Standard Operating Procedure

(version 7.1)

In Vitro Skin Corrosion
including sub-categorisation of corrosive chemicals
with

NOTE: *This SOP is completely adopted for the use of epiCS, although originally written for Epiderm and Skinethic skin corrosion tests (6,7).*

epiCS was formerly known as ***EST****1000 (Epidermal Skin Test 1000)*

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1. RATIONALE AND BACKGROUND

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

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

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

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

In 2002 national co-ordinators of OECD Test Guideline Programme (WNT) endorsed New Draft Test Guidelines TG430 (TER) and TG431 (Human Skin Model; adopted on April 2004, updated July 2013) for *In Vitro* Skin Corrosion Testing. In Guideline TG 431 (paragraphs 9–11) general functional and performance criteria were defined if other (or new) skin or epidermis models are used in the context of this guideline (4). The current SOP is describing a generally applicable method for Skin Corrosion Testing, here applied to the CellSystems model epiCS [formerly known as **EST**1000 (Epidermal Skin Test 1000)].

* *The reconstituted human epidermis* epiCS *consists of an airlifted, living, multi-layered epidermal tissue construct, produced in polycarbonate inserts (0.6 cm^2) in serum-free and chemically defined medium, featuring normal ultra-structure and functionality equivalent to human epidermis in vivo.*

1.1 Changes to the SOP

Since the publication of the former version of this SOP (Version 6.0) new developments necessitate this new version (Version 7.1).

The most important changes are:

a) Introduction of this section
b) Introduction of MTT interference tests (taken from the OECD TG 431 (2016).
c) Assay acceptance criterion 3: Now described for using 2 replicate tissues (as described in OECD TG 431 (2016) or 3 replicate tissues (recommendation for statistical reasons).
d) Prediction model for sub-categorization of corrosive chemicals
e) Annex B is deleted as more and better information are available in the current OECD TG 431
f) Changes in the section how to take epiCS tissues into culture after reception (day prior to testing and day of testing).
g) Note in section 6.6 is removed.

2. SPECIFIC PURPOSE OF THE METHOD

epiCS was developed to reliably discriminate chemicals that are corrosive to skin from non-corrosive chemicals (5), and is therefore meant to be used for the classification of skin corrosion hazard according to the GHS System adopted 2001 by the OECD (1). Further testing qualified epiCS to distinguish between sub-categories of corrosivity. The test is not designed to predict skin irritation potential.

3. BASIC PROCEDURE

Upon reception of the tissues they should be conditioned by pre-incubation in epiCS Culture Medium for release of transport stress related compounds and debris. After overnight pre-incubation tissues are transferred to fresh epiCS Culture Medium and topically exposed with the test chemicals for 3 min and 1 h, respectively. At least two tissues each are used per treatment, negative control and positive control as described in the OECD TG 431 (for statistical reasons we recommend to use three tissue for each treatment.) After exposure tissues are rinsed and blotted and Culture Medium is replaced by MTT-assay medium. After 3 h incubation, tissues are rinsed with PBS, blotted, and the blue formazan salt is extracted with isopropanol. The optical density of the formazan extract is determined spectrophotometrically at 540 - 570 nm, and cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin corrosivity potential of the test materials is classified according to the remaining cell viability obtained after 3 min or 1 h exposure with the test chemical.

4. LIMITATIONS OF THE METHOD

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

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

5. MATERIALS

5.1 epiCS kit components

Examine all components for integrity. If there is a concern please contact CellSystems:

(Telephone +49-2241-25515-0, Fax: +49-2241-25515-30, E-Mail: info@cellsystems.de)

Sealed 24-well plate (contains inserts with tissues on agarose)
epiCS Culture Medium
epiCS MTT Assay Medium
Manual + Certificate of Analysis

5.2 Additionally needed materials and laboratory equipment

Table 1: List of material, not provided within the epiCS Kit

Material / Equipment	Application
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 rinses*

Material / Equipment	**Application**
Sterile disposable pipettes, pipette tips and pipettors	*For diluting, adding, and removing media and test materials. For topically applying test materials to tissues*
37 °C incubator 5 % CO_2, 95% RH	*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*
Mortar and pestle	*For grinding granules*
Adjustable pipette 1 ml	*For pipetting assay medium into 6-well-plate (1 ml)*
Pipette 300 µl	*For pipetting MTT Assay Medium into 24-well plates*
Pipette 2 ml	*For pipetting MTT extraction solution into 24-well plate*
Pipette 200 µl	*For pipetting extracted formazan from 24-well plate into 96 well plate to be used in a plate photometer*
Pipette 50 µl	*For application of liquid test materials*
Positive displacement pipette 50 µl	*For application of semi-solid test materials*
Sharp spoon (NaCl weight: 25 ± 1 mg) Aesculap # FK 623 MEDKA KG	*For application of solids*
(bulb headed) Pasteur pipette	*To aid levelling the spoon*
Laboratory balance	*For pipette verification and checking spoonful weight*
96-well plate photometer 540-570 nm	*For reading OD*
Shaker for microtiter / MTT assay plates	*For extraction of formazan*
Stop-watches	*To be used during application of test materials*
Potassium Hydroxide, 8 N (Sigma # P4494)	*To be used as positive control with each kit*
Dulbecco's PBS	*Use for rinsing tissues* *Use as ready solution* *- or dilute from 10x concentrate* *- or prepare from PBS powder*
MTT (Sigma # M5655)	*For preparing MTT-medium*
HCl	*For pH adjustment of PBS (if applicable)*
NaOH	*For pH adjustment of PBS (if applicable)*

Material / Equipment	**Application**
Mesh, Nylon 150 µm (CellSystems # CS-5010)	*Use as a spreading aid for liquid test materials, provided a pre-test shows the compatibility of test material and nylon mesh*
H_2O, pure (distilled or aqua-pur)	*To be used as negative control with each kit*
Isopropanol	*For MTT extraction*
Two additional 24-well plates	*Use for preparing the "holding plates"*

6. METHODS

6.1 Tissue and Medium Storage

For epiCS tissues and medium refer to this SOP. Prolonged storage (on agarose) is not recommended. Store epiCS Culture Medium in the dark at 4°C. The shelf life is limited (see expiry date on the label). Use cold epiCS Culture Medium, do not pre-warm. Record lot numbers of all components in the Methods Documentation Sheet (see 9. ANNEX A)

6.2 MTT - formazan conversion

In the OECD TG 431 (2015) HPLC / UPLC spectrophotometry is listed as an alternative method to determine MTT / formazan concentration. This SOP describes the spectrophotometric (OD) measurement. For further information on HPLC UPLC spectrophotometry, please consult the current OECD TG 431.

Interference with MTT

Test chemicals may interfere with the MTT assay, either by direct reduction of MTT into blue formazan, and/or by colour interference if the test chemical absorbs, naturally or due to treatment procedures in the same OD range of formazan (570 nm ± 30 nm, mainly blue and purple chemicals). Additional controls should be used to detect and correct for a potential interference from these test chemicals such as the non-specific MTT reduction (NSMTT) control and the non-specific colour (NSC) control. This is especially important if a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the epidermis and is therefore present in the tissues when MTT viability test is performed.

Identification of non-specific MTT reduction (Pretest)

All test materials should be further evaluated for their potential to interfere with MTT assay. To test if a material directly reduces MTT, add 50 µl (liquid) or 25 mg (solid - using sharp spoon) of the test substance to 1 ml of the Assay Medium containing MTT and incubate in the incubator (37±1°C, 5±1% CO_2, 95% RH) for 60 min. MTT Assay Medium containing MTT but no test substance is used as control. If the MTT solution turns blue/purple, the test substance reduces MTT and non-specific MTT (NSMTT) control has to be used.

NSMTT control

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

Following the OECD TG 431 (2015) each MTT reducing chemical is applied to two freeze-killed tissues per exposure time, which undergo the whole skin corrosion test.

Calculation of True viability (OECD TG 431, 2016):

The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the negative control run concurrently to the test being corrected (%NSMTT).

CellSystems' recommendation:

Use two freeze-killed tissues treated with NC in addition per exposure time. *(Note: The NC treated freeze-killed controls may show a small amount of MTT reduction due to residual reducing enzymes within the freeze-killed tissue).*

OD of freeze-killed tissues = OD resulting from MTT reducer treated freeze-killed tissue - OD resulting from - NC treated freeze-killed 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 freeze-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.

MTT interference of test chemicals that become coloured by contact with water or isopropanol

Pre-test

Add 50 µl (liquid) or 25 mg (solid - using a sharp spoon) of the test substance into 0.3 ml of deionised water and into 2 ml Isopropanol.

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 water in the incubator (37±1°C, 5±1% CO_2, 95% RH) for 60 min and with isopropanol for 2 h. 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 colour significantly, interference with the MTT test is presumed. In this case non-specific colour control with living tissues has to be carried out (NSC_{living}).

Interference of coloured test chemicals

In case the test chemical is coloured, non-specific colour control with living tissues has to be carried out (NSC_{living}).

NSC_{living} control

When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissue replicates per exposure time, which undergo the entire skin corrosion test but are incubated with MTT Assay Medium without MTT instead of MTT solution during the

MTT incubation step to generate a non-specific colour (NSC_{living}) control. The NSC_{living} control needs to be performed concurrently per exposure time per coloured test chemical (in each run) due to the inherent biological variability of living tissues.

> ***Note:*** *If the coloured test substance does not completely rinse off, pipette 1 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 inserts and add an additional 1 ml of isopropanol to bring the total volume to 2 ml.*

The true tissue viability is calculate as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with Assay Medium without MTT, run concurrently to the test being corrected (%NSC_{living}).

Test chemicals producing both direct MTT reduction and colour interference

In this case a third set of controls, apart from NSMTT and NSC_{living} controls is required.

In this control, the test chemical is applied on at least two killed tissues replicates per exposure time, which undergo the entire testing procedure but are incubated with MTT-Assay Medium instead of MTT solution during the MTT incubation step. The control should be performed concurrently to the NSMTT control and, where possible, with the same tissue batch.

Calculation of true tissue viability

The true tissue viability is calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus % NSMTT minus % NSC_{living} plus the percent non-specific colour obtained with killed tissues exposed to the interfering test chemical and incubated with medium without MTT, calculated relative to the negative control run concurrently to the test being corrected (%NSC_{killed}).

Data correction procedure

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

> ***OD = OD coloured tissue (MTT assay) – OD coloured tissue (no MTT assay)***

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

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

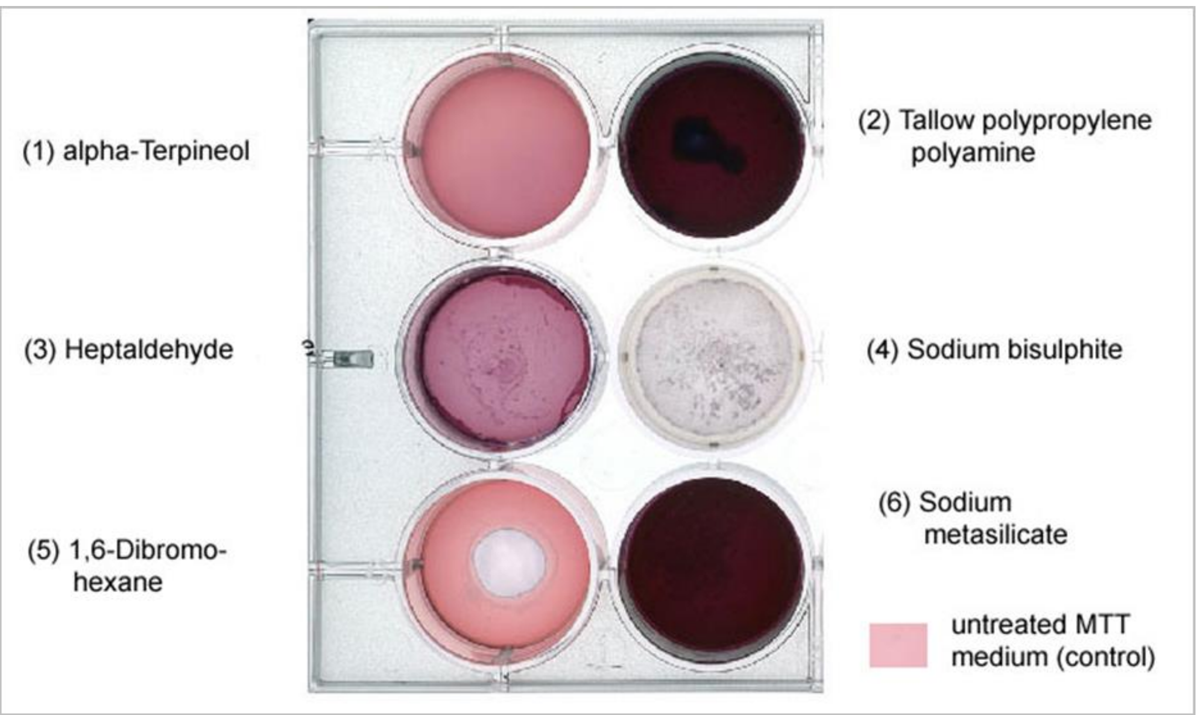


Figure 1: Example of test for direct MTT reduction ability. Test substances (2) (3) and (6) have directly reduced MTT. In these cases, NS MTT control is needed

6.3 Test for Mesh Compatibility (liquid test substances only)

Since the surface of epiCS is hydrophobic, even spreading of aqueous substances is sometimes not possible. Therefore, a nylon mesh (see 5.2) is used as a spreading support. This nylon mesh can be used for all liquids.

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 50 µl test substance. After 60 minutes exposure, check using a microscope: If an interaction between test substance and the mesh is noticed, the test substance has to be applied without using a mesh as a spreading aid.

6.4 Preparations

6.4.1 MTT Assay Medium

Prepare fresh on day of testing.

Per 24 tissues, dissolve 12.5 mg MTT (Sigma # M5655) in 2.5 ml PBS and thoroughly vortex this stock-solution. After filtration (using a sterile 0.45 µm filter) add 2 ml of the stock-solution to 8 ml epiCS MTT Assay Medium (final concentration: 1 mg MTT / ml medium). Keep the MTT Assay Medium in the dark.

6.4.2 Dulbecco's PBS

If PBS is prepared from powder or 10x concentrated PBS is used, prepare according to supplier instructions and adjust to pH 7.0 with either NaOH or HCl. Record the pH adjustment in the MDS.

> ***Note:*** *Alternatively, sterile ready-to-use PBS can be used. About 1 L is sufficient for all rinsing performed with 24 tissues. If PBS is prepared from 10x concentrates or powder it should be sterilised.*

6.4.3 Test substances

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

Liquids: Dispense 50 µl directly atop the tissue, and then gently place nylon mesh (8 mm diameter) on the surface. Record the use of mesh as spreading tool in the MDS.

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

Solids: Crush and grind test material in a mortar with pestle wherever this improves the consistency. Fill 25 mg using a sharp application spoon* (see 5.2) with fine ground test material. Level the spoon by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material. "Packing" can be avoided by using a rod shaped sound instead of a flat spatula. If a bulb headed sound is used the bulb can be used to empty the spoon completely. Add 25 µl H_2O necessary for wetting of the test material to increase tissue surface contact. Increase volume of H_2O in case it is not enough for wetting**. If necessary spread to match size of tissue. Record in the MDS if grinding was not used and the H_2O volume necessary to wet the chemical.

> ***Note:** Since the surface of the solid covering the tissues is more important than the weight, the “levelled spoon technique” is an accepted dosing procedure. The spoon used here has been calibrated to equal 25 mg of fine grinded NaCl. The weight will be different if other materials are used.*
>
> ***Note:** determine in a pre-test a volume of H_2O necessary to wet test chemical.*

For test substances with waxy consistence the spoon application does 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 15 µl H_2O. To improve the contact between test substance and tissue weigh down the “cookie” with a stainless steel aid like that shown in Figure 2.

Figure 2: Stainless steel aid, used for applications with waxy test substances

6.4.4 Exposure

Tissues are topically exposed to the test chemicals for 3 min and 1 h, respectively. Three tissues are used per treatment, negative control and positive control. To avoid possible toxic interference across wells, use one plate per chemical, in particular if volatile substances are tested.

6.5 Assay Quality Controls

6.5.1 Assay Acceptance Criterion 1: Negative Control (NC)

The absolute OD of the H_2O treated NC tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping and storing procedure and under specific conditions of the assay.

Tissue viability is meeting the acceptance criterion if the mean OD of the mean of NC is OD ≥ 0.8 < 2.8.

6.5.2 Assay Acceptance Criterion 2: Positive Control (PC)

8N KOH (Sigma # P4494) is used as PC and has to be tested once on each testing day. The mean viability of the tissue replicates exposed for 1 hour with the PC expressed as % of the negative control, should be < 20%.

6.5.3 Assay Acceptance Criterion 3: Coefficient of Variation (CV) or 30% difference

Except for the freeze-killed control tissues each treatment (NC, PC, and TC {= test chemical}) is performed on at least two tissue replicates per exposure time.

The OECD TG 431 prescribes the use of at least two tissues per exposure time:

a) For statistical reasons, 3 replicates are recommended. In the range between 20% and 100% viability and ODs ≥ 0.3, the coefficient of variation (CV) between tissue replicates should not exceed 30%.
b) In case two tissue replicates are used: the difference of viability between the two tissue replicates should not exceed 30% (see current OECD TG 431)

6.6 Experimental Procedure

Day prior to testing / Day of arrival of epiCS tissues

- Prepare 6-well plates for all epiCS tissues. Pipette 1 ml cold epiCS Culture Medium into each well.
- Remove the shipped multiwell plate containing the epiCS tissues from the inner shipping box and strip off the tape. Open the 24-well plate under a sterile airflow. Carefully take out each insert containing the epidermal tissue, rapidly remove any remaining agarose that might adheres to the outer side of the insert by gentle blotting onto a sterile tissue paper, and immediately place it in a well of the prepared 6-well plate. *Act quickly as the epidermal cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert.*
- Place the 6-well plates containing the tissues in a humidified (37±1°C, 5±1% CO_2, 95% RH) incubator over night.
- If necessary, prepare sufficient amount of rinsing PBS for the next day according to 6.4.2.
- Alternatively, in case the tissue has to be conditioned on the day of testing, follow instructions in the following chapter "Day of testing".

Day of testing

Introductory Note:

Example for 6 test chemicals:

36 tissues are used for testing 6 test chemicals, 6 tissues for negative control and 6 tissues for positive control Per testing day and experimenter, it is recommended to test not more than 6 chemicals plus the concurrent controls. Furthermore, it is recommended to complete first the 1 h exposure, and subsequently perform the 3 min exposure.*

**As stated in TG431 at least 2 tissues should be used per test substance and controls. For statistical reasons CellSystems recommends to use 3 tissues per chemical and control.*

Tissue conditioning (Pre-incubation)

(This applies only, if the tissues have to be conditioned on the day of testing and the steps described in section "Day prior to testing…" have not been done)

- Prepare 6-well plates for all epiCS tissues. Pipette 1 ml cold epiCS Culture Medium into each well. You may already prepare for the following testing. In this case:
- For each chemical and each exposure time use one 6-well plate and place three inserts (in case you work with triplicates) or two inserts (in case you work with duplicates) in the upper row of the prepared 6-well plate. Use this design also for the negative and positive control.
- Remove the shipped multiwell plate from the inner shipping box and strip off the tape. Open the 24-well plate under a sterile airflow. Carefully take out each insert containing the epidermal tissue, rapidly remove any remaining agarose that adheres to the outer side of the insert by gentle blotting onto a sterile filter paper, and immediately place it in a well of the prepared 6-well plate. *Act quickly as the epidermal cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert.*
- Place the 6-well plates containing the tissues in a humidified (37±1°C, 5±1% CO_2, 95% RH) incubator for 2 - 3 h.

Testing

After 2 - 3 h pre-incubation, transfer each insert to fresh Culture Medium (pre warmed to 37°C) in the lower row of the six-well plate as shown in Figure 3.

In case of overnight incubation prepare plates with fresh Culture Medium (pre warmed to 37°C) and place the inserts in 6-well plates. It is important to use one 6-well plate per exposure time and per chemical, to avoid cross contamination of chemicals.

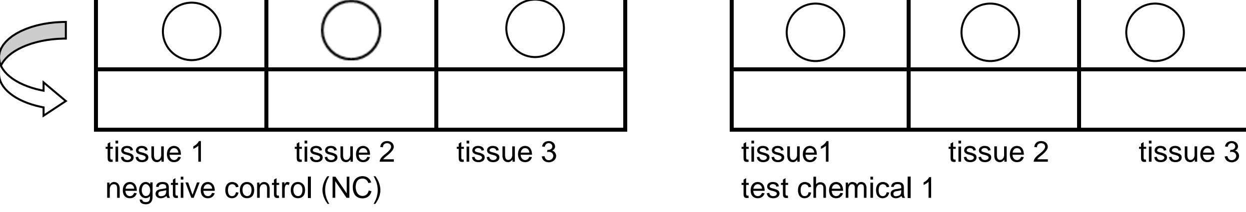


Figure 3: Scheme of the pre-incubation in 6-well plates.

Preparations for the main test

- Prepare MTT Assay Medium according to 6.4.1.
- Prepare two 24-well plates to be used as "holding plates" one for the 3 min experiment, the other for the
  1 h experiment. In addition, prepare two 24-well plates for the MTT assay: Use the plate design shown in Figure 4. Pipette 300 µl of either Culture Medium or MTT Assay Medium in each well. Place the 4 plates in the incubator.

NC	NC	NC	C1	C1	C1
C2	C2	C2	C3	C3	C3
C4	C4	C4	C5	C5	C5
C6	C6	C6	PC	PC	PC

holding plate

NC	NC	NC	C1	C1	C1
C2	C2	C2	C3	C3	C3
C4	C4	C4	C5	C5	C5
C6	C6	C6	PC	PC	PC

MTT assay plate

Figure 4: 24-well plate design (used as "holding plates" and for MTT assay, both for the 1 h experiment and the 3 min experiment). NC = negative control; C1 – C6 = test chemical 1 - 6; PC = positive control.

***Note**: The following time schedule for 1 h and 3 min application is meant for highly experienced technicians and serves as an example. It is recommended to take more time and/or longer intervals between applications if needed.*

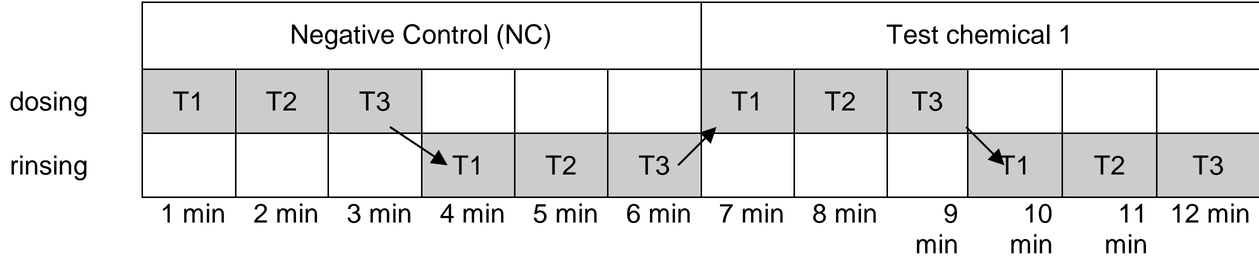
1-hour Application

***Note:** dosing time interval is set by rinsing procedure.*

- Set a timer to 1 h and start it. Add 50 µl H_2O (negative control) onto the first epiCS surface and if needed apply a mesh above. After 1 min repeat the procedure with the second tissue. After 2 min dose the third tissue. Proceed with test material 1 - 6 (50 µl: liquids, 25 mg + 25 µl H_2O: solids) and the positive control in the same manner until all tissues are dosed. Place the 6-well plates into the incubator (37±1°C, 5±1% CO_2, 95% RH) for the rest of the exposure time until 1 hour exposure is reached for first tissue dosed. *Record start time in the MDS.*
- After 1 hour test material exposure remove the first insert from the 6-well plate with forceps. Take off the mesh if used. Using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 6 and the positive control in the same manner until all 24 tissues are rinsed. Rinse all tissues in an interval of 1 min.
- Remove inserts from the holding plate and blot the bottom. Transfer inserts into the 24-well plate, which is prepared for the MTT assay. Place the plate in the incubator and record start time of MTT incubation in the MDS. Incubate for 3 hours (37±1°C, 5±1% CO_2, 95% RH).

3 min Application

Note: *dosing time interval (exactly 1 min) is set by the time needed for the rinsing procedure*



	Negative Control (NC)						Test chemical 1					
dosing	T1	T2	T3				T1	T2	T3			
rinsing				T1	T2	T3				T1	T2	T3
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min	11 min	12 min

Figure 5: Dosing interval scheme for the 3 min experiment. T1, T2, T3 = tissue 1, 2, 3

- Start the timer for 3 min. Add 50 µl H_2O (negative control) into the first insert atop the epiCS and apply a mesh if needed above. After 1 minute repeat the procedure with the second tissue. After 2 minutes repeat the procedure with the third tissue.
- After the 3 min period of exposure (at room temperature) for the first tissue is completed, start the timer for 3 min and remove the first insert from the 6-well plate with forceps. Take off the mesh if used. As shown in Figure 6, using a wash bottle gently rinse the tissue with PBS (= fill and empty insert 20 times in a constant soft stream of PBS) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom on blotting paper. After rinsing the tissue, please control if there are still some remains of the chemical on the surface. If you see remains, remove them very gently with a cotton tip (please, note in MDS). Place insert in the prepared holding plate. After 1 minute repeat the procedure with the second insert, after 2 minutes with the third tissue,etc.

Figure 6: Rinsing epiCS with PBS after treatment (left). Hold epiCS with forceps over a beaker and fill the insert in a constant soft stream of PBS (right). Invert insert to empty PBS into the beaker.

- Proceed with test materials 1 - 6 (50 µl: liquids, 25 mg + 25 µl H_2O: solid) and the positive control in the same manner until all 24 tissues are dosed and rinsed.

- 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 the plate in the incubator, record start time of MTT incubation in the MDS and incubate for 3 hours (37±1°C, 5±1% CO_2, 95% RH).

MTT Assay

- After the 3 hour MTT incubation period is complete, gently aspirate MTT from all wells (e.g. gently using a suction pump), refill wells with PBS and aspirate. Repeat the rinsing twice and make sure that tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.
- Immerse the inserts by gently pipetting 2 ml extracting solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.
- Seal the 24 well plate (e.g. a zip bag or Parafilm™) to inhibit isopropanol evaporation. *Record start time of extraction in the MDS*. Extract either over night without shaking at room temperature (we recommend at 4-8°C to reduce isopropanol evaporation) or, alternatively, 2 hours with shaking (~100 rpm) at room temperature.
- After the extraction period is complete, pierce the inserts with an injection needle and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Place the 24-well plates on a shaker for 2-3 minutes until solution is homogeneous in colour.
- Per each tissue transfer 2 x 200 µl aliquots* of the blue formazan solution into a 96-well flat bottom microtiter plate, both from the 3 min exposure and from the 1 h exposure. For the 96-well plate, use exactly the plate design given in Figure 7, as this configuration is used in the data spreadsheet. Read OD in a spectrophotometer at 540 - 570 nm, without reference filter**.

Note: *In contrast to normal photometers, in plate readers pipetting errors influence the OD. Therefore, 2 formazan aliquots shall be taken from each tissue extract.*

****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%.*

blank	blank	blank	blank	blank	blank	blank	blank					
NC	C1	C2	C3	C4	C5	C6	PC					Tissue 1
NC	C1	C2	C3	C4	C5	C6	PC					2 aliquots
NC	C1	C2	C3	C4	C5	C6	PC					Tissue 2
NC	C1	C2	C3	C4	C5	C6	PC					2 aliquots
NC	C1	C2	C3	C4	C5	C6	PC					Tissue 3
NC	C1	C2	C3	C4	C5	C6	PC					2 aliquots

Figure 7: Fixed 96 well-plate design for OD readings (use one 1 plate for the 3 min exposure, and 1 plate for the 1 h exposure)

6.7 Documentation

6.7.1 Method Documentation Sheet (MDS)

The MDS (see 9. ANNEX A) allows checking the correct set up, calibration and function of the equipment as well as correct weights, applications etc. The MDS is designed as a paper document "in the spirit of GLP". Per each test, make a hardcopy of the MDS, fill in and sign the requested information, starting the day prior to testing and ending after the test has been conducted.

> ***Note (1)***: *If several tests are performed per week, pipette verification is done at the beginning of each week. Nevertheless, if adjustable pipettes are used, the correct adjustment shall be checked and recorded in the MDS before each test.*
>
> ***Note (2):*** *If solids cannot be sufficiently ground to a fine powder, it is recommended to check the weight of the levelled application spoon and record this weight in the MDS.*

6.7.2 MS EXCEL Data Spreadsheets

The MS EXCEL spreadsheets which were made originally by the BfR Biostatistics Research Group are provided by CellSystems. Data of optical densities (ODs) generated by the microplate reader are copied from the Reader software or manually filled into the Windows Clipboard and then pasted into the first map (Import) of the EXCEL spreadsheet in the 96-well format given above in Figure 7.

The spreadsheet consists of three sheets, named Import, Results and Remarks.

The first sheet (*Import*) is used for pasting or manually transposing the OD values. Please fill in all cells highlighted in yellow. All data will be automatically transferred into the part *Results* (2nd sheet).

The second sheet (*Results*) makes blank correction, calculations and provides a column graph of the results automatically.

Any observations and remarks can be written on the third sheet named *Remarks*.

Immediately after performing all tests the completed spreadsheets are submitted for bio statistical analysis.

7. Evaluation, Prediction Model

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 the negative control (NC) H_2O. A chemical is classified "corrosive" if the relative tissue viability after 3 min exposure to a test material is decreased to below 50 % of the NC or after 60 min exposure below 15%. If tissue viability after 3 min exposure is ≥ 50 % and after 60 min exposure ≥ 15 %, the substance is a non-corrosive.

Test items classified as corrosives can be subcategorized into subcategory 1A or into a combination of subcategories 1B and 1C. The 3 min value of the corrosive test item is essential for the subcategorization.

If the viability after 3 min exposure is < 15%, the test item is classified as 1A, if ≥ 15% it is subcategorized as a combination of 1B and 1C. A differentiation between subcategories 1B and 1C by using any reconstructed human epidermis is not possible.

The prediction model works in two steps:

Viability measured after exposure time points (t=3 and 60 minutes)	Prediction to be considered
STEP 1	
< 50% after 3 min exposure	**Corrosive**
≥ 50% after 3 min exposure AND < 15% after 60 min exposure	**Corrosive**
≥ 50% after 3 min exposure AND ≥ 15% after 60 min exposure	**Non-corrosive**
STEP 2	
< 15% after 3 min exposure	**Optional Sub-category 1A**
≥ 15% after 3 min exposure	**A combination of optional Sub-categories 1B and 1C**

(for more details consult OECD TG 431 (2016))

8. REFERENCES

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5. Hoffmann J., Heisler E., Karpinski S,, Losse J., Thomas D., Siefken W., Ahr HJ., Vohr HW., Fuchs HW. (2005). Epidermal-skin-test 1000 (EST-1000) a new reconstructed epidermis for in vitro skin corrosivity testing.Toxicol In Vitro. **2005** Oct;19(7):925-9
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7. Liebsch M., Traue D., Kandarová H. **(2004)**: Standard Operating Procedure (SOP): In Vitro Skin Corrosion: Human Skin Model Test; Specific Model: SkinEthic™, 21p.
8. OECD (2014) Test Guideline 431: In Vitro Skin Corrosion: Human Reconstructed Epidermis (Rhe) Test Method, 2014, Paris
9. OECD (2015) Test Guideline 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method, 2015, Paris
10. OECD (2016) Test Guideline 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method; 2016, Paris

9. ANNEX A: Methods Documentation Sheets (MDS)

This annex is meant to give advice and may act as a checklist. The documentation may be carried out differently. Please refer to the current OECD TG 431 and other relevant documents that describe this matter.

ASSAY No:............................. DATE:...

Corresponding XLS data file name..

Kit receipt

epiCS kit received (day/date):	Day used:
epiCS Lot no.:	Expiration date:
epiCS Culture Medium Lot. no.:	Expiration date:
MTT-Assay Medium Lot. no.:	Expiration date:

ID/ Date:

Incubator verification

Incubator #	CO_2 (%)	Temperature (°C)	Check water in reservoir (✓)

ID/ Date:

Pipette verification (triplicate weightings)

Pipette 3 x H_2O into a small beaker 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.

	2 ml	1 ml	300 µl	200 µl	50 µl	positive displacement pipette
	.....................................H_2O weight in g...					
1.						
2.						
3.						

ID/ Date:

MTT Concentration : 1 mg/ml
ID/ Date:

epiCS Kit remarks:

Microscopic evaluation:

Configuration

PLATE A

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 B

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

ID/ Date:

Dosing procedure

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

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

TEST MATERIAL CODE	Liquid/ Solid (50 µl)/ (25 mg)	Application			Material characterisation *	Remarks
		Pipette (✓)	Mesh (✓)	Spoon (✓)		

* use your own wording, like: "highly viscous" or “waxy”, “sticky”

ID/ Date:

Remarks before MTT incubation

Plate configuration – after MTT incubation

PLATE A

1 hour

PLATE B

3 minutes

Remarks after MTT incubation

PLATE A

1 hour

PLATE B

3 minutes

Time protocols:

Procedure	Pre-incubation of tissues (conditioning)		1 hour substance exposure		3 min test substance exposure +3 min rinsing (3 tissues/chem.)		3 h MTT Assay Medium incubation		formazan extraction	
	Start	Stop	Start	Stop	Start	Stop	Start	Stop	Start	Stop

ID/ Date:

Check plate photometer filter

Tick correct (✓) filter setting

reading filter: nm	
no reference filter	

ID/ Date: