INVITTOX Protocol

epiCS® SKIN IRRITATION TEST

Standard Operating Procedure

(SOP **epiCS** SIT)

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A. Protocol Introduction

This INVITTOX Protocol is based on a SOP kindly provided by Dr. Manfred Liebsch (BfR, Germany). It has been revised and adapted for the use with epiCS by CellSystems GmbH.

epiCS SKIN IRRITATION TEST

epiCS SIT

The epiCS skin irritation test is used for the classification of chemicals concerning their skin irritating properties. Cytotoxic effects on the commercially available reconstructed epidermis epiCS are determined by a standard MTT assay. The method is available as a kit, comprising reconstructed epidermis, media and culture plates.

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

OBJECTIVES & APPLICATIONS

Type of Testing:
Replacement

Level of Toxicity Assessment:
Hazard identification, toxic potential, toxic potency

Purpose of Testing:
Classification and labelling; prediction of skin irritation potential

Context of Use:
In vitro skin irritation tests are regulatory accepted.
Performance standards for applying human skin models to *in vitro* skin irritation testing were defined based on the validated test EpiSkin™ test method (ECVAM SIVS, 2007). After the update of the ECVAM performance standards in 2009 (ECVAM 2008, 2009) the “OECD GUIDELINE FOR THE TESTING OF CHEMICALS In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method” 439 was adopted in 2010 (OECD 2010).

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.

BASIS OF THE METHOD

Dermal irritation is generally defined as “the production of reversible inflammatory changes in the skin” (OECD 2002). This is induced by chemicals which penetrate the *stratum corneum* and lead to damages and cell loss of the underlying cell layers. The epiCS SIT measures the cell damage as reduction of cell viability using a human reconstructed epidermis.

The test consists of a topical exposure of the neat test chemical to a human reconstituted epidermis model followed by a cell viability test. Cell viability is 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 (Mosmann 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. Recent comparative studies in human skin models employing various endpoints to predict skin irritancy of topical formulations have shown that the MTT endpoint had clear advantages, even over mechanistically based endpoints like the release of IL-1α (Faller et al. 2002, Faller & Bracher 2002).

EXPERIMENTAL DESCRIPTION

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.

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

Test System:
epiCS (CellSystems GmbH, Troisdorf, Germany) consists of normal, human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered 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 439 (OECD 2010).
The epiCS tissues (surface 0.6 cm²) are cultured on specially prepared cell culture inserts and shipped world-wide as kits, containing tissues on shipping agarose together with epiCS Culture Medium, epiCS MTT Assay Medium and 6-well plates.

Basic Procedure:
On day of receipt, epiCS are conditioned by incubation for release of transport-stress related compounds and debris at least 4 hrs or overnight. After pre-incubation, tissues are topically exposed to the test chemicals for 20 minutes. Three tissues are used per test chemical, positive control (PC) and negative control (NC). Tissues are then thoroughly rinsed and blotted to remove the test substances, and transferred to fresh medium. After a 24 hrs incubation period medium is changed and the tissues are incubated for another 18 hrs. Afterwards, MTT assay is performed by transferring the tissues to 24 well plates containing 300 µl MTT medium (1 mg/ml). After a 3 hrs MTT incubation the blue formazan salt formed by cellular mitochondria is extracted with 2 ml isopropanol per tissue and the optical density of the extracted formazan is determined in a spectrophotometer at 570 nm. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin irritation potential of the test materials is predicted if the mean relative cell viability of the tissue is below or equal 50 %.

DATA ANALYSIS/PREDICTION MODEL

The test protocol allows predicting the skin irritation potential of test substances according to the United Nations Globally Harmonized System (UN-GHS) for classification and labelling. A reduction of tissue viability of equal or below 50 % of the negative control classifies the substances as “category 2”. Tissue viability of above 50 % results in classification as “no category”.

***In vitro* result**	***In vivo* prediction**
mean tissue viability ≤ 50 %	category 2
mean tissue viability > 50 %	no category

TEST COMPOUNDS & RESULTS SUMMARY

During the multicenter blind trial validation study the 20 reference chemicals (10 *in vivo* irritants and 10 *in vivo* non irritant chemicals) mentioned in the OECD TG 439 In “*Vitro Skin* Irritation: Reconstructed Human Epidermis Test Method” were used. Three different laboratories performed the classification process of all chemicals under GLP like conditions followed by an independent statistical analysis.

MODIFICATIONS OF THE METHOD

None

DISCUSSION

The epiCS SIT is an easy to perform *in vitro* test which only needs none or a minimum of training. The INVITTOX 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 irritation test in rabbits. The material cost for classification of 10 test chemicals + 2 controls is about 1800 €. About 3-4 hrs are needed for preparations (~ 2 hrs), testing (~ 1 h) and data analysis (~ 1 h). In a typical run up to ten chemicals can be tested with 2 technicians. High-throughput testing may be possible if the test will be performed with fully or semi automated processes.

STATUS

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.

Participation in Validation Studies:
The protocol was developed and refined by CellSystems GmbH in compliance with the ECVAM performance standards requirements and was part of a blind trial multicenter validation study.

Regulatory Acceptance:
Data of the multicentre validation study were submitted to EURL-ECVAM. After peer reviewing of the study by the ECVAM's Scientific Advisory Committee EURL-ECVAM published the "ESAC opinion" in 2016. For regulatory acceptance of the epiCS SIT method ESAC recommends training and proficiency testing of users. For training CellSystems proposes the epiCS® Validation Study Irritation (Ref. CS-1018 VI) and individual trainings. (Please contact CellSystems about this.)
Proficiency testing for new users should be done according to the OECD TG 439 before carrying out the epiCS SIT method.

PROPRIETARY ISSUES

The intellectual property rights for the test system are held by CellSystems GmbH.
No IPRs are associated with the present method.

ABBREVIATIONS & 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
SD:	standard deviation
SDS:	sodium dodecyl sulphate
UN-GHS:	United Nations Globally Harmonized System

B. Technical Description

Version: V. 5.1, January 2017

epiCS SKIN IRRITATION TEST

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HEALTH & SAFETY ISSUES

All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial (see below), 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:

a) Wear gloves during handling with the skin and kit components
b) 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).
c) Examine all kit components for integrity and deviation from standard appearance.

Please contact CellSystems if you have any questions or concerns.

MATERIALS AND PREPARATIONS

TEST SYSTEM

Standard epiCS Kit Components

Sealed 24-well plate (epiCS)	*Contains up to 24 inserts with epidermis tissues (origin human keratin 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*

Expiration and Kit Storage

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

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

EQUIPMENT

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 (NaCl weight: 30 mg)	*For application of solids -(Aesculap, ref. FK 623)*

Consumables:

96-well plate	
24-well plate	For the MTT assay
Sterile paper towel	For blotting of cell culture inserts
Meshes (Nylon) (CellSystems, 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 (NeoLab ref. 7-2220 or ref. 2-5082) or Parafilm M	Covering plates during formazan extraction
Injection needle	

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

PREPARATIONS

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 hrs).
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.

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”.

Positive Control Solution:

5 % (w/v) (aq) SDS

Weigh out 0.50 g SDS (analytical grade, see Equipment) into an appropriate calibrated 10 ml flask and complete with distilled water to 10 ml (final volume). For better solubilisation of SDS place solution in a water bath (37±1°C) for 10 minutes. Solution can be stored up to one month (2-8°C). Record the preparation in the MDS.

Negative Control Solution:

Dulbecco´s PBS (DPBS)

Sterile ready-to-use DPBS should be used. If DPBS is prepared from 10x concentrates or powder, pH needs to be adjusted to 7.0±0.1 and solution must be sterilized.

INTERFERENCE TESTS

The current OECD TG 439 makes provisions for interference tests. (See chapter 27ff (OECD TG 439, 2015)).

Test for interference of chemicals with MTT endpoint and correction procedures

A test substance may interfere with the MTT endpoint if: a) it is coloured and/or b) able to directly reduce MTT (for possible combination of interactions, see Annex D). The MTT assay is affected only if the test material is present in or on the tissues when the MTT viability test is performed.

Some non-coloured test materials may change into coloured materials in wet or aqueous conditions and thus stain tissues during the 20 min exposure. Therefore, before exposure, a functional check for this possibility should be performed (Step 1).

Step 1:

Add 30 µl (liquid) or 30 mg (solid) of the test substance into 0.3 ml of deionised water. 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 the incubator (37±1 °C, 5±1 % CO_2, 90±10 % RH) for 60 min. 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, the test substance is presumed to have the potential to stain the tissue. A functional check on viable tissues should be performed (Step 2).

Step 2:

To check the tissue-binding of a coloured test article (or a chemical that changes into a coloured substance) the following test should be performed ideally with 3 tissue replicates (at least 2) concurrently tested with each and every test performed with the coloured chemical (using the same tissue batch): Expose three tissues replicates to 30 µl of liquid test substance or 30 mg of solid test substance. In parallel, expose a tissue to DPBS (negative control). Follow all procedures as described in this protocol in the Method Section except incubate the tissue for 3h in epiCS MTT Assay Medium without MTT (37±1°C, 5±1 % CO_2, 90±10 % RH) instead of incubating in epiCS MTT Assay Medium containing MTT. After the 3 hrs incubation, rinse the tissues and extract the tissues using 2.0 ml of isopropanol and measure the optical density (OD) at 570 nm.

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 (treated with DPBS), the chemical should be concurrently tested with each and every test performed with the coloured material using the procedure described above. The 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 DPBS treated control tissue and the tissue viability (determined in MTT assay) is not close to the classification cut-off (50 %), 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 DPBS treated control tissue, additional steps and expert judgment must be performed to determine if the test substance must be considered as incompatible with the test.

Step 3:

All test materials (including those already evaluated in Step 1 and Step 2) should be further evaluated for their potential to interfere with MTT assay. To test if a material directly reduces MTT, add 30 µl (liquid) or 30 mg (solid) of the test substance to 1 ml of the epiCS MTT Assay Medium containing MTT and incubate in the incubator (37±1°C, 5±1 % CO_2, 90±10 % RH) for 60±2 min. As negative control use 1 ml epiCS MTT Assay Medium containing MTT. If the MTT solution turns blue/purple, the test substance reduces MTT and additional functional check (Step 4) must be performed.

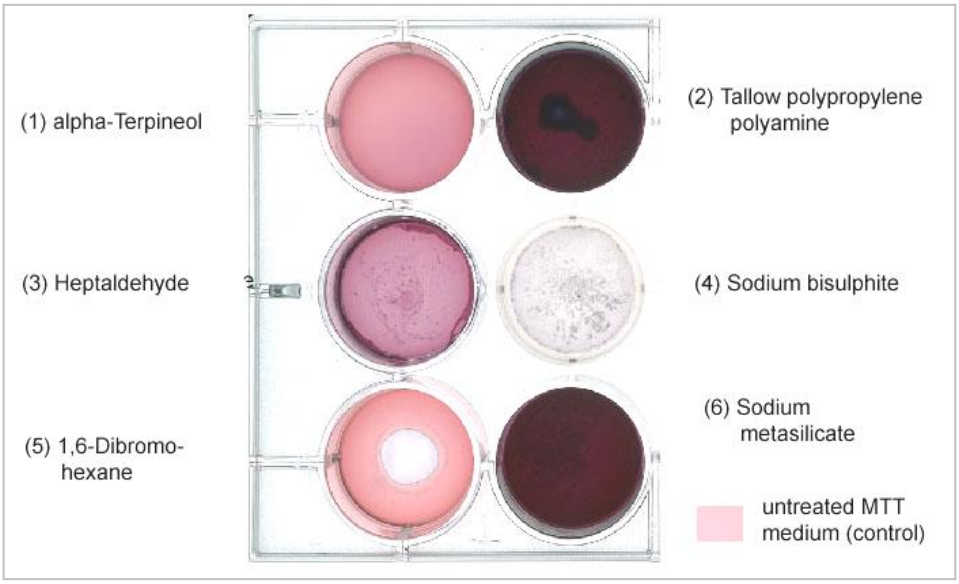

Figure 1: Example of test for direct MTT reduction ability (Step 3). Test substances (2) (3) and (6) have directly reduced MTT. In these cases, Step 4 must be performed.

Step 4:

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

Prepare freeze-killed tissues:

a) transfer epiCS in 24-well plates
b) freeze tissue at -20°C (or -80°C) for at least 48 hrs (3 tissues / MTT-interacting test substance)
c) thaw tissue 1 h (±10 min) before use
d) keep at RT

Correction of non specific MTT reduction should be performed with 3 tissue replicates in a single run, independently of the number of independent tests performed with the direct MTT-reducing chemical:
Each MTT reducing chemical is applied to three freeze-killed tissue replicates. In addition, three freeze killed tissues are left untreated (Note: The untreated killed controls will show a small amount of MTT reduction due to residual reducing enzymes within the killed tissue). The entire assay protocol is performed with the frozen tissues in parallel to the viable epiCS tissues. Data are then corrected as follows:

Data correction procedure

True viability = Viability of treated tissue – MTT conversion by chemical

= OD tvt – OD kt

OD kt = (mean OD tkt – mean OD ukt)

tvt = treated viable tissue kt = killed tissues

tkt = treated killed tissue ukt = untreated killed tissue (NC treated 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

If the interference by the test substance is ≤ 30 % of the negative control value, the net OD of the test substance treated 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.

Note 1:	*If the coloured test material or the MTT reducing chemical is classified as irritant by the Skin Irritation Test (tissue viability ≤50 %), the correction procedures are not necessary.*

Test for Mesh Compatibility

Capillary effects (surface tension effects) were observed if low volumes of lipophilic liquid test chemicals were topically applied on epiCS tissues. Therefore, a nylon mesh is used as a spreading support for all substances (both liquids and solids)

Some chemicals may react with the mesh and therefore the compatibility of each 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 30 µl test substance or 50 µl of DPBS and 30 mg of a solid test substance. After 60 minutes exposure, check using a light microscope, if an interaction between test substance and the mesh is noticed (Figure 2, right side). In that case the test substance has to be applied without using a mesh as a spreading aid.

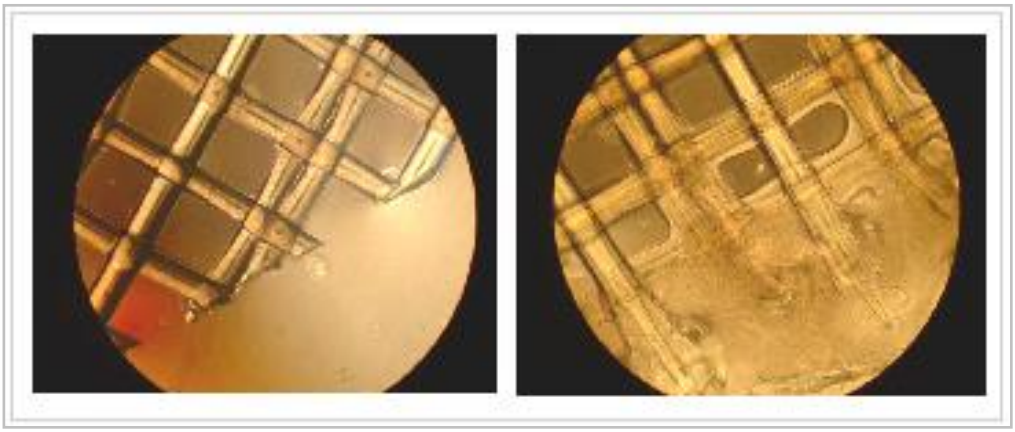

Figure 2: The mesh compatibility test

TEST SUBSTANCES

Safety Instruction

1. For handling of known test substances follow instructions given in the Material Safety Data Sheet.
2. If coded materials or unknown samples 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 irritating and toxic and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).
3. Store all test substances according to recommendations. Respect special storage conditions (special temperature, protection from light, protection from oxidization by nitrogen, etc.)

Liquids: Dispense 30 µl directly atop the tissue. Avoid contact with the tissue surface. Afterwards place carefully the nylon mesh on the tissues surface. If necessary, gently positions the mesh using a pipette tip or tweezers/forceps.

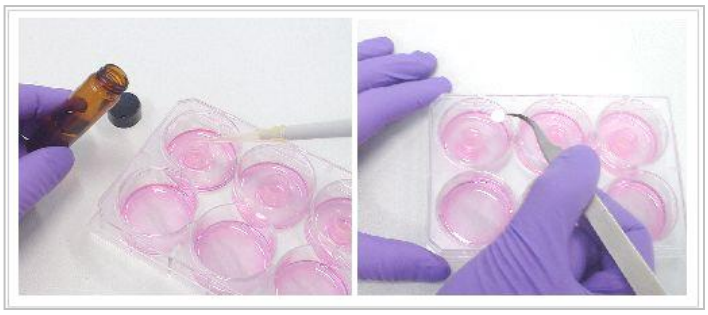

Figure 3: Application of liquids

Semisolids: Dispense 30 µl using a positive displacement pipette directly atop the tissue. If necessary spread the chemical with a pipette tip. Afterwards place carefully the nylon mesh on the tissues surface. If necessary, gently positions the mesh using a pipette tip or tweezers/forceps.

Figure 4: Application of semisolids (positive displacement pipette - detail)

Solids: If necessary, crush and grind test material in a mortar with pestle. Shortly before application of the solid substance, moisten the tissue surface with 50 µl of sterile DPBS to improve contact of the tissue surface with the test chemical. Fill 30 mg sharp application spoon (see fig. 5) with fine ground test material. A pipette tip can be used to empty the spoon completely. It is essential that the whole surface of the epiCS tissue is covered with the test substance. If this is not the case apply additional test substance to secure the coverage of the surface.

Do not press onto the tissue surface. Document all observations concerning the solubility of the material in the MDS. Record in the MDS if grinding was not necessary. Afterwards place carefully the nylon mesh on the tissues surface. If necessary, gently positions the mesh using a pipette tip or tweezers/forceps

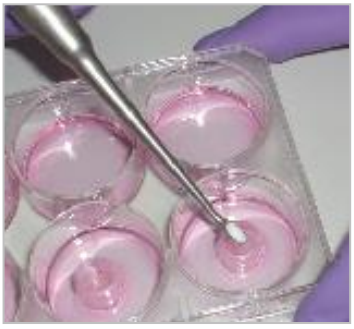

Figure 5: Application of solids

Waxes: For test substances with waxy consistency the spoon application will not work. In these cases try to form a flat “cookie like” piece of about 8 mm diameter and place it atop the tissue, which is pre-wetted with 50 µl sterile DPBS. To improve the contact between test substance and tissue weigh down the “cookie” with a stainless steel aid like that shown in Figure 6.

Figure 6: The stainless steel aid used application of waxy materials

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.*

METHOD

TEST SYSTEM PROCUREMENT

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ROUTINE CULTURE PROCEDURE

Day prior to testing (day 0)

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

   Contact person:

   Dr. Oliver Engelking
   CellSystems Biotechnologie Vertrieb GmbH
   phone: +49 (0) 2241-25515-0
   email: oliver.engelking@cellsystems.de

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).

Tissue conditioning:

1. *Do not pre-warm epiCS Culture Medium!*
2. Pipette exactly 1.0 ml of the cool epiCS Culture Medium into each well of sterile 6-well plates. (For 24 inserts prepare eight 6-well plates. Use one 6-well plate for pre-incubation of three inserts).
3. Remove the shipped multi well plate from the package. Under sterile conditions carefully (using sterile tweezers) take out each insert containing the epiCS tissue. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile blotting paper, and place the tissues in one well of the prepared 6-well plate. Avoid air bubble formation underneath the tissue culture insert.
4. Perform visual inspection of the inserts within next 5 min. Record any tissue defects and excess of moisture on the surface. Do not use defect tissues or tissues with excessive moisture on the surface.

Note: For detailed evaluation a dissecting stereoscope (magnification 5 X) can be used.

5. Incubate the plates at least for 4 hrs or overnight 37±1°C, 5±1 % CO_2, 90±10 % RH).
6. Store remaining epiCS MTT Assay Medium refrigerated (2-8°C).

Note 1: *Air bubbles trapped underneath the insert should be released.*

Note 2: *The visual quality check of the tissues has to be done quickly*

Do not use tissues which are completely covered with liquid!!!

7. If necessary, prepare sufficient amount of rinsing DPBS for the next day (approximately 1L per 24 inserts).
8. Prepare and sterilize all devices which will be used in the assay:
   - Micropipette
   - washing bottles (UV/ gamma irradiation or 70 % ethanol)
   - blotting paper (autoclave)
   - sharp spoon for application of solids (70 % ethanol)

TEST MATERIAL EXPOSURE PROCEDURES

Chemical exposure (day 1)

1. Place all devices, solution and chemicals needed for the test in the sterile hood.

   Checklist:
   - sterile meshes
   - Micropipette
   - sterile washing bottles pre-filled with sterile DPBS
   - sterile blotting paper
   - sterile tips and pipettors
   - vials with chemicals set to room temperature, including negative control (NC) sterile DPBS and as positive control (PC) 5 % SDS
   - timer
   - sharp, pointed tweezers
   - sharp spoon
   - beaker for waste material

Note: *Wipe all non-sterile material bottles with 70 % ethanol.*

2. Prepare sufficient amount of 6-well plates pre-filled with 1 ml of pre-warmed (37±1°C) epiCS Culture Medium in the upper row

Note: *Use one plate per one chemical during exposure and later on during the post-incubation phase to avoid cross contamination !*

3. Remove 6-well plates from the incubator approximately 5 min before exposure to chemicals.
4. Check the surface of tissues and exclude completely wet tissues or tissues with any visible defects.
5. Before test substance exposure, label all 6 well plate lids with the test material codes or names.

Test Substance Exposure

1. Apply 30 µl or ~30 mg of the undiluted chemical, NC or PC to three single tissues (triplicates) each according to the PREPARATIONS section. Apply chemicals at the time intervals needed later for rinsing off the chemical (recommended intervals are 1,5 to 2 min). Use 50 µl DPBS to moisten tissues before applying a solid chemical. Apply each epiCS tissue with a sterile mesh.

2. Keep plates with applied chemical in the laminar hood at RT (20±2°C) expose test chemical to the tissue for 20±1 min.

> ***Note**: Good rinsing tissues is a very important step that removes residual chemicals from the tissue surface. Chemical residues may have large influence on the outcome of the test method. So, rinsing and washing have to be carried out thoroughly and as described in this protocol in the following paragraphs.*

3. Rinse tissues with sterile DPBS using a squeeze wash bottle. Fill and empty the tissue insert 20 times to remove any residual test material (Figure 7). Use constant stream of DPBS applied from ~1.5 cm distance from the tissue surface. The stream of DPBS should not be too soft; otherwise, the test chemical may not be removed. Optimal wash bottle, with pointed endings as shown in Figure 7.

   Ensure that the mesh is removed after washing three to five times. If the mesh still sticks to the tissue remove it carefully by using tweezers or forceps without damaging the epiCS tissue.

4. Completely submerge and swirl the insert 3 times in 150 ml DPBS (separate beaker per test or control article) to remove remaining test chemicals.

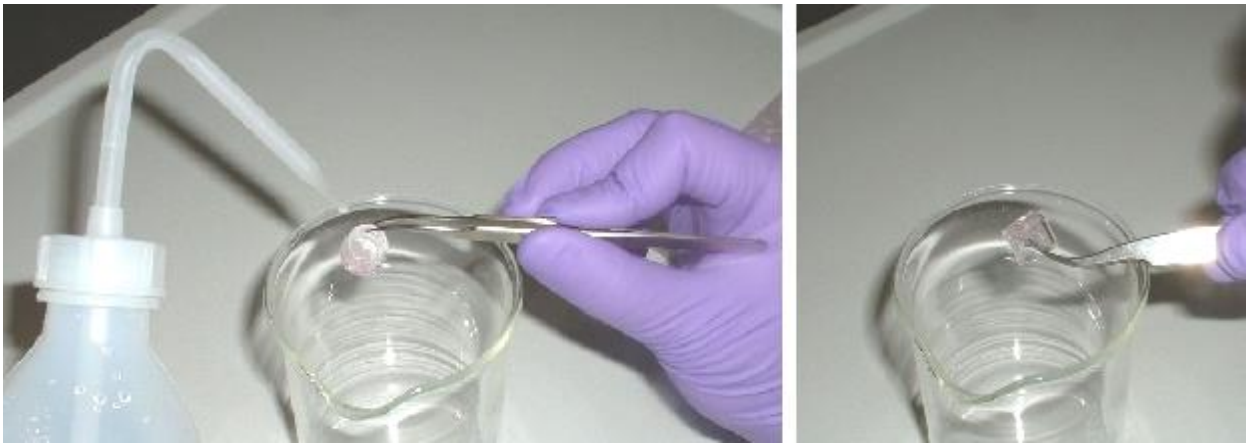

Figure 7: Washing procedure

5. Finally, rinse the tissue once again by repeating step 3 (with 20 more rinses) and after this once from outside with sterile DPBS. Remove excess DPBS by gently shaking the insert, blot insert on sterile blotting paper (Figure 8).

6. Transfer blotted tissue inserts to new 6 well plates pre-filled with fresh pre-warmed (RT-37°C) epiCS Culture Medium. Use one 6-well plate per test substance to avoid potential cross contamination within one plate.

7. Incubate tissues in the incubator for another 24±2 hrs (37±1°C, 5±1 % CO_2, 90±10 % RH). Record starting time of incubation in the MDS.

Figure 8: Blotting and drying of the surface

Medium change (day 2)

Exchange the medium for each tissue 24 hrs after the application of the test samples and place the 6 well plates back into the incubator for additional 18±2 h post-incubation (37±1°C, 5±1 % CO_2, 90±10 % RH).

ENDPOINT MEASUREMENT

MTT viability test (day 3)

MTT assay

1. Prior to the MTT assay, label sufficient amount of 24-well plates.
2. Prepare MTT medium as described above and pipette 300 µl of MTT medium to each well.
3. Remove inserts from the 6-well plates, blot bottoms, and transfer them into the 24-well plates, prefilled with 0,3 ml of MTT (1 mg/ml). Place the plates in the incubator (37±1°C, 5±1 % CO_2, 90±10 % RH), record start time of MTT incubation in the MDS and incubate for 3 hrs ±5 min.

***Note**: Keep 3 hrs ±5 min MTT incubation time strictly. Deviation from the 3 hrs time for MTT incubation will result in different MTT values.*

4. After MTT incubation is completed, gently aspirate MTT medium from all wells. Immerse the inserts by gently pipetting 2 ml extracting solution (isopropanol) into each insert. The level will rise above the upper edges of the insert, thus completely covering the tissues from both sides.
5. Seal the 24 well plates (e.g. with parafilm or gas non-permeable adhesive tape) to inhibit isopropanol evaporation. Record start time of extraction in the MDS and extract formazan for

at least 2 hrs (maximum 2,5 hrs) at room temperature with gently shaking on a plate shaker (~ 100 rpm).

6. After the extraction period is complete, pierce the inserts with an injection needle (~gauge 20, ~ 0,9 mm diameter, Fig. 9) and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Before transferring the extract to 96 well plates pipette up and down 3 x until solution is homogenous.

Figure 9: Piercing the inserts

7. Per each tissue transfer 2 × 200 µl aliquots of the blue formazan extracting solution into a 96-well flat bottom microtiter plate according to fixed plate design given in spreadsheet (example is given in Figure 10). Use isopropanol as blanks. Read OD in a 96-well plate spectrophotometer using a wavelength between 550 and 570 nmpreferably at 570 nm, without using a reference filter.

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
	BL	BL	BL	BL	BL	BL	E	E	E	E	E	E	A
Tissue 1	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	B
	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C
Tissue 2	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	D
	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	E
Tissue 3	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	F
	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	G
	E	E	E	E	E	E	E	E	E	E	E	E	H

NC - Negative control; PC – Positive control; C1, C2 - Chemical No. 1, No. 2

BL – Blank; E - Empty

Figure 10: Fixed 96 well-plate design (for OD reading in plate spectrophotometer, 2 aliquots per tissue)

In contrast to normal spectrophotometers, in plate readers pipetting errors may influence the OD. Therefore, 2 formazan extract aliquots will be taken from each tissue extract. In the Excel data sheet, these 2 aliquots will be automatically reduced to one value by calculating the mean of the two aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.

The plate map is according to the plate design used in the spreadsheet, which is used in the validation study for data collection and preliminary calculations. It is necessary to strictly keep the plate design given here. Otherwise, the calculation of results will be incorrect.

***Note**: Readings are performed <u>without</u> 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 30 %.*

ACCEPTANCE CRITERIA

Assay Acceptance Criterion 1: Negative Control

The absolute OD of the negative control (NC) tissues (treated with sterile DPBS) in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

The assay is meeting the acceptance criterion if the mean OD_{570} of the NC tissues is ≥ 1.0 and does not exceed 2.8

Assay Acceptance Criterion 2: Positive Control

A 5 % SDS (in H_2O) solution (see Preparations**)** is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95±1 % confidence interval of the historical data.

The assay is meeting the acceptance criterion if the mean viability of PC expressed as % of the negative control is ≤ 20 %.

Assay Acceptance Criterion 3: Standard Deviation (SD)

Since the test skin irritancy 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 %

Retesting

A single test run composed of three replicate tissues should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements and/or mean percent viability equal to 50±5 %, a second run should be considered, as well as a third run in case of discordant results between the first two runs.

DATA ANALYSIS

A blank, MS EXCEL spreadsheet Annex_III_epiCS_SIT_Spreadsheet.XLS can be provided by CellSystems. A copy should be made before the first data entry. The spreadsheet consists of two single tabs named: IMPORT and SPREAD.

Data files of optical densities (OD) generated by the microplate reader (without blank subtraction) are copied from the reader software to the Windows Clipboard and then pasted into the first tab of the EXCEL spreadsheet (IMPORT). The blank corrections, calculation of results and statistical parameters are done automatically in the second part of the spreadsheet (SPREAD).

Use the fixed 96-well plate design as specified in the SOP (see Figure 10). In addition to the entry of the reader raw data, some requested information has to be filled in the first map of the spreadsheet: tissue lot numbers, test material codes, date and lab personnel.

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_SIT_Spreadsheet.XLS"
- Fill in the requested information in "Annex_III_epiCS_SIT_Spreadsheet.XLS "
- In addition, per each experiment, keep signed hardcopies of "Annex_III_epiCS_SIT_Spreadsheet.XLS" together with the signed hardcopy of the MDS.

PREDICTION MODEL

The test protocol allows predicting the skin irritation potential of test substances according to the United Nations Globally Harmonized System (UN-GHS) for classification and labelling. A reduction of tissue viability of equal or below 50 % of the negative control classifies the substances as category 2. Tissue viability of above 50 % results in classification as no category.

In vitro result	***In vivo prediction***
mean tissue viability ≤ 50 %	*category 2*
mean tissue viability > 50 %	*no category*

ANNEXES

ANNEX A:

epiCS Skin Irritation Test

FLOWCHART

Transfer tissues from agarose to epiCS Culture Medium

↓

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

↓

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

↓

Expose 20 min at room temperature

↓

Stop exposure after 20 min by rinsing thoroughly with DPBS

↓

Transfer tissues to fresh epiCS Culture Medium

↓

Incubate (37±1°C, 5±1 % CO_2, 90±10 % RH) for 24 ±2 hrs

↓

Change epiCS Culture Medium

↓

Incubate (37±1°C, 5±1 % CO_2, 90±10 % RH) for another 18 ±2 hrs

↓

Blot tissues and perform MTT assay

↓

Read OD in a plate spectrophotometer at 550-570 nm

ANNEX B:

METHOD DOCUMENTATION SHEET (MDS)

Assay:……

Date: …….

Corresponding XLS data file name: ……

Performed by: .....Signatures: .....

TIME PROTOCOL

Receipt of epiCS (date, day, hour**):**
…….
ID:
Experimental schedule

Procedure	**Date (dd-mm-yy)**	**Set 1**		**Set 2**		**Remark**
		start	**stop**	**start**	**stop**	
Pre-incubation 2 (18 ±3 hrs)						
Exposure (20 ±1 min)						
Washing						
Post-incubation 1 - start (24 ±2 hrs)						
Medium change (24 hrs a exposure ±2 hrs)						
Post-incubation 2 - start (18 ±2 hrs)						
MTT test (3 hrs ±5 min)						
Extraction (2-2,5 hrs)						
Measurement						

DEVICES VERIFICATION

Incubator verification

Incubator #	CO_2 < 5 ±1 % >	Temperature < 37 ±1°C >	Check water in reservoir (✓)

ID/ Date:

Refrigerator verification

Refrigerator #	Temperature < 2-8°C >

ID/ Date:

Water bath verification

Water bath #	Temperature < 37 ±1°C >

ID/ Date:

In case that your devices are controlled by central computer, fill in the following table instead of the fields above:

Name of the device	**device #**	**reference**

ID/ Date:

Pipette verification (triplicate)

Pipette 3 x H_2O into a small beaker on a laboratory balance and record readings in grams (g)
Perform pipette verification once per week and refer to it in all assays of that week. If adjustable pipettes are used, check adjustment daily.

	0.9 ml	2 ml	300 µl	200 µl	25 µl	30 µl
	.....H_2O weight in g.....					
1.						
2.						
3.						
Mean						
SD						

ID/ Date:

epiCS KIT COMPONENTS

epiCS (CS-1001), tissue Lot no.:		Production date:
epiCS Culture Medium (CS-3050, CS-3051, CS-3052, C 3053) Lot no.:		Expiration date:
epiCS MTT Assay Medium 3030, CS-3031)	Lot no.:	Expiration date:
Sterile Meshes (CS-5010)	Lot no.:	Expiration date:
Position of ice-packs: (direct contact of the ice-packs with the skin must be avo		
Other remarks		

ID/ Date:

VISUAL QUALITY CONTROL OF THE SKIN

Use scores: 1- very good, 2- good, 3- acceptable, 4- not acceptable

APPEARANCE	**KIT 1**	**KIT 2**
MACROSCOPICALLY		
No of excluded tissues with: - edge defects - air bubbles - extensive moisture on the surface		

Specific observations:

SOLUTIONS

POSITIVE CONTROL

SDS 5 % solution in distilled sterile water (w/v) :

- SDS reference, batch no.:.....
- Weight:.....
- Distilled water, volume added:.....
- Preparation date:.....
- Expiration date:.....
- Storage: Refrigerator no.:.....

Note: *In case that if you are preparing your own MTT stock solution and/or DPBS fill in the following* forms

DPBS solution preparation:

- Preparation date:.....
- pH adjustment (to 7.0):.....
- Type of sterilisation:.....
- Preparation date:.....
- Expiration date:.....

DOSING PROCEDURE

Tick (✓) in the columns the type of application (pipette, patch, spoon...),
(**P**: pasty; **V:** viscous; **W**: waxy; **ST**: sticky; **C**: creamy)

Code	Liquid/ Solid (L/S)	Application (30 mg / 30 µl)					Remarks
		Pipette (✓)	Mesh (✓)	Spoon (✓)	Pin (✓)	Wetting (✓)	

ID/ Date:

REMARKS TO SINGLE TISSUES

If you observe any abnormality during the assay, you have to substitute tissues, or deal with any technical problems, fill in the following table.
Record the tissue number, substance code and your observation or remark.

Substance co	Tissue no.	Remark

ID/ Date:

MTT PLATE CONFIGURATION

PLATE 1

PLATE 2

ID/ Date:

SPECTROPHOTOMETRICAL MEASUREMENT

PLATE CONFIGURATION FOR READING *(for transfer to Spreadsheet epiCS-SI-Spreadsheet.xls):*

Record the positions of substances on 96-well plate.

Strictly adhere to the fixed plate design of this SOP.

		3										
BL	BL	BLA	BL	BL	BL	EM	EM	EM	EM	EM	EM	
N N	P P											Tissue1
N N	P P											Tissue2
N N	P P											Tissue3
BL	BL	BLA	BL	BL	BL	EM	EM	EM	EM	EM	EM	

Note: switch on the reader 10 min before reading

Check plate photometer filter
Tick correct (✓) filter setting

reading filter: 570 (550-570) nm	
no reference filter	

ID/ Date:

ARCHIVING

Raw data saved in/as:

Spreadsheet saved in/as:

MDS saved in/as:

ANNEX C:

CHARACTERISATION OF TEST SUBSTANCES

Code	Physical state (solid (S) /	Colour	MTT reduction	Reaction with)	Remarks			
					known R-phrases	known S-phrases	special storage requirements	other

ANNEX D:

Test for interference of chemicals with MTT and correction procedures

Water colouration (Step 1, Section 7.3)	Tissue staining (Step 2, Section 7.3)	MTT interaction (Step 3, Section 7.3)	Test conditions
-	-	-	A
+	-	-	A
-	+	-	B
+	+	-	B
-	-	+	C
+	-	+	C
-	+	+	B+C
+	+	+	B+C

Test conditions obtained
A
Perform all steps according to the basic SOP. Correction of results using additional controls is not needed
B
Perform Step 2, Section 7.3 in addition to the basic SOP. Correction of results with viable tissue is needed
C
Perform Step 4, Section 7.3 in addition to the basic SOP. Correction of results with freeze-killed tissue is needed

B + C

A coloured chemical (or a chemical that may turn coloured after interaction with water) may cause both tissue staining and false MTT reduction. If the experimenter is interested in the correction of final results, combination of test condition C is sufficient for this purpose, since the frozen tissues absorb approximately the same amount of chemicals (and colour) as the viable tissues. However, since the frozen tissues are more hydrated than viable tissues, tissue staining by water soluble colourant (or colourless chemicals that will turn in aqueous conditions into coloured) can be overestimated. In addition, conditions may arise when information on the amount of non-specific colouration is required. For increased precision of the above mentioned case, Steps 2 & 4 (Section 7.3) need to be performed.

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