DB-ALM Protocol n° 119 : EpiDerm™ Skin Corrosivity Test

Skin Irritation and Corrosivity

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EPIDERM™ human epidermal model. *(Prevalidation study protocol)*

Objective & Application

TYPE OF TESTING	:	replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency, hazard identification
PURPOSE OF TESTING	:	classification and labelling

The test method was granted regulatory approval as a replacement for the *in vivo* skin corrosivity test (Method B.40 bis, EU 2000, 2008; OECD Test Guideline 431, OECD 2004) and it is used for hazard identification and classification of corrosive potential in order to fulfil the regulatory requirements concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (EU, 2008). Furthermore, the test method is recommended for its use within the context of the sequential skin corrosivity testing strategy (OECD Test Guideline 404, OECD 2002 and Method B.4, EU 2004 and 2008).

The test allows the identification of corrosive chemical substances and mixtures and enables the identification of non-corrosive substances and mixtures when supported by a weight-of-the-evidence determination using other existing information (OECD, 2004 and EU, 2008).

Résumé

The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The two major mechanisms of skin corrosion are the destruction (erosion or solubilisation) of the skin penetration barrier (stratum corneum) including the viable skin cells underneath, and the rapid penetration of highly cytotoxic chemicals through the skin barrier without involving its destruction. The determination of the skin corrosion potential is included in international regulatory requirements for the testing of chemicals, such as the U.S. Code of Federal Regulations (US DOT, 1991), the updated OECD Test Guideline No 404 (OECD, 2002) and the Method B.4 of the Annex to Commission Regulation 440/2008/EC (EU, 2008). Corrosivity was usually determined *in vivo* using the Draize rabbit skin test (Draize *et al*., 1944).

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The test is designed to predict and classify the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted three-dimensional human epidermis model. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Liebsch *et al*., 2000).

Experimental Description

Endpoint and Endpoint Measurement:

CELL VIABILITY: Cell viability as determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT

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 day of receipt EPIDERM™ tissues are placed in the refrigerator. Next day, at least one hour before starting the assay, the tissues are transferred to 6-well plates with assay medium, which is immediately replaced before the test is started. The test is performed on a total of 4 tissues per test material, together with a negative control and a positive control.
Two tissues are used for a three-minute exposure to the test chemical and two for a one-hour exposure. 50 µl of the undiluted test material (liquids, semi-solids) or ~ 25 mg solid +25 µl H_2O are added into the MILLICELL® insert on top of the Epi-200 tissues. The remaining tissues are concurrently treated with 50µl distilled water (negative control) and with 50µl 8N-KOH (positive control). After the exposure period, the tissues are washed with phosphate buffered saline (PBS) to remove residual test material. Rinsed tissues are kept in 24-well plates (holding plates) in 300 µl serum free assay medium until 12 tissues (= one application time) have been dosed and rinsed. The assay medium is then replaced with 300 µl MTT-medium and tissues are incubated for three hours (37° C, 5% CO_2). After incubation, tissues are washed with PBS and formazan is extracted with 2 ml isopropanol (either for 2 hrs or overnight). The optical density of extracted formazan is determined spectrophotometrically at 570 nm (or 540 nm) and cell viability is calculated for each tissue as a % of the mean of the negative control tissues.

The skin corrosivity potential of the test materials is classified according to the remaining cell viability following exposure to the test material for either of the two exposure times.

Data Analysis/Prediction Model

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after exposure compared to the negative control tissues concurrently treated with H_2O. Chemical is classified "corrosive", if the relative tissue viability after 3 min exposure to a test material is decreased below 50% (PM1). In addition, those materials classified "non corrosive" after 3 min (viability >=50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15% (PM2).

For details see the section 4. **"Evaluation, Prediction Models (PM1 and PM2)"** reported in the present standard operating procedure.

Test Compounds and Results Summary

A total of 24 test compounds were chosen from the 60 chemicals tested in the ECVAM International Validation Study on *In Vitro* Tests for Skin Corrosivity in 1996/1997 (Liebsch *et al*., 2000).
These compounds included: 4 organic acids, 6 organic bases, 4 neutral organics, 2 phenols, 3 inorganic acids, 2 inorganic bases, 2 electrophiles and 1 soap/surfactant.

Discussion

In 1996-1997, international pre- and validation studies on four *in vitro* tests for skin corrosivity were performed by ECVAM (ECVAM, 1996, Botham *et al.*, 1995, Barratt *et al.*, 1998 and Fentem *et al.*, 1998). The evaluated tests were: Rat Skin Transcutaneous Electrical Resistance (TER) assay; CORROSITEX™ test; Skin² ZK 1350 and EPISKIN™ test (*DB-ALM* protocol numbers: 115, 116, 117, 118 respectively).
As an outcome of this validation study, two tests (TER assay and EPISKIN™) have been validated as a replacement to the respective animal test (Fentem *et al.*, 1998).
Following the conclusion of this study, the production of two *in vitro* 3-D models of reconstructed human skin/epidermis (Skin² ZK 1350 and EPISKIN™) was interrupted by the manufacturers. Therefore, at that time, the present EPIDERM™ assay was evaluated and proposed as an alternative human skin model.

Status

Participation in Validation Studies:

In 1996-1997, international pre- and validation studies on four *in vitro* tests for skin corrosivity were performed by ECVAM (ECVAM, 1996, Botham *et al.*, 1995, Barratt *et al.*, 1998 and Fentem *et al.*, 1998). The evaluated tests were: Rat Skin Transcutaneous Electrical Resistance (TER) assay; CORROSITEX™ test; Skin² ZK 1350 and EPISKIN™ test (*DB-ALM* protocol numbers: 115, 116, 117, 118 respectively).
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at that time, the present EPIDERM™ assay was evaluated and proposed as an alternative human skin model.

Following the successful conclusion of the ECVAM Skin Corrosivity Validation Study in 1998, a small catch up study of the EPIDERM™ test has been carried out (1997-1998) (Liebsch *et al.*, 2000). Based on the positive outcome of the study (Botham & Fentem, 1999), the ECVAM Scientific Advisory Committee unanimously endorsed the statement that the EPIDERM™ human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the EU and draft OECD test guidelines on skin corrosion (ESAC, 2000).

Regulatory Acceptance:

In 2000 the human skin model assays, which meet certain criteria (such as EPIDERM™ and EPISKIN™), have been included into "Annex V. Part B.40 on Skin Corrosion" of the "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" (EU, 2000).

The test method was further recommended to be used as one of the *in vitro* methods for skin corrosivity testing in the OECD Test Guideline 404 (OECD, 2002) and in the Method B.4 of Annex V of the Directive 67/548/EEC (EU, 2004) laying down the step-wise testing strategy for classifying skin corrosives by the sequential application of three alternative methods: structure-activity relationships, pH measurements and a single *in vitro* method (Worth *et al*., 1998).

In 2004, *In Vitro* Skin Corrosion: Human Skin Model Test was adopted as the OECD Test Guideline No 431 which is applicable to the assays for skin corrosion employing reconstituted human skin (EPISKIN™ and EPIDERM™) models (OECD, 2004).

In May 2008, all the test methods of Annex V of the Directive 67/548/EEC have been incorporated into the Annex to the Commission Regulation 440/2008/EC, 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) (EU, 2008).

Last update: December 2008

PROCEDURE DETAILS, 24 October 1997*

EpiDerm™ Skin Corrosivity Test

DB-ALM Protocol n° 119

The protocol presents the standard operating procedure used in the Prevalidation of EPIDERM™ Skin Corrosivity test (1997-1998) (Liebsch *et al.*, 2000)

* The accuracy of the SOP has been confirmed in October 2000.
During the preparation of the regulatory test guidelines some refinements have been introduced into the test method. Therefore, the proposed update of the SOP has been sent to the person responsible for the method for review and can be provided on request. As soon as new information will become available this version of the protocol will be updated.

Contact Details

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1. Introduction

The SOP is based on a method developed at Procter & Gamble in 1996. The SOP was drafted at ZEBET in Phase I of the prevalidation study and a database comprising 96 tests with 50 chemicals was produced using the first Draft SOP. The SOP was then refined according to discussions with P&G and with the partner laboratories participating in phases II and III (Huntingdon Life Sciences, UK and BASF AG, D) which lead to the attached final SOP.

2. Materials

2.1. MATERIALS, NOT PROVIDED WITH THE KITS

Sterile, blunt-edged forceps	For transferring tissues from agarose
500 ml wash bottle	For rinsing tissue after test material exposure
200 ml beaker	For collecting PBS washes
Sterile disposable pipettes, pipette tips and pipetters	For diluting, adding, and removing media and test materials. For topically applying test materials to tissues
37°C incubator 5% CO_2	For incubating tissues prior to and during assays
Vacuum source/trap (optional)	For aspirating solutions
Laminar flow hood (optional)	For transferring tissues under sterile conditions
37°C water bath	For warming Media and MTT solution
Mortar and Pestle	For grinding granulars
Adjustable Pipet 1 ml	For pipetting assay medium under inserts (0.9 ml)
Pipet 300 µl	For pipetting MTT medium into 24-well plates

Pipet 2 ml	For pipetting MTT extraction solution into 24-well plate
Pipet 200 µl	For pipetting extracted formazan from 24-well plate into 96 well plate to be used in a plate photometer
Pipet 50 µl	For application of liquid test materials
Positive displacement pipet 50 µl	For application of semi-solid test materials
Sharp spoon (NaCl weight: 25±1 mg) Aesculap, Purchase No.: FK623	For application of solids
(bulb headed) sound	To aid levelling the spoon (spoonful)
Laboratory balance	For pipette verification and checking spoonful weight
96-well plate photometer 570 or 540 nm	For reading OD
Shaker for microtiter/MILLICELL® plates	For extraction of formazan
Stop-watches	To be used during application of test materials
Potassium Hydroxyde, 8 N (Sigma # 17-8)	To be used as positive control with each kit
Dulbeccos PBS (ICN # 196 0054) or (ICN # 196 1054) or (ICN # 176 0020) or (ICN # 176 0022)	Use for rinsing tissues Use as ready solution or dilute from 10x concentrate or prepare from PBS powder
HCl	For pH adjustment of PBS
NaOH	For pH adjustment of PBS
H2O, pure (distilled or aqua pur)	To be used as negative control with each kit
Two additional 24-well plates	Use for preparing the "holding plates"

2.2. EPI-200 KIT COMPONENTS

Examine all kit components for integrity. If there is a concern call MatTek Corporation immediately (Mitch Klausner, telephone number +1-508-881-6771, Fax +1-508-879-1532).

1	Sealed 24-well plate	Contains 24 inserts with tissues on agarose
2	24-well plates	Use for MTT viability assay
4	6-well plates	Use for storing inserts, or for topically applying test agents
1 bottle	Maintenance Medium	Do not use in present assay
1 bottle	Serum-Free Assay Medium	DMEM-based medium
1 bottle	PBS Rinse Solution (100 ml)	Use for rinsing the inserts in MTT assay
1 vial	1% Triton X-100 Solution (10 ml)	Skin irritant reference chemical Do not use in the present assay

1	MTT Assay Protocol	MatTek Corporation: steps are included in the present protocol

2.3. MTT-100 ASSAY KIT COMPONENTS

1 vial, 2 ml	MTT concentrate	-
1 vial, 8 ml	MTT diluent (supplemented DMEM)	For diluting MTT concentrate prior to use in the MTT assay
1 bottle, 60 ml	Extractant Solution (Isopropanol)	For extraction of formazan crystals

3. Methods

3.1. EXPIRATION AND KIT STORAGE

Epi-200 kits are shipped from Boston on Monday. If possible, make sure that they are arriving in the laboratory on Tuesday. Upon receipt of the EPIDERM^{TM} tissues, place the sealed 24 well plates and the assay medium into the refrigerator (4°C). Place the MTT concentrate containing vial in the freezer (-20°C) and the MTT diluent in the refrigerator (4°C).

part #	description	conditions	shelf life
Epi-200	EPIDERM^{TM} cultures	refrigerator (4°C)	until Friday, of the week of delivery
Epi-100	assay medium	refrigerator (4°C)	7 days
MTT-099	MTT diluent	refrigerator (4°C)	7 days
MTT-100	MTT concentrate	freezer (-20°C)	2 months

3.2. QUALITY CONTROLS

3.2.1. Assay Acceptance Criterion 1: Negative Controls

The absolute OD $_{570}$ or OD $_{540}$ of the negative control tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping and storing procedure and under specific conditions of the assay.
Tissue viability is meeting the acceptance criterion if the mean OD of the two tissues is OD> or =0.8.

3.2.2. Assay Acceptance Criterion 2: Positive Control

Potassium Hydroxyde as 8.0 normal ready made solution (Sigma # 17-8) is used as positive reference and has to be tested with each kit according to section 3.4 **"Experimental Procedure"**. A 3 minutes application of 8.0 n KOH will reveal a mean relative tissue viability of ~20%.
An assay is meeting the acceptance criterion if mean relative tissue viability of the 3 min Positive Control is < or =30%.

3.2.3. Maximum inter tissue viability difference

In the present test protocol each chemical is tested on 2 tissues per application time (3 min and 1 hr). Thus, in contrast to the first test version (which used only 3 min application on 4 tissues) statistically outlying tissues cannot be identified any more. According to the historical data base existing at ZEBET the mean difference between untreated tissue duplicates is 9%±7% (S.D.).
A difference > 30% between two tissues treated identically should be regarded as a rejection criterion, and re-testing of the chemical is recommended if the resulting viability is near to a classification cut-off.

Note: If necessary, calculate % difference between the mean of the 2 tissues (= 100%) and one of the two tissues. If this difference is > 15% then rejection should be considered.

3.3. PREPARATIONS

3.3.1. MTT solution (prepare freshly on day of testing)

Thaw the MTT concentrate (MTT-100) and dilute with the MTT diluent (MTT-099). Store the remaining MTT solution in the dark at 4°C for later use on the same day (do not store until next day).

Note: Some test chemicals may reduce MTT, which will result in a blue colour without any involvement of cellular mitochondrial dehydrogenase.
Although in the present assay the test chemicals are rinsed off and the DMEM medium beneath the tissues is changed before contact with MTT medium, some amount of a test chemical may be released by the tissues into the MTT medium and directly reduce the MTT, which would be interpreted as "tissue viability".
To check MTT reducing capability a solution of MTT in DMEM (1.0 mg/ml) can be prepared and ~100 µl (liquid test material) or 30 mg (solid test material) added to 1 ml MTT medium. If the mixture turns blue/purple after about 1 hr at room temperature, the test material is presumed to have reduced the MTT. This check can only be used to explain unexpected results, but it can not be used for quantitative correction of results.

3.3.2. Dulbecco's PBS

Using ICN FLOW 10x DPBS (Cat. no. see section 2.1 **"Materials, not provided with the kits"**) dilute 1 in 10 with distilled water and adjust to pH 7.0 with either NaOH or HCl.

Record the pH adjustment in the MDS. If PBS powder is used: prepare PBS according to supplier instructions.

Note: 1 litre is sufficient for all rinsing performed with one kit. If PBS is prepared from 10x concentrates or powder and not sterilised after preparation do not use PBS for more than one week.

3.3.3. Test materials

Safety Instruction

1. For handling of non-coded test chemicals follow instructions given in the Material Safety Data Sheet.
2. If coded chemicals are supplied from BIBRA, no information regarding the safe handling will be provided. Therefore, all test materials must be treated as if they were corrosives and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).

Except solids all test materials are applied neat (undiluted):

Liquids	:	Dispense 50 µl directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Semi-solids	:	Dispense 50 µl using a positive displacement pipet directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Solids	:	Crush and grind test material in a mortar with pestle wherever this improves the consistency. Fill 25 mg application spoon (see section 2.1. **"Materials not provided with the kits"**) with fine ground test material. Level the "spoonful" by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material [#]. Add 25 µl H20 for wetting of the test material (increase volume of H20 in case of materials where this is not enough for wetting). If necessary spread to match size of tissue. Record in the MDS if grinding was not used and if spreading or increasing H20 volume was necessary.

Note: "Packing" can be avoided by using a rod shaped sound instead of a flat spatula. If a bulb headed sound is used the bulb can be used to empty the spoon completely.

3.4. EXPERIMENTAL PROCEDURE

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

Day prior to testing

1) Upon receipt of the EPIDERM™ kit(s), place the sealed 24 well plates containing the tissues and the assay medium into the refrigerator (4° C). Place the vial containing the MTT concentrate in the freezer (-20°C).

2) Preparation of PBS according to section 3.3.2 "Dulbecco's PBS".

Day of testing

Introductory note: One kit is used for testing 4 test chemicals, negative control and positive control, each of them applied both for 3 min and 1 hr to two tissue replicates. Thus, the experimental design can be either that the 3 min applications are completed first and subsequently the 1 hr experiment is performed, or, alternatively, that the 3 min applications are performed during the exposure period of the 1 hr experiment. The following steps are describing the latter option.

1) Before treatment pre-warm the assay medium in a 37°C waterbath.

2) Pipet 0.9 ml of the assay medium into each well of four sterile 6-well plates.

3) At least 1 hour before dosing, remove the EPIDERM™ tissues from the refrigerator. Under sterile conditions using sterile forceps, transfer the inserts into four 6-well plates containing the pre-warmed assay medium.

Note: Care should be taken to remove all adherent agarose sticking to the outside of the inserts. Any air bubbles trapped underneath the insert should be released. Label the 6 well plates (lid and bottom) indicating the test material.

4) Place the four 6-well plates containing the tissues into a humidified (37°C, 5% CO_2) incubator for at least 1 hour prior to dosing (pre-incubation).

5) Prepare MTT solution according to section 3.3.1 **"MTT solution"**.

6) Before pre-incubation is complete, prepare two 24-well plates to be used as "holding plates", one for the 3 min experiment, the other for the 1 hr experiment. In addition, prepare two 24-well plates for the MTT assay: Use the plate design shown below. Pipette 300 µl of either pre-warmed assay medium or MTT medium in each well. Place the 4 plates in the incubator.

24-well plate design (used as "holding plates" and for MTT assay)

NC	C1	C2	C3	C4	PC		NC	C1	C2	C3	C4	PC
NC	C1	C2	C3	C4	PC		NC	C1	C2	C3	C4	PC
3 min 1 hour												

NC= Negative Control

C1-C4= Test Chemical 1, 2, 3, 4

PC= Positive Control

7) After pre-incubation is completed (at least 1 hr) replace medium by 0.9 ml fresh assay medium in all four 6-well plates. Place two 6-well plates (3 min experiment) back into the incubator, the other two 6-well plates are used for the 1 hour experiment. Use the following plate design:

6-well plate design (chemical treatment and incubation)

negative control	test material 1	test material 2		negative control	test material 1	test material 2
negative control	test material 1	test material 2		negative control	test material 1	test material 2
plate A (3 min) plate C (1 hour)						
test material 3	test material 4	positive control		test material 3	test material 4	positive control
test material 3	test material 4	positive control		test material 3	test material 4	positive control
plate B (3 min) plate D (1 hour)						

Note: To avoid experimental errors it is recommended to use NC and PC at identical positions in all experiments. In contrast, test chemicals should be positioned differently in the two independent experiments.

8) **1 hour experiment:** Add 50 µl H_2O (negative control) into the first insert atop the EPIDERM™ tissue. Set the timer to 1 hr and start it, repeat the procedure with the second tissue. Proceed with test material 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed. Place both 6-well plates into the incubator (37°C, 5 % CO_2). Record start time in the MDS.

9) **3 minutes experiment**: Add 50 µl H_2O (negative control) into the first insert atop the EPIDERM™ tissue. Set the timer to 3 min and start it. Repeat the procedure with the second tissue. Important: keep a constant time interval between dosing (e.g. 40 sec.). After 3 min of application, with forceps, remove the first insert immediately from the 6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed.

10) **3 minutes:** once all tissues have been dosed and rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).

11) **1 hour:** after the 1 hour period of test material exposure (in the incubator) is completed with forceps remove the first insert from the

6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are rinsed.

12) **1 hour:** once all tissues have been rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).

13) **3 minutes:** After the 3 hour MTT incubation period is complete, aspirate MTT medium from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.

14) **3 minutes:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.

15) **3 minutes:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.

16) **1 hour:** After the 3 hour MTT incubation period is complete, aspirate MTT medium from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.

17) **1 hour:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.

18) **1 hour:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.

Second day of testing (only if formazan has been extracted over night!)

19) After the extraction period is complete for both, the **3 min** and the **1 hr** experiment, 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. Place the 24-well plates on a shaker for 15 minutes until solution is homogeneous in colour.

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

* **Note:** In contrast to normal photometers, in plate readers pipetting errors influence the OD. Therefore, 3 formazan aliquots shall be taken from each tissue extract. In the data sheet these 3 aliquots will be automatically reduced to one value by calculating the mean of the three aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.

Note: Readings are performed without reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters may have a ± tolerance in some cases the reference filter reduces the dynamics of the signal (OD) up to 40%.

Fixed 96 well-plate design (for OD reading in plate photometer, 3 aliquots per tissue)

NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	***3 min***
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	***1 hour***
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
tissue 1	**tissue 2**	**tissue 1**	**tissue 2**	**tissue 1**	**tissue 2**	**tissue 1**	**tissue 2**	**tissue 1**	**tissue 2**	**tissue 1**	**tissue 2**	

3.5. DOCUMENTATION

3.5.1. Method Documentation Sheet, MDS (see ANNEX)

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

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

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

3.5.2. Data Spreadsheet

The MS EXCEL spreadsheet "C-SPREAD.XLS" is provided by ZEBET. Data files of optical densities (ODs) generated by the microplate reader are copied from the reader software to the Windows Clipboard and then pasted into the first map of the EXCEL spreadsheet in the 96-well format given above (Note: Only 72 wells of the 96 wells are used!).

The spreadsheet consists of three maps, named Import, MDS Information and Spread. The first map (Import) is used for pasting the OD values (cursor position: A20!). Use the second map (MDS information) for the entry of the requested information (tissue lot-no., test material codes, date...), they will be copied from there to the other maps. The third map (Spread) does the calculations and provides a column graph of the results.

File names to be used in prevalidation phase III:

Since each single XLS file contains the data of 4 test chemicals, each of them coded by BIBRA with a four digit number there is no way to use "intelligent" file names which would allow to recognise the test

chemicals from the file names. Therefore, file names should first give the testing laboratory name (3 digits), then a dash (1 digit) and then the test number (2 digits):

BAS-01.XLS, BAS-02.XLS, ...BAS-12.XLS
HLS-01.XLS, HLS-02.XLS, ...HLS-12.XLS
ZEB-01.XLS, ZEB-02.XLS, ...ZEB-12.XLS

4. Evaluation, Prediction Models (PM 1 and PM 2)

Note: The mathematical rule for the prediction or classification of *in vivo* skin corrosivity potential from the *in vitro* data is called Prediction Model (PM). For the present test two prediction models are defined, one definitive model (PM 1), based on published data (Perkins et al., 1996) which have been confirmed by extensive testing at ZEBET during Phase I of the present prevalidation study.

Nevertheless, the data base obtained in Phase I indicated that sensitivity was a bit too low (71%) to be used as a full animal replacement test, whereas the specificity of the test was very high (89%).
Since a shift of the cut-off for classification would not have sufficiently increased the sensitivity, the test design was changed by including a second, longer application time of 1 hr for the test chemicals. This changed test design was experimentally tested at ZEBET when the prevalidation study had already proceeded to Phase II.
Therefore, ZEBET was able to test only those chemicals again, which were classified negative with the 3 min EPIDERM™ protocol. The data indicated that the sensitivity was increased (some false negatives were predicted now correct as corrosives) but the influence of this change on the total predictive capacity of the assay could not be sufficiently investigated.
Therefore, a second, tentative prediction model (PM 2) was defined, which has to be verified / falsified by the data obtained in Phase III of the present prevalidation study.

4.1. PREDICTION MODEL 1

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

Mean tissue viability (% negative control)	**prediction C / NC**
< 50	corrosive
>= 50	non-corrosive

4.2. PREDICTION MODEL 2

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 min treatment compared to the negative control tissues concurrently treated with H20. A chemical is classified "corrosive" in any case, if the relative tissue viability after 3 min treatment with a test material is decreased below 50 %.
In addition, those materials classified "non corrosive" after 3 min (viability >=50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15 %.

Mean tissue viability (% negative ctrl.)	**prediction C / NC**
3 min: < 50	corrosive
3 min:. >=50 and 1 hour: < 15	corrosive
3 min:. >= 50 and 1 hour: >= 15	non-corrosive

ANNEX: METHODS DOCUMENTATION SHEET (MDS)

ASSAY No:..... DATE:.....
XLS file name:.....

Kit receipt

EPIDERM™ kit received (day/date):	Day used:
EPIDERM™ Lot no.:	Production date:
Epi-100 Assay medium Lot no.:	Expiration date:
MTT concentrate Lot no.:	Date:
MTT diluent Lot no.:	Date:
MTT extractant Lot no.:	Date:
Booked in by (ID):	

PBS preparation

DPBS Lot no.:	Expiration date:	
Vol 10x DPBS:	Vol water:	Initial pH:
NaOH used to adjust pH:		Final pH:
HCl used to adjust pH:		Final pH:
Prepared by (ID):		

Incubator verification

Incubator #	CO2 (%)	Temperature (°C)	Check water in reservoir (✓)

ID / date:

Pipette verification (triplicate weightings)

Note: Perform pipette verification only once per week and refer to it in all assays of this week. But: If adjustable pipettes are used, check correct adjustment daily and mark with (✓).

Verification	0.9 ml	300 µl	200 µl	25 µl	50 µl
	H_2O weight (mg)				
1. 2. 3.					

ID / date:

Dosing procedure

Please mark (✓) the type of application. Also, mark (✓) wetting with H_20. If significantly more than 25 µl of H20 had to be used for wetting solids record ~ volume. REMARKS: record, if spreading was necessary or if crushing and grinding was not used (because it did not improve consistence of test material).

Test Material CODE	LIQUID SEMI-SOLID 50 µl (✓)		SOLID spoon (✓) +x µl H_2O	Material Characterisation §	Remarks
Neg. Control					
Pos. Control					

§ use your own wording, like: "highly viscous"

Record experimental design of the 6-well plates

plate A (3 min) plate C (1 hour)						
negative control				negative control		
negative control				negative control		
plate B (3 min) plate D (1 hour)						
		positive control				positive control
		positive control				positive control

(record code numbers of test materials) ID / date:

Time protocols:

Procedure	**Start**	**Stop**
1 hr pre-incubation of tissues		
1 hr chemical application (incubator)		

3 hrs MTT incubation (1 hr experiment)		
3 hrs MTT incubation (3 min experiment)		
Formazan extraction		

ID / Date:

Check plate photometer filter (✓)

reading filter: 570 nm	
reading filter: 540 nm	

ID / Date:

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