Standard Operating Procedure (Version 7.3)

published on EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM) as Protocol n° 213: epiCS® Skin Corrosion Test (epiCS® SCT)

epiCS® Skin Corrosion Test (epiCS® SCT) including sub-categorisation of corrosive chemicals

***NOTE**: This SOP is completely adopted for the use of epiCS.*

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

SkinInVitro GmbH was formerly known as "CellSystems Biotechnologie Vertrieb GmbH"

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A Protocol Introduction

epiCS® Skin Corrosion Test

1. Abstract

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 (OECD (2001)).

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*.

**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 7.2) new developments made this new version (Version 7.3) necessary.

The most important changes are:

a) CellSystems Biotechnologie Vertrieb GmbH changed name in „SkinInvitro GmbH“as of April 1, 2019
b) page 28 Assay Acceptance Criterion 2: Positive Control (PC) „Mean viability should be < 15 % “
c) page 17 footnote after Prediction Model „for more details consult OECD TG431 (2019) “
d) page 18 References: new 11. OECD (2019) Test Guideline 431

2. Objectives & Applications

2.1 Type of Testing:

Replacement

2.2 Level of Toxicity Assessment:

Hazard identification, toxic potential, toxic potency

2.3 Purpose of Testing:
Classification and labelling; prediction of skin corrosions potential.

2.4 Context of Use:
epiCS was developed to reliably discriminate chemicals that are corrosive to skin from non-corrosive chemicals (Hoffmann. J., et al. 2005) and is therefore meant to be used for the classification of skin corrosion hazard according to the GHS System adopted 2001 by the OECD (OECD (2001)). Further testing qualified epiCS to distinguish between sub-categories of corrosivity. The test method is not designed to predict skin irritation potential, see Skin Irritation Test (SIT).

2.5 Applicability Domain:
The test described in this protocol is designed for the classification of chemicals and was established for liquids, viscous and solid test substances.

3. Résumé
Skin corrosion refers to the production of irreversible damage to the skin; namely visible necrosis through the epidermis. The potential to induce skin corrosion is an important consideration in establishing procedures for safe handling, packing and transports of chemicals. In the year 1998 the first in vitro corrosivity test (EPISKIN) were successfully validated and met the acceptance criteria previously defined by the Management Team of the ECVAM International Validation Study (Fentem, et al. 2008).
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 (OECD (2003)). As a result, the performance of the epiCS skin model (formerly known as EST-1000) was evaluated under OECD Test Guideline No 431. In June 2008 the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) accepted EST-1000 (now known as epiCS- models) for in vitro corrosivity test. The current protocol is describing a generally applicable method for Skin Corrosion Testing, here applied to the SkinInVitro epiCS-model test method (epiCS SCT) [formerly known as EST1000 (Epidermal Skin Test 1000)].

3.1 Endpoint & Endpoint Detection:
Cell viability is used as endpoint and measured by dehydrogenase conversion of MTT [*(3-4,5-dimethyl thiazole 2-yl)-2,5-diphenyltetrazoliumbromide*], present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues (Mossmann 1983). The reduction of viability of tissues exposed to chemicals in comparison to negative controls (treated with DPBS) is used to predict skin irritation potential.

3.2 Endpoint Value:
The cell viability is expressed as percentage of the negative control.

3.3 Test System:

epiCS [SkinInVitro GmbH, Troisdorf, Germany (formerly known as CellSystems GmbH)] consists of normal, human-derived epidermal keratinocytes, which have been cultured to form a multi-layered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. A generic description of general and functional conditions that skin models need to comply with can be found in the OECD Test Guideline 431 (OECD 2019).

The epiCS tissues (surface 0.6 cm^2) are cultured on specially prepared cell culture inserts and shipped world-wide as kits, containing tissues on agarose together with epiCS Culture Medium, epiCS MTT-assay medium and 6-well plates.

3.4 Basic Procedure:

Upon receive 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 the 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 (OECD, 2019) (for statistical reasons we recommend using 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 transfer in 2ml isopropanol. 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. Data Analysis/Prediction Model

The test protocol allows predicting the skin corrosivity potential of test substances according to the United Nations Globally Harmonized System (UN-GHS) for classification and labelling.

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

Figure 1: Prediction model

Test items are classified as non-corrosives and corrosives. Corrosives can be subcategorized into subcategory 1A or into a combination of subcategories 1B and 1C. For further information see Prediction Model.

5. Test Compounds & Results Summary

Evaluation of the performance of the epiCS skin model in the context of the OECD Test Guideline No. 431. This study showed that 11 out of 12 Reference Chemicals of the OECD TG 431 were correctly predicted by EST-1000 (now known as epiCS) in all four participating laboratories when considering the final prediction of each laboratory derived from the mode of the individual laboratory predictions (the mode of 3 test predictions per Reference Chemical per laboratory). Following its 30th meeting, held on March 9 and 10 March 2009, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) endorsed on 12 June 12, 2009 by consensus and written procedure the statement, confirming that the EST-1000 (now known as epiCS®) Skin Corrosion Test method for skin corrosion testing can be used for reliably predicting the corrosive potential of chemical substances.Modification of the Method
None

6. Discussion

The epiCS Skin Corrosion Test (SCT) is an easy to perform *in vitro* test which only needs none or a minimum of training. The protocol is self-explaining. Besides a standard cell culture and chemicals laboratory no special equipment is required.
In contrast to the use of laboratory animals or excised human skin the method offers a high reproducibility due to standardised materials and processes during the production. Furthermore, the epiCS is based on human cells to predict effects on humans and serves as a complete replacement of the *in vivo* acute skin corrosions test.

7. Status

7.1 Known Laboratory Use:

epiCS is used in industry and academia for research, efficacy testing and regulatory toxicology testing (fully validated and accepted for skin corrosivity testing), listed in the OECD TG 431.

7.2 Participation in Validation Studies:

The protocol was developed and refined by CellSystems Biotechnology Vertrieb GmbH (now known as SkinInVitro GmbH) in compliance with the ECVAM performance standards requirements and was part of a validation study.

7.3 Regulatory Acceptance:

After the successful validation, the epiCS (formerly known as EST-1000) model achieved regulatory acceptance as OECD Test Guideline 431 and the EU test method B.40 BIS: In vitro skin corrosion: human skin model test of Council Regulation (EC) No 440/2008 of 30 May 2008

8. Proprietary Issues

The intellectual property rights for the test system are held by SkinInVitro GmbH (formerly known as CellSytems Biotechnologie Vertrieb GmbH). No IPRs are associated with the present method.

9. Abbreviations and Definitions

MDS: method documentation sheet
MTT: 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide
NC: negative control
OD: optical density
PC: positive control
PS: performance standards
ref.: reference
RH: relative humidity
RT: room temperature
SCT: Skin Corrosion Test
SD: standard deviation
SDS: sodium dodecyl sulphate
UN-GHS: United Nations Globally Harmonized System

Last update: Oct. 2019

B Procedure Details, Latest Version: October 2019

epiCS® Skin Corrosion Test

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1. Health and Safety Issues

1.1 General Precautions:

All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination.

The epidermal cells are taken from healthy volunteers negative for HIV, and Hepatitis B+C virus. Nevertheless, handling procedures for biological materials should be followed:

Wear gloves during handling with the skin and kit components

After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using 10 % bleach, special containers or autoclaving).

Examine all kit components for integrity and deviation from standard appearance.

Please contact SkinInVitro GmbH if you have any questions or concerns.

1.2 MSDS Information:

- Non-coded test chemicals should be handled following chemical safety datasheet.
- 8N KOH (H290, H302, H314)

  Isopropanol (R11, R36, R67 –H225, H319, H336)

  MTT (R68, R36/38 –H315, H319, H335, H341)

Work in ventilated cabinets: to prevent accidental contact wear protective gloves, and if necessary, safety glasses.

- Unknown and coded test chemicals with no or incomplete safety handling information should be considered as irritant and toxic and must be handled with maximum care. In accordance with chemical safety guidelines: use safety ventilated cabinet, wear gloves, eye and face protection

2. Materials and Preparations

2.1 Test systems:

2.2 Standard epiCS Kit Components:

Sealed 24-well plate (epiCS)	*Contains up to 24 inserts with epidermis tissues (origin human keratinocytes) on agarose*
6-well plates (sterile)	*For assay / culture*
epiCS Culture Medium	*For tissue culture*
epiCS MTT Assay Medium	*For diluting MTT reagent prior to use in the MTT assay*

2.3 Expiration and Kit Storage:

reference	description	conditions	shelf life
CS-1001	*epiCS (reconstructed epidermis)*	*refrigerator (2-8°C)*	*72 h*
CS-3050 *CS-3051* *CS-3052* *CS-3053*	*epiCS Culture Medium:* *50 ml* *75 ml* *100 ml* *125 ml*	*refrigerator (2-8°C)*	*(see label)*
CS-3030 *CS-3031*	*epiCS MTT Assay Medium:* *25 ml* *50 ml*	*refrigerator (2-8°C)*	*(see label)*

Note: *Examine all kit components for integrity. If there is a concern call immediately. Phone: + 49 (0)2241-25515-0*

2.4 Fixed Equipment:

Laminar flow hood	*For safe work under sterile conditions*
Humidified incubator (37±1°C, 5±1 % CO_2, 90±10 % relative humidity (RH))	*For incubating tissues prior to and during assays*
Vacuum source/trap (optional)	*For aspirating media and solutions*
Laboratory balance	*For pipette verification and checking spoonful weight*
96-well plate spectrophotometer	*For reading OD*
Plate shaker	*For extraction of formazan*
Timers	*To be used during application of test materials*
Sterile, forceps or tweezers	*For handling tissue inserts*
500 ml plastic wash bottle	*For rinsing tissue with DPBS*

200 ml beaker	*For collecting DPBS rinses*
150 ml beaker	*For swirling of cell culture inserts*
37±1°C water bath	*For warming Media and MTT solution*
Mortar and pestle	*For grinding granular solids*
Adjustable pipette	*For pipetting 2 ml MTT isopropanol*
Adjustable micropipette	*For application of 30 µl liquid test materials and 50 µl of DPBS when wetting the tissue surface before application of solid substances*
Positive displacement pipette 30 µl	*For application of semi-solid test materials*
Sharp spoon	*For application of solids -(Aesculap, ref. FK 623)*

2.5 Consumables:

96-well plate	
24-well plate	*For the MTT assay*
Sterile paper towel	*For blotting of cell culture inserts*
Meshes (Nylon) (CellSystems GmbH ref. CS-5010)	*Use as a spreading aid for liquid test materials*
Extra 6-well plates – sterile	*To transfer tissue inserts to fresh media (instead of replacing the media using the same plate)*
Adhesive tape or Parafilm M	*Covering plates during formazan extraction (NeoLab ref. 7-2220 or ref. 2-5082)*
Injection needle	

2.6 Media, Reagents, Sera, Others:

Dulbecco´s PBS (DPBS) Ca/Mg-free (sterile)	*Use for rinsing tissues*
5 % (w/v) SDS in sterile deionised water; [151-21-3] (Sigma ref. L-4509, purity min. 98.5±1 %)	*To be used as positive control*
MTT - Thiazolyl Blue Tetrazolium Bromide (Sigma, ref. M-5655, cell culture tested, purity min. 97,5 %	*For MTT assay*
Isopropanol	*For extraction of the formazan crystals*

3. Preparations

3.1 Media and Endpoint Assay Solutions:

MTT solution (prepare freshly on day of testing)

Per 24 tissues, dissolve 12.5 mg MTT (Sigma ref. M5655) in 12.5 ml epiCS MTT-assay medium and thoroughly vortex this stock-solution. Keep the MTT medium in the dark and warm to 37°C prior to usage (Use within 2 h).
Safety precaution: MTT is toxic (Risk phrases: R26, R68, R22, R36, R37, R38).
Wear protective gloves during manipulation with MTT solution!

Dulbecco´s PBS (DPBS) for rinsing

Sterile ready-to-use DPBS should be used. About two litres are sufficient for all rinsing performed with one kit. If DPBS is prepared from 10x concentrates or powder, pH needs to be adjusted to 7.0±0.1 and solution must be sterilized. Record the preparation in the MDS.

3.2 Test Compounds:

Follow safety instructions for respective compounds.
The mode of application onto the tissues differs depending on substance consistency (liquid / viscous / solid); see chapter “test substances”.

3.3 Positive Control Solution:

8N potassium hydroxide

3.4 Negative Control Solution:

Distilled H_20

3.5 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.

4. Interference Tests

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 MTT-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 must be used.

NSMTT control

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

Following the OECD TG 431 (2019) each MTT reducing chemical is applied to two freeze-killed tissues (EpiCS Frozen (CellSystems # CS-1001F)) per exposure time, which undergo the whole skin corrosion test.

Calculation of True viability (OECD TG 431, 2019):

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).

SkinInVitro' 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 considered, 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 must be carried out (NSC_{living}).

Interference of coloured test chemicals

In case the test chemical is coloured, non-specific colored control with living tissues must 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.

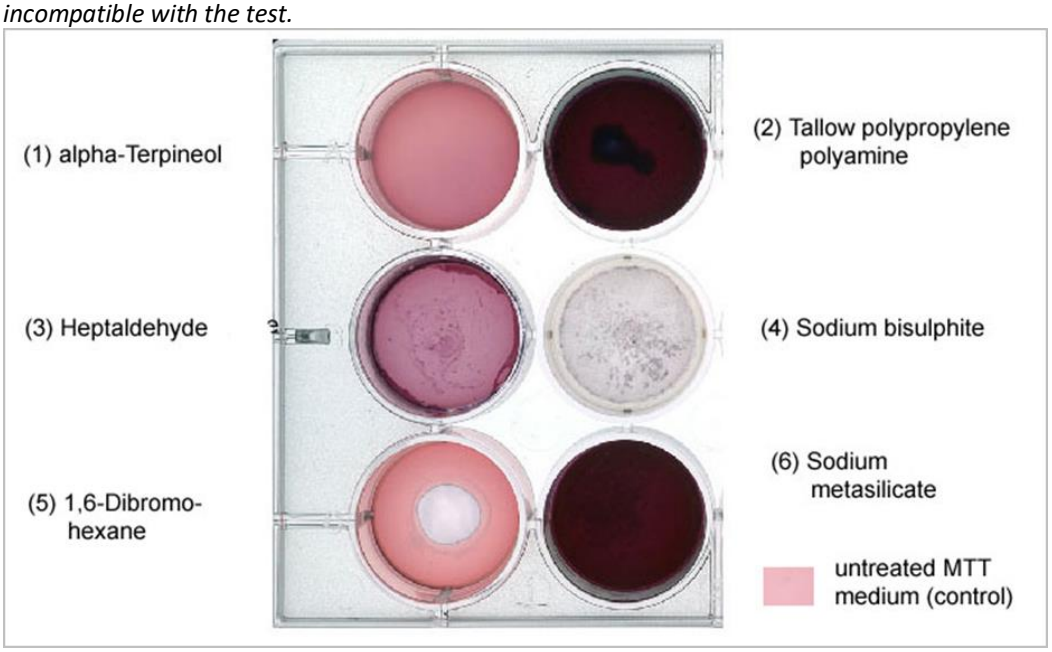

Figure 2: Example of test for direct MTT reduction ability. Test substances (2) (3) and (6) have directly reduced MTT. In these cases, MSMTT control is needed

5. 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 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 must 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 must be applied without using a mesh as a spreading aid.

6. Test Substances

6.1 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).

6.2 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.

6.3 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 1.1) 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 H2O necessary for wetting of the test material to increase tissue surface contact. Increase volume of H2O 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 H2O 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.*

6.4 Semi-solids:

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.

6.5 Waxes:

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

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

C Method

Experimental System Procurement

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1. Routine Procedures:

Day prior to testing (day 0)

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

Contact person:

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SkinInVitro GmbH

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email: bernadette.thiede@skininvitro.com

2. Document all information about supplied material in the MDS.
3. Place the DPBS into the refrigerator (2-8°C) and the vial containing the MTT concentrate in the freezer (-20±5°C).
4. Prepare 6-well plates for all epiCS tissues. Pipette 1 ml cold epiCS Culture Medium into each well.
5. 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.*
6. Place the 6-well plates containing the tissues in a humidified (37±1°C, 5±1% CO_2, 95% RH) incubator overnight.

7. If necessary, prepare enough rinsing PBS for the next day according (Media and Endpoint Assay Solutions:).
8. Alternatively, in case the tissue must be conditioned on the day of testing, follow instructions in the following chapter "Day of testing".

2. Exposure:

Tissues are topically exposed to the test chemicals for 3 min and 1 h, respectively. To avoid possible toxic interference across wells, use one plate per chemical, if volatile substances are tested.

2.1 Test Material Exposure Procedures:

Chemical exposure (day 1)

Introductory Note:
As stated in TG431 at least 2 tissues should be used per test substance and controls. For statistical reasons SkinInVitro recommends using 3 tissues per chemical and control.

Example for 6 test chemicals:
3 tissues per chemical and control*: 48 tissues are used for testing 6 test chemicals. 6 tissues for one test chemical, 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.*

Tissue conditioning (Pre-incubation)

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

1. 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.
2. 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.

   Note: *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.*
3. 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.

2.2 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 4.

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.

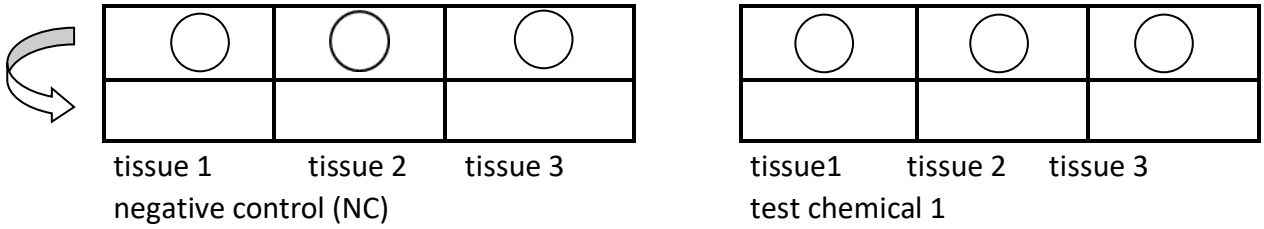

Figure 4: Scheme of the pre-incubation in 6-well plates.

Preparations for the main test

Prepare MTT-assay medium according to (Media and Endpoint Assay Solutions).

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 5. Pipette 300 µl of either Culture Medium for the “holding plates” or 300 µl MTT-assay medium for MTT-assay, 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 5: 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-h Application

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

1. 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 h exposure is reached for first tissue dosed. *Record start time in the MDS*.

2. After 1 h test material exposure removes 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.
3. 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 h (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*

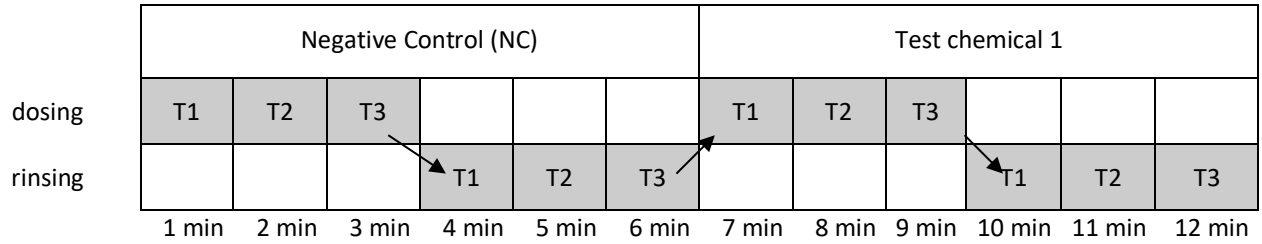

Figure 6: Dosing interval scheme for the 3 min experiment. T1, T2, T3 = tissue 1, 2, 3

1. 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.
2. 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 7, 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 7: 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.

3. 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.
4. 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 h (37±1°C, 5±1% CO_2, 95% RH).

2.3 Endpoint Measurement:

MTT Assay

1. After the 3 h 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.
2. 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.
3. 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 h with shaking (~100 rpm) at room temperature.
4. 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.
5. 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 8, 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 8: 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)

3. Acceptance Criteria

The OECD TG 431 prescribes the use of at least two tissues per exposure time: For statistical reasons, 3 replicates are recommended.

3.1 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.

3.2 Assay Acceptance Criterion 2: Positive Control (PC)

8N KOH (Sigma # P4494) is used as PC and must be tested once on each testing day. The mean viability of the tissue replicates exposed for 1 h with the PC expressed as % of the negative control, should be < 15%.

3.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

Each treatment (NC, PC, and TC {= test chemical}) is performed on at least three tissue replicates per exposure time.

Since the test skin corrosivity potential is predicted from the mean viability determined from three single tissues, the variability of tissue replicates should be acceptably low.
The assay is meeting the acceptance criterion if the SD calculated from individual tissue viabilities of the 3 identically treated replicates is <18%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) (OECD,2019). If the difference of the viability is > 30%, a re-testing of the chemical is recommended.

3.4 Retesting:

A single test run composed of three replicate tissues should be enough for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second run should be considered, as well as a third run in case of discordant results between the first two runs.

4. Data Analysis

The MS EXCEL spreadsheets which were made originally by the BfR Biostatistics Research Group are provided by SkinInVitro. 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 8.
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

After data entry, the following calculations will be performed on the spreadsheet:

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

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

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

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

5. 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**

Figure 9: Prediction model

D Annex

1. Annex A

Skin Corrosions Test

FLOWCHART

Transfer tissues from agarose to epiCS Culture Medium

↓

Incubate (37±1°C, 5±1 % CO_2, 90±10 % RH) at least 4 h or overnight

↓

Dose 3 tissues each with 30 µl / 30 mg TS, or PC, or NC

↓

Expose 60 min at room temperature

↓

Stop exposure after 60 min by rinsing thoroughly with DPBS

↓

Transfer tissues to Holding-Plate

↓

Expose 3 min at room temperature

↓

Stop exposure after 3 min by rinsing thoroughly with DPBS

↓

Transfer tissues to Holding-Plate

↓

Blot tissues and perform MTT assay

↓

Read OD in a plate spectrophotometer at 550-570 nm

2. Annex B

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

ID/ Date:

Remarks after MTT incubation

PLATE A

1 hour

PLATE B

3 minutes

ID/ Date:

Time protocols:

Procedure	Pre-incubation of tissues (conditioning)		1 h substance exposure		3 min test substance exposure		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:

E Bibliography

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