Standard Operating Procedure
(Version 1.1)

SKIN IRRITATION TEST
FOR MEDICAL DEVICES EXTRACTS

***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

This Protocol is based on a SOP of epiCS® Skin Irritations Test [SOP epiCS® Skin Irritations Test, SkinInVitro GmbH (formerly known as CellSytems GmbH)] and Protocol n° 212: [epiCS® Skin Irritation Test (epiCS® SIT) published on EURL ECVAM DataBase service on Alternative Methods to animal experimentation (DB-ALM)] and the Round Robin Study (Round Robin study, 2017). It has been revised and adapted for the use for medical Devices with epiCS. This Protocol is for the testing of extracts of medical products. The epiCS Skin Irritation Test (SIT) method for determining skin irritation of chemicals is included in OECD Technical Guideline 439 (OECD, 2019).The evaluation of the catch-up validation study is based and guided by the performance standards (PS) of the OECD TG 439 (OECD, 2015), EC Testing Method B.46. (In vitro skin irritation: reconstructed human epidermis model test. Council Regulation (EC) No 761/2009) was tested in each laboratory (OECD, 2013) (B 46. (EC) No 761/2009), following ESAC opinion in 2016 (ESAC Opinion Number 2016-5) and independent peer review in 2018 (Peer Review Report of the Validation of the Skin Irritation Tests 2018).

The epiCS Skin Irritation Assay is applicable for evaluating the potential presence of irritants in medical device extracts (with very low concentrations) with an exposure time of 24 h. The negative control is Dulbecco's Phosphate Buffered. The saline and sesame oil vehicles used for extraction of the medical device. The positive control is a 1 % solution of Sodium dodecyl sulfate (SDS) in polar and non-polar vehicles. The Vehicle controls have undergone the ISO 10993-12 medical device extraction procedure. The irritations test for medical devices extracts witch epiCS-models was evaluated form SkinInVitro GmbH, based on the study designs of Round Robin Study.

epiCS® Skin Irritation Test for Medical Devices Extracts

1. Abstract

The endpoints considered depend up on the type of device, the contact of the device with the patient, and duration of the contact. Nevertheless, cytotoxicity, irritation and sensitization testing are recommended for almost all devices (Round Robin Study, 2012). The epiCS skin irritation test for medical devices extracts is designed for the prediction of acute skin irritation of medical devices by measurement of cytotoxic effects of extract [prepared according to ISO10993-12 (ISO/TC 194 10993-12, 2012)]. Cytotoxic effects on the commercially available reconstructed epidermis epiCS are determined by a standard MTT assay. The test is developed to be proposed as replacement method of the animal method recommend in the ISO 10993 part 10 (Biological evaluation of medical devices - Part 10: Tests for irritation and skin sensitization).

2. Objectives & Applications

1.1 Type of Testing:

Replacement

1.2 Level of Toxicity Assessment:

Hazard identification, toxic potential, toxic potency

1.3 Purpose of Testing:

Classification and labelling; prediction of skin irritation potential of Medical Devices Products

1.4 Applicability Domain:

The test described in this protocol is designed for extracts of medical devices liquid (polar and non-polar solutions). The method is not applicable to gases and aerosols.

2. Basis of Method

Dermal irritation is generally defined as "the production of reversible inflammatory changes in the skin" (OECD 2002). This is induced by extracts of medical devices polymers, with a low concentration of irritant which penetrate the *stratum corneum* and lead to damages and cell loss of the underlying cell layers. The epiCS irritations test of medical devices measures the cell damage as reduction of cell viability using a human reconstructed epidermis.

The test consists of a topical exposure of the extracts of Medical Devices polymers (Medical Devices extracts in saline and sesame oil), for 24h, 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 PBS) 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).

3. Experimental Description

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 measur

ed 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. The acceptance criteria that skin models need to comply with can be found in 4.5.1 Quality Control Performed by SkinInVitro
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 shipping agarose together with epiCS Culture Medium, epiCS MTT assay medium and 6-well plates.

4. Basis Procedure

Samples are prepared according to ISO10993-12 by extraction (37 +/-1 C° for 72 +/- 2 h with agitation) using polar (saline) and non-polar (sesame Oil of pharmaceutical grade) solvents to be used 24h maximum after extraction. Vehicle controls should include saline solution and sesame oil that have undergone the ISO 10993-12 medical device extraction procedure. On day of receipt, epiCS are conditioned by incubation for release of transport-stress related compounds and debris at least 4 h -24 h. On the day of experiments, spike the positive control (SDS) into polar (saline) and non-polar (sesame oil) solvents at the concentration specified. After pre-incubation, tissues are topically exposed to the extracts of test polymers for 24 h. Three tissues are used per test polymer extracts, positive control (PC), negative control (NC) and vehicle control (VC). After a 24 h incubation period the tissues are then thoroughly rinsed and blotted to remove the test substances and transferred to a holding plate. Afterwards, MTT assay is performed by transferring the tissues to 24 well plates containing 300 µl MTT medium (1 mg/ml). After a 3 h 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 %.

Note 1: *Vehicle controls should include saline solution and sesame oil that have undergone the ISO 10993-12 medical device extraction procedure.*

Note 2*: On the day of experiment spike the positive control (SDS) into polar (0.9% NaCl) and non-polar (sesame oil) solvents at the concentrations specified*

5. Data Analysis/Prediction Model

The test protocol allows predicting the skin irritation potential of extracts of test polymers, from the field of medical products, 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

SKIN IRRITATION TEST FOR MEDICAL DEVICES

EXTRACTS

6. Modification of the Method

None

7. Discussion

The various toxicological endpoints for the safety evaluation of medical devices are described in the (EN/ISO 10993 series of standards. The tests to consider for the safety evaluation of medical devices are described in ISO 10993-1:2009 Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process). For determining skin irritation potential of medical devices (ISO10993-10:2010 Biological evaluation of medical devices –Part 10: Tests for irritation and skin sensitization, provides information on the testing of medical devices and medical device extracts) (Round Robin Study, 2017).

The epiCS irritation test for medical devices extracts is an easy to perform *in vitro* test which only needs a minimum of training. 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.

8. Status

8.1 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 and fully validated and accepted for skin irritation testing, listed in the OECD TG 439.

8.2 Proprietary Issues:

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

9. 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
SO:	sesame oil
UN-GHS:	United Nations Globally Harmonized System
VC:	vehicle control

B Procedure Details, Latest Version: August 2019

epiCS® Skin Irritation Test for Medical Devices Extracts

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1. Health & Safety Issues

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.

1.1 Safety Precautions:

Irritant materials are dangerous: It is thus recommended to work in laminar flow hood with chemical filter or in ventilated cabinets and wear gloves, coat, as necessary.

MTT (R26 R68 R22 R36 R37 R38), isopropanol (R11 R36 R67) and SDS (R11 R21 R22 R36 R37 R38 S26 S36 S37) are dangerous.

Please contact SkinInVitro if you have any questions or concerns.

2. Materials and Preparations

2.1 Test System:

2.2 Standard epiCS 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*
	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: *MTT solution is light sensitive. Protect it from light*

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

2.4 Equipment:

2.5 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, Extraction of polymers*
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, Extraction of polymers medical devices samples*
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, for better solubilisation of SDS*
shaker	*For extraction of polymers*
Adjustable pipette	*For pipetting 2 ml MTT isopropanol*
Adjustable micropipette	*For application of 100 µl liquid*
Lab vials	*For extraction*

2.6 Consumables:

96-well plate	*For Optical Density readings (MTT)*
24-well plate	For the MTT assay
Sterile paper towel	For blotting of cell culture inserts
6-well plates – sterile	To transfer tissue inserts to fresh media (for preincubation and exposition)
Adhesive tape (NeoLab ref. 7-2220 or ref. 2-5082) or Parafilm M	Covering plates during formazan extraction
Injection needle	

2.7 Media, Reagents, Others:

Dulbecco´s PBS (DPBS) Ca/Mg-free (sterile)	Use for rinsing tissues and negative control
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
0,9% NaCl	For extraction of polymers, vehicle control, positive control
Sesame oil	For extraction of polymers, vehicle control, positive control

3. Test Substances

A short description of the various steps involved in performing the *in vitro* skin irritation test is presented in **Annex A**.

4. 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.)

Follow safety instructions for respective compounds.

5. Preparation of Samples, Controls and Solution

5.1 Preparation of Medical Devices Extracts:

Document the information on the test substances/medical devices in the table of **Annex C**: Characterisation of test substances.

Day prior to testing (day -3)

Samples (Polymers of medical devices) are prepared according to ISO10993-12 by extraction (37 +/- 1 °C for 72 +/- 2 h with agitation) using polar [saline (0.9%NaCl)] and non-polar (sesame oil of pharmaceutical grade) solvents to be used 24h maximum after extraction. For more information see annex D: Iso 10993-12 EXTRACTION PROCEDURE.
The vehicle controls be subjected to the identical extraction procedure as the test materials. Details of this controls in preparation of control.

5.2 Preparation of Vehicles Control Solution:

Saline and Sesame oil

The vehicle controls (0.9% NaCl and sesame oil) should be placed into the (amber glass vial) extraction vessels and be subjected to the identical extraction procedure (72 h at 37°C) as the test materials. Details of this ISO 10993-12 extraction procedure are provided in **Annex D.**

Chemical exposure (day 1)

5.3 Preparation of Control:

For testing of in vitro skin irritation for medical devices, 5 controls are used. See **Figure 1.**

Controls (3 Replicates per Control) = 15 EpiCS		
Negative Controls (NC)	**PBS**	
	polar solvent:	**non-polar solvent:**
Extraktion Solution	**0,9% NaCl**	**sesame oil**
Vehicle Controls (VC)	**0,9% NaCl**	**sesame oil**
Positive Controls (PC)	**1% SDS, NaCl**	**1% SDS, sesame oil**

Figure 1: Controls for irritation test of medical devices products

On the day of experiment spike the positive control (SDS) into polar (0,9% NaCl) and non-polar (sesame oil) solvents at the concentrations specified. The Vehicle controls be subjected to the identical extraction procedure as the test materials.

5.4 Positive Control Solution:

10 % (w/v) (aq) SDS

Weigh out 250 mg SDS (analytical grade, see Equipment) into an appropriate calibrated 2,5 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 min. Solution can be stored up to one month (2-8°C). Record the preparation in the MDS.

Positive Controls (1% v/v SDS)

500 µL of 10% SDS (Fluka /Sigma) should be mixed with 4,5 mL of the vehicle (0,9% NaCl or sesame oil) and thoroughly vortexed.

5.5 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.

6. Preparation of Solutions

6.1 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 enough 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.

C Method

Experimental System Procurement

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Fax: **+49 (0) 2241-25515-26**
E-mail: **info@skininvitro.com**

1. 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 SkinInVitro immediately.

Contact person:

M.Sc Bernadette Thiede
SkinInVito GmbH
phone: +49 (0) 2241-25515-0
email: bernadette.thiede@skininvitro.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).

1.1 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.
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 h or overnight (24h) 37±1°C, 5±1 % CO2, 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 enough rinsing DPBS for the washing day 2 (approximately 1L per 24 inserts). See preparation of solutions

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)

2. Test Material Exposure Procedures

Chemical exposure (day 1)

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

    Checklist:

    - micropipette
    - sterile tips and pipettors
    - vials with extracts from polymers from Medical Devices set to room temperature, including negative control (NC) sterile DPBS and as positive control (PC) 1 % SDS in 0,9% NaCl or sesame oil. Vehicle control (VC) (identical extraction procedure as the samples).
    - timer
    - sharp, pointed tweezers
    - beaker for waste material

> ***Note:*** *Wipe all non-sterile material bottles with 70 % ethanol.*
> ***Note:*** *Use one plate per one extraction during exposure! One plate for the polar extraction and one plate for the non- polar extraction.*

2. Remove 6-well plates from the incubator approximately 5 min before exposure to chemicals.

3. Check the surface of tissues and exclude completely wet tissues or tissues with any visible defects.
4. Before test substance exposure, label all 6 well plate lids with the test material codes or names.

2.1 Test Substance Exposure:

1. Apply 100 µl of the extracts, NC, PC or VC 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).
2. Keep plates with applied extracts or controls in the laminar hood at RT (20±2°C).
3. Incubate the treated epiCS (37±1°C, 5±1 % CO2, 90±10 % RH) for 24h ± 1h.

Note: Dispense 100 µl directly atop the tissue. Avoid contact with the tissue surface.

Figure 2: Application of liquids

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 considered, like testing the volatile substances on separate plates.*

2.2 Washing:

Washing (day 2)

1. After 24h incubation, 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 3). 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 3.
2. After the 20 times rinse from washing bottle, completely submerge the inserts 3 times in 150ml DPBS (shake to remove all rests of test material).

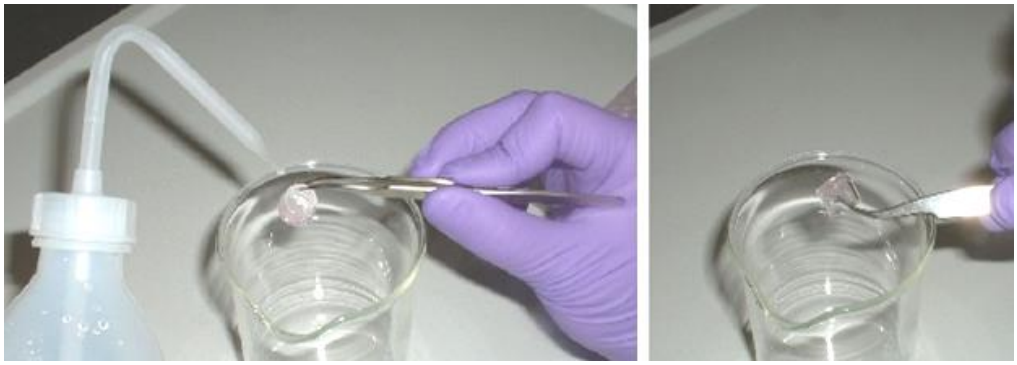

***Figure 3**: Washing procedure*

> ***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 must be carried out thoroughly and as described in this protocol in the following paragraphs.*

3. 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 4).
4. Transfer tissues in the holding plate

***Figure 4:** Blotting and drying of the surface*

3. ENDPOINT MEASUREMENT

MTT viability test (day 2)

3.1 MTT assay:

1. Prior to the MTT assay, label enough 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 h ±5 min.

***Note**: Keep 3 h ±5 min MTT incubation time strictly. Deviation from the 3 h 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 h (maximum 2,5 h) at room temperature with gently shaking on a plate shaker (~ 100 rpm).

6. As an alternative, overnight extraction (18-24 h) is also possible. Seal plates as described above and extract at room temperature or in the refrigerator in the dark, without shaking. Before using the extracts, shake for at least 15 min on plate shaker.

7. After the extraction period is complete, pierce the membranes of the inserts with an injection needle (~20 gauge, ~0.9 mm diameter) and allow the extract to run into the well from which the insert was taken. Afterwards the insert with the tissue can be discarded. Before transferring the extract from the wells of the 24-well plate into 96-well plates, pipette up and down at least 3x until the extractant solution is homogenous.

***Figure 5:** Piercing the inserts*

8. Per each tissue transfer 3 × 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 6). Use isopropanol as blanks. Read OD in a 96-well plate spectrophotometer using a wavelength between 550 and 570 nm preferably at 570 nm, without using a reference filter.

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank	blank	blank	empty	empty	empty	empty	empty	empty
B	NC	NC	NC	NC	NC	NC	NC	NC	NC	empty	empty	empty
C	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	empty	empty	empty
D	SO	SO	SO	SO	SO	SO	SO	SO	SO	empty	empty	empty
E	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	empty	empty	empty
F	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	empty	empty	empty
G	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	empty	empty	empty
H	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	empty	empty	empty
	Tissue 1			Tissue 2			Tissue 3					

Blank, isopropanol solution for background measurement; NC, negative control; NaCL 0,9 % vehicle control; SO, sesame oil vehicle control; PCNACL, positive sample control in NaCl extraction vehicle; PCSO, positive sample control in sesame oil extraction vehicle. NaCL 1 and SO 1 isopropanol samples of 200 µl in triplicate for each tissue incubation for test sample 1; Additional test samples can be measured in additional 96 well plates. For the EpiCS RHE model the OD is determined in triplo (3x 200µl) according to the manufacturer

Figure 6: Fixed 96 well-plate design (for OD reading in plate spectrophotometer, 3 aliquots per tissue)

In contrast to normal spectrophotometers, in plate readers pipetting errors may influence the OD. Therefore, 3 formazan extract aliquots will be taken from each tissue extract. In the Excel data sheet, these 3 aliquots will be automatically reduced to one value by calculating the mean of the 3 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 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 30 %.

4. ACCEPTANCE CRITERIA

4.1 Quality Control Performed by SkinInVitro:

The epiCS RHE models are free of bacteria, fungi, HIV and Hepatitis B, C. The quality of the epiCS tissues is assessed by an MTT-assay and by histological examination.
Cell viability MTT OD ≥ 0,80 and ≤ 2.8
Barrier function integrity 2h > ET50 <5h

4.2 Negative Control Acceptance Criteria: (NC)

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 ≥ 0,8 and does not exceed 2.8

4.3 Positive Control Acceptance Criteria: (PC)

A 1 % SDS (in extraction medium) (see Preparations) is used as positive control (PC) and tested concurrently with the test devices. Concurrent means here the PC must 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.

The assay is meeting the acceptance criterion if the mean viability of PC expressed as % of the negative control is ≤ 40 %

4.4 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 < 20 %

4.5 Batch Acceptance Criterion:

All extraction data from one batch are considered as valid if both the negative, the vehicle and the positive controls data fulfil the above criteria requirements.

4.6 Extraction Data Acceptance Criterion:

The value is considered as valid if it the Standard Deviation of the mean viability of the three exposed tissues after incubation with the extraction sample is <20%. The three independent runs must predict the same class of irritation.

4.7 Retesting:

For a given extract, if only one batch (among the 3 batches used) gives an SD is > 20, then the extract is retested once (possible technical problem or error). The three independent runs must predict the same class of irritation. If this requirement is not met the test shall be repeated.

5. Data Analysis

5.1 Data Calculation Step:

Blanks:
- Calculate the OD mean from the 9 replicates for each plate ODblank

Negative PBS--treated controls:
- Calculate the blank corrected value ODNC = ODNCraw – ODblank
- Calculate the OD mean per tissue (3 replicates)
- The mean OD for all tissues corresponds to 100% viability =mean ODNC

Positive SDS-treated control:
- Calculate the blank corrected value ODPC = ODPCraw – ODblank
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue %PC = [ODPC / mean ODNC] x 100
- Calculate the mean viability for all tissues Mean PC = Σ %PC / number of tissues

Tested compound:
- Calculate the blank corrected value ODTT = ODTTraw – ODblank
- Calculate the OD mean per tissue (3 replicates)
- Calculate the viability per tissue %TT = [ODTT / mean ODNC] x 100
- Calculate the mean viability for all tissues Mean TT = Σ %TT / number of tissues

Standard deviations are calculated on OD and % viabilities.

6. 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*

D ANNEXES

1. Annex A

Skin Irritation Test for Medical Devices Extracts

Flowchart

Prepared unknown dives extract in a polar (0,9%) and non-polar (sesame oil) solvents. Prepare the VC. Extraction according to ISO10933-12. Use for the extraction 37°C +/- 1°C for 72h +/- 1h with continuous agitation/shaking

↓

Transfer tissues from agarose to epiCS Culture Medium

↓

Incubate for 60 min (37±1°C, 5±1 % CO_2, 90±10 % RH) then transfer epidermis into fresh medium

↓

Incubate 4h- 24h (37±1°C, 5±1 % CO_2, 90±10 % RH)

↓

On the day of experiment spike the positive control (SDS) into polar (saline) and non-polar (sesame oil) solvents at the concentrations specified

↓

Dose 3 tissues each with 100 µl NC, or VC, or PC; or extracts from the tested materials

↓

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

↓

Stop exposure by rinsing thoroughly with DPBS

↓

Transfer tissues to holding Plate (300µl Culture Medium)

↓

Blot tissues and perform MTT assay

↓

Read OD in a plate spectrophotometer at 550-570 nm

2. Annex B

2.1 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)**	**Time**		**Remark**
		start	**stop**	
Extraction of samples Start				
Extraction of samples Stop				
Pre-incubation 1 (1 h)				
Pre-incubation (4 h – 24 h)				
Exposure (24 h+/- 1 h)				
Washing				
MTT test (3 h ± 5 min)				
Extraction Isopropanol (minimum 2 h)				
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 Medical 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, CS-3053) Lot no.:		Expiration date:
epiCS MTT Assay Medium (CS-3030, CS-3031)	Lot no.:	Expiration date:
Position of icepacks: (direct contact of the icepacks with the skin must be avoided)		
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**
MACROSCOPICALLY	
No of excluded tissues with: - edge defects - air bubbles - extensive moisture on the surface	

Specific observations:

SOLUTIONS

Sesame oil:

- Reference Batch N° .....
- Expirate date: .....

Saline (0.9 % NaCl):

- NaCl supplier /Reference .....
- Saline Batch N° .....

POSITIVE CONTROL

SDS 1% (v/v) solution in saline and sesame oil, prepared from 10% solution of SDS:

On a day of experiment, prepare fresh solutions of 1% SDS in saline and sesame oil by mixing 200 µL of 10% SDS with 1.8 mL of vehicle. Vortex thoroughly after preparation and shortly before application to the tissue surface. Expiration is on the same day.

SDS 1 % solution in saline or sesame oil (w/v):

- 100% SDS reference, batch no.: .....
- Expiration date: .....
- Weight: .....
- Distilled water, volume added: .....
- Preparation date: .....
- Expiration date: .....
- Storage: Refrigerator no.: .....
- Sesame oil Batch N° .....
- Sesame oil volume added: .....
- Saline Batch N° .....
- Saline volume added: .....

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

DPBS solution preparation:

- (D)PBS concentrate reference, batch N°: .....
- pH adjustment (to 7.0): .....
- Type of sterilisation: .....
- Preparation date: .....
- Expiration date: .....

REMARKS TO SINGLE TISSUES

If you observe any abnormality during the assay, you must 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 code	Tissue no.	Remark

ID/ Date:

MTT PLATE CONFIGURATION

PLATE 1

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 2

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:

SPECTROPHOTOMETRICAL MEASUREMENT

PLATE CONFIGURATION FOR READING Record the positions of substances on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	blank	blank	blank	blank	empty	empty	empty	empty	empty	empty
B	NC	NC	NC	NC	NC	NC	NC	NC	NC	empty	empty	empty
C	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	empty	empty	empty
D	SO	SO	SO	SO	SO	SO	SO	SO	SO	empty	empty	empty
E	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	PCNaCl	empty	empty	empty
F	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	PCSO	empty	empty	empty
G	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	NaCl 1	empty	empty	empty
H	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	SO 1	empty	empty	empty
	Tissue 1			Tissue 2			Tissue 3					

Strictly adhere to the fixed plate design of this SOP.

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:

3. Annex C

3.1 Characterisation of Test Substances:

Test Material Name or Code	Amount of sample used:	Volume of extract added:	Incubator:	Time in:	Time out:	Pre- and post-extraction appearance

ID/ Date:

4. Annex D

4.1 ISO 10993-12 Extraction Procedure:

ISO 10993-12:2012

NOTE: In this protocol an extraction procedure is used following the procedures in ISO 10993-12:2012. This text of ISO 10993-12:2012 is added for information only.

4.2 Preparation of extracts of Medical Devices samples:

4.3 Abstracts:

If extracts of the medical device are required for a test procedure, the extraction vehicles and conditions of extraction used shall be appropriate to the nature and use of the final product and to the purpose of the test, e.g. hazard identification, risk estimation or risk assessment. The physicochemical properties of the device materials, leachable substances or residues shall be considered when choosing the extraction conditions.

4.4 Extraction Conditions and Methods:

The Extraction conditions are based on common practice and are carried out based on providing a standardized approach.

a) (37 ± 1) °C for (72 ± 2) h;

b) (50 ± 2) °C for (72 ± 2) h;

c) (70 ± 2) °C for (24 ± 2) h;

d) (121 ± 2) °C for (1 ± 0,1) h.

NOTE Extraction at (37 ± 1) °C for (24 ± 2) h in tissue culture media is acceptable for cytotoxicity testing. Extraction temperatures greater than (37 ± 1) °C can adversely impact chemistry and/or stability of the serum and other constituents in the culture medium.
Extraction is a complex process. The effects of other conditions on extraction and the identity of the extraction vehicle(s) should be considered carefully.
Liquid extracts shall, if possible, be used immediately after preparation. If an extract is stored for longer than 24 h, then the stability and homogeneity of the extract under the storage conditions shall be verified
Extractions shall be performed with agitation or circulation.
Extract pH shall not be adjusted unless a rationale is provided.

4.5 Extraction Vehicles:

The extraction vehicle and conditions of extraction shall be selected based on physicochemical properties of the material and/or predicted low-molecular-weight chemicals that might be extracted. Extraction using polar and non-polar extraction vehicles shall be performed. The following are examples of extraction vehicles:

a) polar extraction vehicle: saline or culture media without serum;

b) non-polar extraction vehicle: sesame oil

c) additional extraction vehicles: ethanol/water, ethanol/saline, polyethylene glycol, dimethyl-sulfoxide and culture media with serum.

4.6 Containers for Extraction:

To ensure that the extraction vessels do not adulterate the extract of the test sample, the extraction be be performed in clean, chemically inert, closed containers with minimum dead space.

4.7 Changed Extraction Conditions:

The control of the manufacturing process must always be ensured. Possible residues such as trace elements and cleaning and disinfectants agents should be considered.
Where the toxic potential is shown to be within the requirement for a product tested by exaggerated and/or exhaustive extraction, there shall be no need to further challenge the device by simulated-use extraction.
For test methods that use these materials directly (e.g., direct contact or agar overlay cytotoxicity, implantation, some genotoxicity tests, and direct contact haemolysis) the material shall be used as in clinical use, with in situ cure in the test system.

4.8 Documentation of Samples for the Extraction:

The Documentation of the sample and its preparation shall include:
lot or batch number
type and, if known, composition of material, source of material, device, device portion or component.
description of processing, cleaning or sterilization treatments, if appropriate;
extraction techniques, as appropriate, including documentation of conditions for extraction, extraction vehicle, extraction ratios, means of agitation, deviations from the conditions specified in this part of ISO 10993, for example filtration of the extract or extraction media.

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