chemicals: optimisation of the EPISKIN prediction model within the framework of the ECVAM validation process.
  Alternatives to Laboratory Animals (ATLA) 33, 329-349
- Draize J.H., Woodard G.K. and Calvery H.O. (1944)
  Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes.
  Journal of Pharmacology and Experimental Therapeutics 82, 377-390
- ECVAM (2007)
  Skin Irritation Validation Study Phase II: Analysis of the primary endpoint MTT and the secondary endpoint IL-1 alpha.
  European Union Reference Laboratory for Alternatives to Animal Testing
- ESAC (2009)
  Performance Standards for in vitro skin irritation test methods based on Reconstructed human Epidermis (RhE).
  ECVAM Scientific Advisory Committee.
- ESAC (2008)
  Statement on the Scientific Validity of In Vitro Tests for Skin Irritation Testing, Issued by the ECVAM Scientific Advisory Commitee (ESAC 29th meeting, 4-5th November, 2008)
  ECVAM Scientific Advisory Committee.
- ESAC (2007)
  Statement on the validity of *in vitro* tests for skin irritation, 26th meeting of ECVAM Scientific Advisory Committee (ESAC), 26-27 April, 2007.
  ECVAM Scientific Advisory Committee.
- EU (2009)
  Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical

progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). **Link to document** (last access 27.05.2013)
Official Journal of the European Communities L 220, 1-94

- EU (2008)
  Commision Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). **Link to document** (last access 04.03.2013)
  Official Journal of the European Communities L 142, 31.05.2008, 1-739
- EU (2008)
  Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP). Entered into force on the 20 January 2009 which will stepwise replace the Council Directive 67/548/EEC (substances), Directive 1999/45/EC (preparations), amending Regulation (EC) No 1907/2006 and subsequent amendments and adaptations. **Link to document** (last access 27.03.2013)
  Official Journal of the European Communities L 353, 1-1355
- EU (2006)
  Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). **Link to document** (last access 16.09.2013)
  Official Journal of the European Communities L396
- Faller, C., & Bracher, M. (2002)
  Reconstructed skin kits: reproducibility of cutaneous irritancy testing.
  Skin Pharmacology and Physiology S1, 74-91
- Faller, C., Bracher, M., Dami, N., & Roguet, R. (2002)
  Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics.
  Toxicology In Vitro 16(5), 557-572
- Kandárová H, Liebsch M, Gerner I, Schmidt E, Genschow E, Traue D, Spielmann H. (2005)
  The EpiDerm test protocol for the upcoming ECVAM validation study on in vitro skin irritation tests--an assessment of the performance of the optimised test.
  Alternatives to Laboratory Animals (ATLA) 33(4), 351-367
- Kandárová, H., Hayden, P., Klausner, M., Kubilus, J., Kearney, P., & Sheasgreen, J. (2009)
  In vitro skin irritation testing: Improving the sensitivity of the EpiDerm skin irritation test protocol.
  Alternatives to Laboratory Animals (ATLA) 37(6), 671-689
- Mosmann T. (1983)
  Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays.
  Journal of Immunological Methods 65, 55-63
- OECD (2015)
  OECD Test Guideline No. 404: Acute Dermal Irritation/Corrosion. First adopted in 1981. Latest update adopted on the 28th of July 2015. **Link to document** (last access: 16.06.2016)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2010)
  OECD Test Guideline No. 439: *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method, adopted on the 22nd of July 2010. **Link to document** (last access 16.08.2012)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- OECD (2005)
  OECD Series on Testing and Assessment No. 34: Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment.
  OECD Environmental Health and Safety Publications
- OECD (2004)
  OECD Test Guideline No. 431: *In Vitro* Skin Corrosion: Human Skin Model Test, adopted on the 13th of April 2004.
  Link to document (last access 16.08.2012)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects
- Portes, P., Grandidier, M.H., Cohen, C., Roquet, R. (2002)
  Refinement of the EPISKIN protocol for the assessment of acute skin irritation of chemicals: follow-up to the ECVAM pravalidation study.
  Toxicology In Vitro 16, 765-770
- Spielmann, H., Hoffmann, S., Liebsch, M., Botham, P., Fentem, J. H., Eskes, C., Roguet, R., et al. (2007)
  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.
  Alternatives to Laboratory Animals (ATLA) 35(6), 559-601
- United Nations (2013)
  Globally Harmonised System of Classification and Labelling of Chemicals (GHS); New York and Geneva. Fifth revised

edition. **Link to document** (last access 15.01.2014)

- Welss, T., Basketter, D. A., & Schröder, K. R. (2004)
  In vitro skin irritation: facts and future. State of the art review of mechanisms and models.
  Toxicology In Vitro 18(3), 231-243