International Standards Organization
ISO/TC 194 WG 8 Irritation and Skin Sensitization
Protocol for a Round Robin Study:

Evaluation of a Method to Detect Skin Irritation of Medical Device Extracts using Reconstructed human Epidermis (RhE).

Test using EpiDerm™ RhE, MatTek Corporation

Version 9 Final

CONTENTS

EpiDerm™ SKIN IRRITATION TEST FOR MEDICAL DEVICE EXTRACTS

The irritation potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, using a reconstructed human epidermis (RhE) as a model.
This protocol describes the use of the EpiDerm™ (EPI-200) RhE available from MatTek Corporation, USA and MatTek In Vitro Life Science Laboratories (Slovakia).

General

TYPE OF TESTING**:**
Skin Irritation of Medical Device Extracts using Reconstructed human Epidermis (RhE).

LEVEL OF ASSESSEMENT**:**
Identification of skin irritation hazard to inform safety assessment

PURPOSE OF TESTING**:**
Evaluation of a method to detect skin irritation of Medical Device Extracts using Reconstructed human Epidermis (RhE) to replace the acute skin irritation test in rabbits.

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1. RATIONALE AND BACKGROUND

The test is designed to predict and classify the skin irritation potential of medical device extracts by measuring the effect of the extracts on the viability of reconstructed human epidermis (RhE). As previous studies were performed with the EpiDerm™ tissues (EPI-200) provided by MatTek Corporation (Casas J.W. et al., 2013), these will be used as a reference test system in this round robin (RR) study.

The EpiDerm™ Skin Irritation Test (SIT) method for determining skin irritation of chemicals has been validated by ECVAM and is included in OECD Technical Guideline 439 and EU Guideline B.46. This method was validated with neat industrial chemicals for the purpose of classification and labeling. The primary objective of this study is to determine whether the modified EpiDerm™ Skin Irritation Assay is applicable for evaluating the potential presence of irritants in medical device extracts that may be present at very low concentrations. The negative control is Dulbecco's Phosphate Buffered Saline (DPBS) and/or vehicles used for extraction of the medical device (saline, sesame oil) while the positive control is a 1% solution of Sodium dodecyl sulfate (SDS) in polar and non-polar vehicles. Vehicle controls are saline solution and sesame oil that have undergone the ISO 10993-12 medical device extraction procedure.

2. SPECIFIC PURPOSE OF THE METHOD

The present test is designed to predict and classify the skin irritant potential of extracts from medical devices according to the requirements of ISO standards 10993-1:2009, 10993-5:2009, 10993-10:2010, and 10993-12:2012 using the RhE model EpiDerm™ (EPI-200) and parameters related to skin irritation. In the present study the modification of the validated EpiDerm™ SIT assay will be evaluated for the determination of the presence of irritant chemicals in extracts of medical devices.

3. BRIEF EXPERIMENTAL DESCRIPTION

3.1. Test System

Epidermis model
The reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, MA, USA and MatTek IVLSL, Bratislava, Slovakia) 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*.

The EpiDerm™ tissues (surface 0.63 cm^2) are cultured on specially prepared cell culture inserts and shipped world-wide as kits, containing 24 tissues on shipping agarose together with the necessary amount of culture media, DPBS, 6-well plates, and 24-well plates. For the purpose of the current validation study, specialized kits will be provided by MatTek under the part # EPI-200-SIT-MD.

In addition, the MTT kit (containing MTT concentrate, diluent, and extractant) is provided by MatTek for this validation study to avoid variation and mistakes that can happen during the preparation of the MTT solutions by single laboratories.

Quality Control
EpiDerm™ kits are manufactured according to defined quality assurance procedures (GMP). All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (1%) for each EpiDerm lot. The ET-50 must fall within a range established based on a historical database of results.

3.2. Basis of the Method

Endpoints: Cell viability determination is based on cellular reduction of MTT, and subsequent conversion to a purple formazan salt that is quantitatively measured after extraction from tissues (Faller et al., 2002; Mosmann, 1983). The reduction of cell viability in treated tissues is compared to negative controls and expressed as a percentage. The percent reduction in viability is used to predict the irritation potential.
Interleukin-1 alpha (IL-1α) can be measured in the basal media as a secondary endpoint that may help to increase sensitivity, therefore media from the exposure will be collected and kept frozen at -20°C for possible analysis after the completion of the main study.

Brief Procedure

Studies performed with biomaterials specifically manufactured to contain irritant chemicals at low concentrations indicated that a prolonged incubation is needed compared to the OECD TG 439 protocol. Initially, 4, 18, 24 and 48 hours exposure times were evaluated and it was concluded that 18 hours exposure to low concentrated irritants and extracts from biomaterials is sufficient to predict irritation in vitro by reduction of tissue viability below 50% (Casas et al, 2013; Kandarova et al. 2015).

In this validation study, 100 µLextracts from polymer samples spiked with known irritants will be applied to the epidermal model and incubated at 37^{o}C for 18 hours. Samples will be extracted with polar and non-polar solvent vehicles. Three tissue replicates will be used per test material and three independent runs will be performed with different EPI-200 tissue lots. Tissues will be incubated at 37°C, 5% CO_2 and 95% relative humidity (RH) following the addition of the test extract.

Exposure to the test sample extract will be terminated by rinsing with Dulbecco's phosphate buffered saline (DPBS). The viability is assessed immediately after the washing step by incubating the tissues for 3 hours with MTT solution in a 24-well plate (1 mg/mL; 0.3 mL per well). The formazan is then extracted using 2 mL of isopropanol (MTT-100-EXT). Two aliquots per tissue of extracted formazan will be added to 96-well plates (200 µl/well) and quantified spectrophotometrically at 570 nm.

Aliquots of culture media collected after 18 hours exposure will be stored frozen (-20 °C) for cytokine (IL-1α) measurements. A 1% (v/v) solution of SDS in sesame oil and in saline solution will be used as positive controls and DPBS-treated epidermis will be used as the negative control, respectively.

Vehicle controls should include saline solution and sesame oil that have undergone the ISO 10993-12 medical device extraction procedure. For each treated tissue the viability is expressed as a percent relative to negative DPBS treated control tissues (mean).

Known limitations of the method: The method is not applicable to gases and aerosols.

Known cases of test-compounds requiring specific controls: Some chemicals can directly reduce the MTT reagent (e.g., electrophiles, test articles with high pH), while other chemicals can directly color the tissue or the cells. Such test substance properties can only interfere if sufficient amounts of the chemical are still present on the tissue at the end of the exposure period. In these cases a special procedure allowing the quantification of the "true" MTT reduction should be applied. The use of specific and adapted controls will enable the calculation of true tissues viability after subtracting the unspecific Optical Densities due to direct chemical MTT reduction and/or chemical residual color extracted from the tissues. Conditions for use of specific controls are described in the procedure (EPI-200-SIT protocol, 2012).

3.3. Prediction Model (PM)

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This prediction model is based on the prediction model of the OECD TG 439 and data further generated during the optimization of the medical device (MD) protocol (Casas et al., 2013; Kandarova et al., 2015). While the prediction model (PM) seems to be applicable for the MD protocol, data from this project will allow a more accurate prediction model to be developed.

If cell viability at 18 hours exposure time is ≤ 50%: the chemical is classified as Irritant (I).

If cell viability at 18 hours exposure time > 50%: the chemical is classified as Non-Irritant (NI)

4. MATERIALS

4.1. EpiDerm™ EPI-200

Product Description

Precautions
The epidermal cells are taken from healthy volunteer donors negative to anti-HIV 1 and 2, and to hepatitis C antibodies, and to hepatitis B antigens. Nevertheless, normal handling procedures for biological materials should be followed:

(a) It is recommended that gloves be worn during handling:

(b) After use, the epidermis, the material and all media in contact with it, should be decontaminated (for example, by using a 10% solution of bleach or appropriate containers), prior to disposal.

Examine all kit components for integrity. If there is a question or concern, call MatTek Corporation.

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DESCRIPTION	USE/COMMENT
EPI-200-SIT-MD kit	
1x EPI-200 plate containing 24 reconstructed epidermis units (surface area: 0.63 cm^2)	Each reconstructed epidermis is attached to the base of a tissue culture insert and maintained on nutritive agar for transport
2 x 24-well assay plates	For assays
8 x 6-well plates	For assays and pre-incubations
1x 100 mL bottle of sterile Assay Medium (EPI-100-NMM)	Basic medium for use in MTT assays
1 x 100 mL bottle of sterile DPBS	For negative control and initial rinsing tissues
MTT-100 kit (MTT assay components for 24 tissues)*	
1 X MTT-CON	MTT concentrate (5 mg/mL), 2mL
1 X MTT-DIL	MTT diluent, 8mL
1x MTT-EXT	MTT extractant (isopropanol), 60 mL

*needs to be ordered separately

4.2. Other Materials Needed

Microbiological safety cabinet (laminar flow hood)	For safe work under sterile conditions
Non-sterile ventilated cabinet	For safe work with chemicals, applications, washes
Cell incubator 37 °C, 5% CO_2, 95% humidity	For incubating tissues
Plate reader (96-well) with a 570 nm filter	For Optical Density readings (MTT)
Water bath at 37^{o}C	For warming media
Vacuum source/trap and Pasteur pipettes	For aspirating media and solutions
Laboratory balance (accuracy 0.1 mg)	For checking chemicals weight and spatula weight
Shaker for 96-well plates	For shaking tissues before media sampling
Wash bottle (500 mL)*	For rinsing tissues
Sterile DPBS and/or PBS (without Ca and Mg)	For Rinsing, About two liters is sufficient for use as the negative control (DPBS) and for all rinsing performed with one kit
Flasks and 200 mL beakers	For rinsing tissues and submerging rinsed tissues
Adjustable micro-pipette – 0 to 200µL	For pipetting 200 µL formazan extracts (96-well plate)
Adjustable micro-pipette – 0 to 1000µL	For assay and maintenance media
Adjustable positive displacement micro-pipette - 0 to 100 µL	For application of test samples and controls
Stop-watches/Timers	For controlling contact and step times
Bulb headed glass Pasteur Pipettes	For spreading samples on tissue
Small sterile blunt-edged forceps	For handling tissue inserts
20% SDS [151-21-3], Cat # 05030, Fluka /Sigma	To be used as stock solution for preparing 1% SDS (positive control) in sesame oil and saline
Sesame oil Sigma-Aldrich 85067 (was Fluka 85067), CAS Number 8008-74-0 or Spectrum Chemicals, Sesame Oil, NF, Nr: SE130, CAS Number: 8008-74-0.	Pharma grade (pretested on RhE) to be used as nonpolar vehicle
Saline (0.9% solution of NaCl)	Pretested on RhE, to be used as polar vehicle
96-well plates	For reading Optical Density
Adhesive tape or Parafilm M	For covering plates during extraction to minimize
Sterile cotton swabs	For drying the tissue surfaces
Sterile Water	For preparation of 1% SDS
Incubator capable of 37±1°C	For sample extractions
Orbital shaker	To agitate sample extractions during incubation

4.3. Preparation of Medical Device Extracts

Document the information on the test substances/medical devices in the table of **Annex 2**: Characterisation of test substances. The preparation of polymer material extracts will be done according to ISO 10993-12 guidelines and will be based on surface area/weight to volume ratios described in this standard.

- Polar extracts will be prepared in 0.9% saline solution (0.9g NaCl in 100 mL ultrapure or deionized water).
- Non-polar extracts will be prepared in sesame oil of pharmaceutical grade.

Note: Use for the extraction (37 ± 1) °C for (72 ± 2) hours with continuous agitation/shaking.

5. METHODS

A short description of the various steps involved in performing the *in vitro* skin irritation test is presented in Section 11. The text below provides a detailed description of all steps and handling in the assay.

5.1. Precautions

Safety precautions: MTT (R26 R68 R22 R36 R37 R38) and isopropanol (R11 R36 R67) are dangerous.

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

5.2. MTT Preparation

The MTT-100 kit is available from MatTek Corporation. It is strongly recommended that this kit be used in the validation study to overcome variations and mistakes that can happen during the preparation of the MTT solution.

Note: MTT solution is light sensitive. Protect it from light.

MTT Ready to Use Solution Preparation

Dilute 2 mL of MTT concentrate solution (MTT-100-CON) with 8 mL of MTT diluent (MTT-100-DIL). The final concentration will be 1 mg/mL. Protect from light until use (do not exceed 2 hours stocking before use).

5.3. Test Material and Control Preparation

Record the main information about the test chemicals and control chemicals, including codes or numbers, physical consistence, volumes or weight, expiration date and stocking conditions.

Specific Procedure for the Negative Control (DPBS):

The negative control is Dulbecco's Phosphate Buffered Saline (DPBS). Sterile ready-to-use DPBS should be used. If DPBS is prepared from 10x concentrates or powder, the pH needs to be adjusted to 7.0 and solution must be sterilized. Record the preparation in the Methods Documentation Sheet (MDS). A volume of 100 µL will be added to the tissues. .

Specific Procedure for the Positive Controls (1% v/v SDS):

500 µL of 20% SDS in water should be mixed with 9.5 mL of particular vehicle and thoroughly vortexed. From these preparations a volume of 100 µL will be removed using a positive displacement pipette and added to the tissues.
For the 1% SDS in sesame seed oil, that forms emulsion after preparation from stock solution, if needed, spread the PC solution with the bulb headed glass Pasteur pipette to ensure a correct contact with the entire epidermis

Note: 20% SDS solution from a commercial supplier should be used (Fluka, Cat # 05030 is recommended). The PC in both vehicles has to be prepared freshly before each use on the day of the experiment.

Specific Procedure for the Vehicles
The vehicle controls (0.9% saline and sesame oil) should be placed into the (amber glass vial) extraction vessels and be subjected to the identical extraction procedure (72 hours at 37°C) as the test materials. Details of this ISO 10993-12 extraction procedure are provided in **Annex 3**.

5.4. Receipt of the EPI-200 Tissues (Day 0)

Kit details and assay procedures should be registered on the Methods Documentation Sheet (MDS).

Check the date of sending written on the package as well as some critical points before opening the EPI-200 kit.

(a) Place the MTT concentrate at -20°C and DBPS supplied with the kits at 2-8°C (refrigerator).

(b) Let the assay medium reach room temperature (20-25°C). Do not pre-heat to 37°C.

5.5. Preparation and Pre-incubation (Day 0)

(a) Pipette 0.9 mL of the assay 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.

(b) Under sterile conditions, open the plastic bag containing the 24-well plate with epidermal tissues. Under a sterile airflow, remove the sterile gauze and carefully (using sterile forceps) take out each insert containing the epidermal tissue. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on the sterile filter paper or gauze, and place the tissues in the empty, sterile 24-well plate (Figure 1).

Note: the 24-well plate used for shipping may be kept/stored sealed at RT to look for signs of possible contamination at the end of the week.

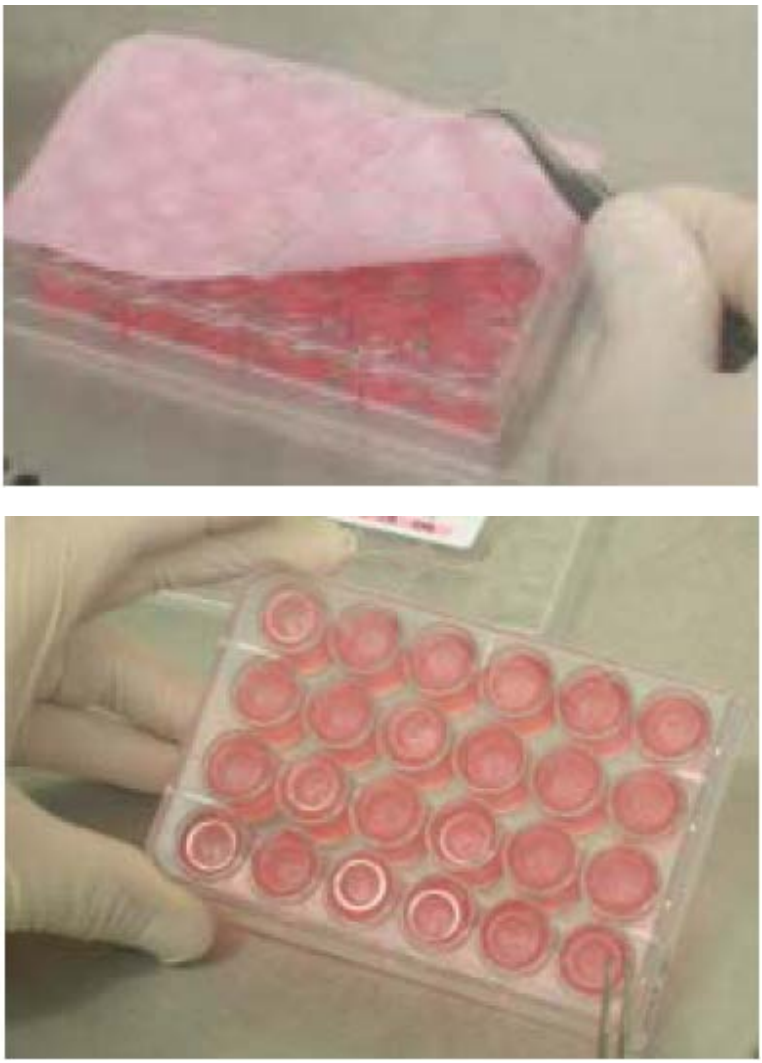


Figure 1. Visual inspection of the epidermal tissues.

(c) Perform visual inspection of the inserts within the next 5 minutes. Record any tissue defects and excess moisture on the surface. Do not use tissues with defects or tissues with excessive moisture on the surface.

(d) Dry the surface of the tissues with a sterile cotton tip swab and transfer inserts with the tissues to a 6-well plate pre-filled with 0.9 mL medium. Place the plates for 60 ± 5 minutes into the incubator (37±1°C, 5±1% CO_2, 95% RH).

(e) At the end of the first pre-incubation period, transfer the inserts from upper wells into the lower wells of the 6-well plate (Figure 2). Incubate the tissues (37±1°C, 5±1% CO_2, 95% RH) overnight for approximately 18-24 hour.

Note: Alternatively six tissues could be pre-incubated in one 6-well plate and the medium carefully refreshed after the pre-incubation of 60 minutes.

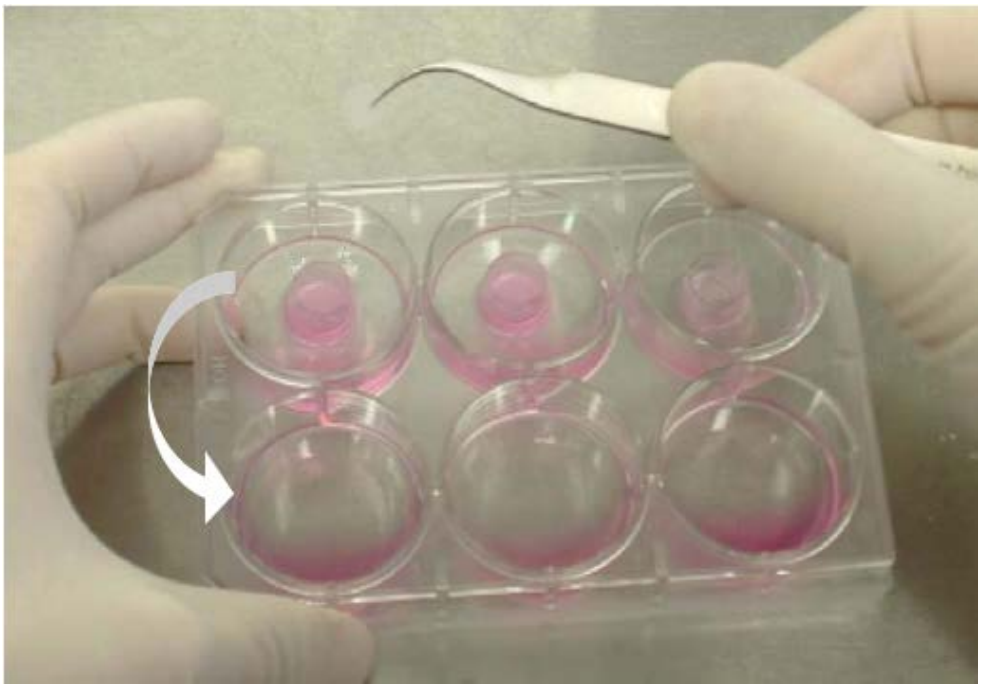


Figure 2. Pre-incubation plate design.

5.6. Application of the Test Extract and Rinsing (Day 1)

Safety precautions: Irritant materials are dangerous. It is thus recommended to Work in ventilated cabinets and wear protective gloves, a mask and safety glasses, as necessary.

5.6.1. Preparation

(a) Remove the 6 well plates from the incubator approximately 5 minutes before exposure to the extracts.

(c) Evaluate the surface of tissues and exclude completely wet tissues or tissues with any visible defects.

(d) Remove any moisture using sterile cotton tip.

(e) Before test extract exposure:
* label all 6-well plate lids with the test material codes.
* refresh medium below the inserts (add 0.9 mL medium) or transfer inserts to a new 6-well plate with fresh medium (0.9 mL)

Note: Deviations from this SOP may cause different outcomes.

5.6.2 Test extract and controls exposure

(a) Apply 100 µL of the undiluted medical device extracts, NC or PC to three single tissues each. Dose tissues at the time intervals needed later for rising off the test extracts (optimal and highly recommended is 1 minute interval).

(b) Keep plates with dosed tissues in the laminar flow hood, until the last tissue is dosed.

(c) After dosing the last tissue, transfer all plates to the humidified incubator (37±1°C, 5±1% CO_2, 95% RH) for the necessary exposure time (18 hours ± 30 minutes).

(d) After the exposures, rinse the tissues with sterile (D)PBS, filling and emptying the tissue insert 15 times to remove any residual test material (Figure 3). Use a constant stream of (D)PBS applied from 1.5 cm distance from the tissue surface. The stream of (D)PBS should not be too gentle otherwise the test article will not be removed. Optimal wash bottle, with pointed endings is shown in Figure 3.

(e) After the 15th rinse from washing bottle, completely submerge the insert 3 times in 150 mL (D)PBS (shake to remove all rests of test material).

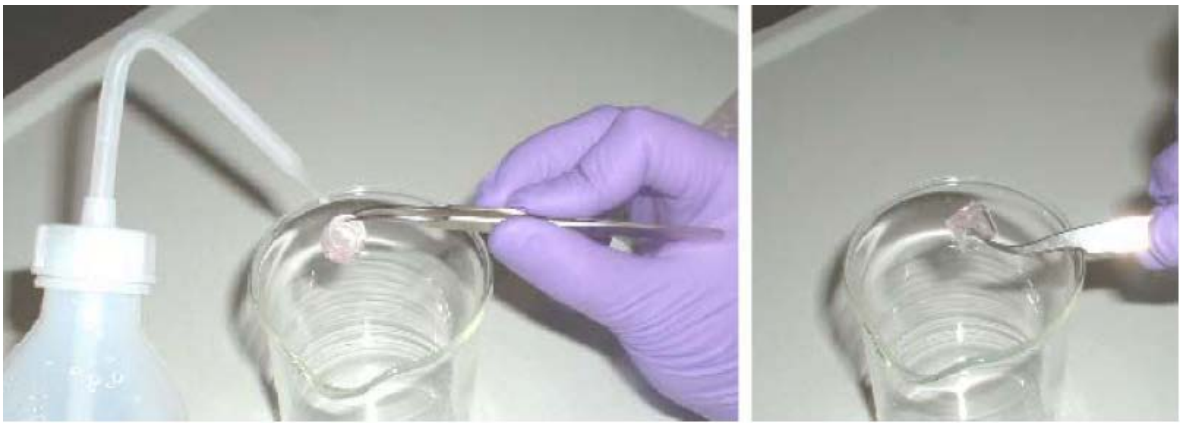


Figure 3. Removal of test articles and controls – washing procedure.

(f) Finally, rinse the tissue once from inside and once from outside with sterile (D)PBS. Remove excess of (D)PBS by gently shaking the insert, dry the insert by placing on sterile adsorbent paper (Figure 4).

(g) Transfer the blotted tissue inserts into a 24-well plate pre-filled with fresh assay medium (0.3 mL/well) until all tissues are rinsed. Proceed to MTT incubation.

(h) After all inserts are washed, DO NOT FORGET to carefully dry the surface of each tissue with a sterile cotton tipped swab (Figure 4). In case that traces of the extract are still present on the surface, try to remove it with a sterile wetted cotton swab. Record this procedure in the MDS. You may evaluate visually tissue surface under a dissecting stereoscope.

Figure 4. Completion of tissue washing – blotting and drying the tissue surface

5.7 Basal Media Collection for IL-1α Analysis

(a) Before the incubation procedures are finished, prepare the empty sterile 24-well plate.

(b) Label the 24-well plate with test material codes. Use a water resistant marker.

(c) After removing the insert for MTT measurement, collect 0.8 mL from the 6-well plates used for the exposures (see 5.6.2 (c)) into the pre-labeled 24-well plates.

Note: Before collecting the media, for IL-1α, place the plates on the plate shaker for 10 minutes (500 rpm/min). Alternatively, pipette the medium in the plates up and down 3 times. Change the tip between the different extracts. Avoid air bubbles or foam formation.

(d) Do not forget to write on the lids of the 24-well plate the tissue lot number, number of tissue replicate and date of the experiment(plates should be sealed with Parafilm). Samples can be stored at -20 ± 5°C until the analysis. Samples can be stored for 12 months.

5.8 MTT Test After the Exposure Period

Note: MTT assay is performed immediately after the 18 hour exposures are finished. There is no post-exposure time as in the EpiDerm SIT (OECD TG 439).

Note: The preparation of the MTT-solution and pre-filling of 24- well plate (0.3 mL/well) must be performed before starting with the washing procedure.

(a) Prior to the MTT assay, label a sufficient number of 24-well plates.

(b) Prepare MTT medium (section 5.2) and prepare 24-well plates with assay medium. Pipette 300 µL of MTT medium (concentration 1 mg/mL) in each well of

the required number of 24-well plates.

(c) Remove inserts from the temporary storage plates, dry the bottom of each insert by pressing it against sterile adsorbent paper, and transfer them into the 24-well plates, pre-filled with 0.3 mL of MTT (1 mg/mL). Place the plates in the incubator (37±1°C, 5±1% CO_2, 95% RH), record the start time of MTT incubation in the MDS and incubate for 3 hours ± 5 minutes.

(e) **Note:** The 3 hours ± 5 minutes MTT incubation time must be strictly adhered to. Deviations from the 3 hour time for MTT incubation will result in different MTT readings.

5.9 Formazan Extraction

(a) After MTT incubation is complete, blot any residual MTT media off of the tissue inserts and transfer the tissues to 24-well plates prefilled with 2 mL isopropanol (extractant solution MTT-100-EXT) per plate. Submerge the tissues fully.

(b) Seal the 24-well plates (e.g., with Parafilm or place into a sealable plastic bag) to inhibit extractant evaporation. Record start time of extraction in the MDS and extract formazan for at least 2 hours at room temperature with gentle shaking on a plate shaker (~ 120 rpm).

(c) As an alternative, overnight extraction (18-24 hours) 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 minutes on plate shaker. 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.

6. ABSORBANCE MEASUREMENTS

(a) Transfer per tissue 2 x 200 µL sample/well (= 2 wells per tissue) from each tube into a 96-well flat bottom microtiter plate (labeled appropriately). A single 96-well plate is to be used per time point.

Note: Be careful to avoid isopropanol evaporation in 96-well plates. Additionally, VC1 and VC2 in the plate maps below are always 0.9% saline and sesame oil vehicle controls.

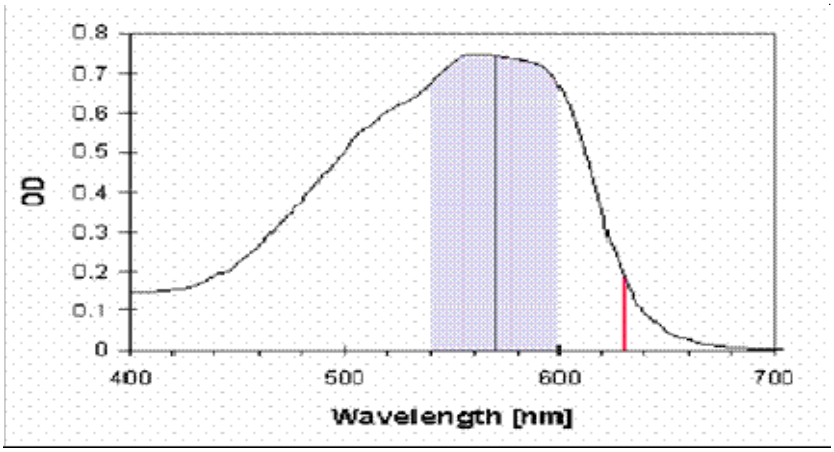
Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12	
	BL	BL	BL	BL	BL	BL							A
Tissue 1	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	B
	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	C
Tissue 2	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	D
	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	E
Tissue 3	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	F
	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	G
													H

BL Blank, Isopropanol
NC Negative Control, DPBS.
PC1. Positive Control 1, 1% SDS in saline.
PC2. Positive Control 2, 1% SDS in in sesame oil.
VC1. Vehicle Control 1, Saline.
VC2. Vehicle Controls 2, Sesame oil.
TA1-TA7 Test sample 1-7 in saline and/or sesame oil.

(b) Read the Optical Densities (**OD**) in a 96-well plate spectrophotometer using wavelength filter centered at **570 nm** pass-band ± 30nm and isopropanol solution as blank **without using a reference filter**.

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 have a tolerance their use can lead to reduction of the dynamics of the signal (OD).



(c) Record results on ***prepared templates***. Templates can be link to specific readers program. Thus slight differences in presentation can occur. A direct print of the readings should be made immediately and identified with date and signature and kept as raw data.

7. VIABILITY EXPRESSION AND ACCEPTABILITY CRITERIA

7.1 Data Report Forms and Calculations (per time point)

General Information:	
Study no.:	
Tissue-lot no.:	
Date:	
Operator:	

Negative control	NC
Positive control 1	PC1
Positive control 2	PC2
Vehicle control 1	VC1
Vehicle control 2	VC2
Test Article No. 1	TA1
Test Article No. 2	TA2
Test Article No. 3	TA3
Test Article No. 4	TA4
Test Article No. 5	TA5
Test Article No. 6	TA6
Test Article No. 7	TA7

FIXED DESIGN OF 96 WELL PLATE

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	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	Tissue1
C	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	
D	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	Tissue2
E	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	
F	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	Tissue3
G	NC	PC1	PC2	VC1	VC2	TA1	TA2	TA3	TA4	TA5	TA6	TA7	
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	

IMPORT:

PLATE 1

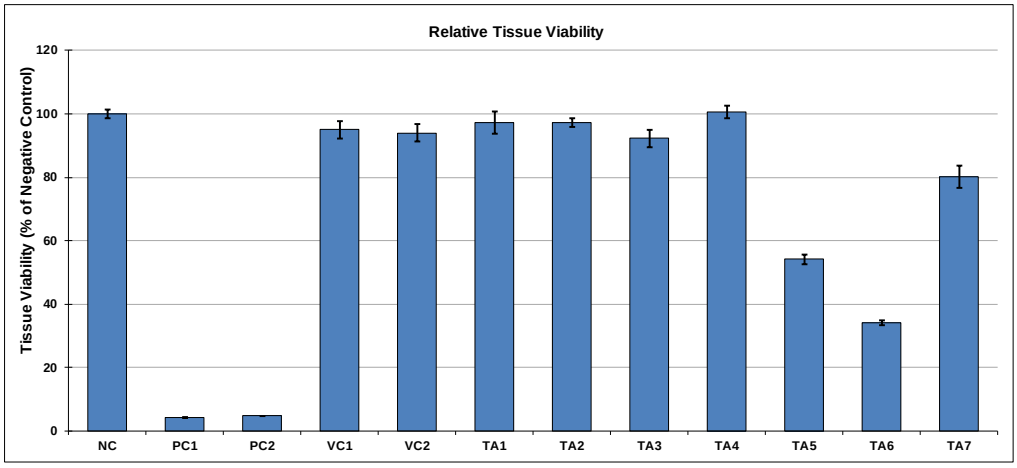
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.047	0.046	0.044	0.045	0.045	0.046	0.033	0.035	0.033	0.035	0.034	0.036	
B	2.136	0.132	0.150	2.062	2.000	2.001	2.114	1.915	2.280	1.224	0.789	1.896	Tissue1
C	2.195	0.134	0.147	2.108	2.000	2.088	2.152	1.976	2.191	1.219	0.788	1.755	
D	2.210	0.133	0.151	2.130	1.959	2.059	2.147	1.971	2.214	1.225	0.769	1.763	Tissue2
E	2.210	0.141	0.146	2.105	2.100	2.163	2.023	2.063	2.080	1.198	0.746	1.756	
F	2.163	0.139	0.152	2.018	2.103	2.180	2.144	2.063	2.182	1.156	0.756	1.659	Tissue3
G	2.136	0.135	0.144	1.990	2.121	2.203	2.133	2.067	2.178	1.166	0.778	1.689	
H	0.035	0.033	0.035	0.034	0.036	0.037	0.037	0.033	0.035	0.034	0.033	0.034	

REMARKS

RESULTS

exp. no.:	0
tissue-lot no	0
date:	0
operator:	0

Blank	0.047
	0.046
	0.044
	0.045
	0.045
	0.046
Mean	0.0455



Code N°	Tissue n	Raw data Aliq. 1	Raw data Aliq. 2	Blank corrected data Aliq. 1	Blank corrected data Aliq. 2	mean of aliquots	% viability
NC	1	2.136	2.195	2.091	2.150	2.120	99.6
	2	2.21	2.21	2.165	2.165	2.165	101.6
	3	2.163	2.136	2.118	2.091	2.104	98.8
PC1	1	0.132	0.134	0.087	0.089	0.088	4.1
	2	0.133	0.141	0.088	0.096	0.092	4.3
	3	0.139	0.135	0.094	0.090	0.092	4.3
PC2	1	0.15	0.147	0.105	0.102	0.103	4.8
	2	0.151	0.146	0.106	0.101	0.103	4.8
	3	0.152	0.144	0.107	0.099	0.103	4.8
VC1	1	2.062	2.108	2.017	2.063	2.040	95.8
	2	2.13	2.105	2.085	2.060	2.072	97.3
	3	2.018	1.99	1.973	1.945	1.959	92.0
VC2	1	2	2	1.955	1.955	1.955	91.8
	2	1.959	2.1	1.914	2.055	1.984	93.2
	3	2.103	2.121	2.058	2.076	2.067	97.0
TA1	1	2.001	2.088	1.956	2.043	1.999	93.9
	2	2.059	2.163	2.014	2.118	2.066	97.0
	3	2.18	2.203	2.135	2.158	2.146	100.8
TA2	1	2.114	2.152	2.069	2.107	2.088	98.0
	2	2.147	2.023	2.102	1.978	2.040	95.8
	3	2.144	2.133	2.099	2.088	2.093	98.3
TA3	1	1.915	1.976	1.870	1.931	1.900	89.2
	2	1.971	2.063	1.926	2.018	1.972	92.6
	3	2.063	2.067	2.018	2.022	2.020	94.8
TA4	1	2.28	2.191	2.235	2.146	2.190	102.8
	2	2.214	2.08	2.169	2.035	2.102	98.7
	3	2.182	2.178	2.137	2.133	2.135	100.2
TA5	1	1.224	1.219	1.179	1.174	1.176	55.2
	2	1.225	1.198	1.180	1.153	1.166	54.8
	3	1.156	1.166	1.111	1.121	1.116	52.4
TA6	1	0.789	0.788	0.744	0.743	0.743	34.9
	2	0.769	0.746	0.724	0.701	0.712	33.4
	3	0.756	0.778	0.711	0.733	0.722	33.9
TA7	1	1.896	1.755	1.851	1.710	1.780	83.6
	2	1.763	1.756	1.718	1.711	1.714	80.5
	3	1.659	1.689	1.614	1.644	1.629	76.5

	mean of OD	SD of OD	mean of viabilities [%	SD of viabilities	CV % [%]
NC	2.130	0.031	100.0	1.47	1.47
PC1	0.090	0.002	4.2	0.11	2.56
PC2	0.103	0.000	4.8	0.01	0.28
VC1	2.023	0.058	95.0	2.74	2.89
VC2	2.002	0.058	94.0	2.73	2.90
TA1	2.070	0.074	97.2	3.46	3.56
TA2	2.073	0.029	97.4	1.38	1.42
TA3	1.964	0.060	92.2	2.82	3.06
TA4	2.142	0.045	100.6	2.10	2.09
TA5	1.153	0.032	54.1	1.52	2.81
TA6	0.726	0.016	34.1	0.75	2.19
TA7	1.708	0.076	80.2	3.57	4.45

Classification

NC	NI	Qualified
PC1	I	Qualified
PC2	I	Qualified
VC1	NI	Qualified
VC2	NI	Qualified
TA1	NI	Qualified
TA2	NI	Qualified
TA3	NI	Qualified
TA4	NI	Qualified
TA5	NI	Qualified
TA6	I	Qualified
TA7	NI	Qualified

All data/calculations should be recorded on prepared ***Data Report Forms***. Excel prepared spread-sheets provided by the management team should be used.

All data generated by the microplate reader should be printed after each reading and considered as raw data, dated and signed.

Blank data and compound data (OD) are copied and pasted in the prepared Excel tables named: “IMPORT sheet” (see example above).

7.2. Data Calculation Steps

These calculation steps are applicable to the majority of test-substances characterized as follows: no interaction with the MTT reagent, non-colored, with a low ability to stain the tissues and measured non-specific color value ≤ 5% relative to negative control.

Main steps automatically followed by the prepared form:

(a) Blanks: calculate the OD mean from the 6 replicates for each plate.

(b) Negative DPBS-treated controls: Subtract blanks mean value from individual tissues OD. Corrected OD mean for the 3 tissues corresponds to 100% viability.

(c) Positive control (SDS 1%): Subtract blanks mean value from individual tissues OD. Calculate the OD mean for each individual tissue.

(d) Tested extract sample: Subtract blanks mean value from individual tissues OD. Calculate the OD mean for each individual tissue.

(e) The viability percent for each treated epidermis, are calculated relative to the mean of negative controls (see formula below).

(f) Standard deviations are calculated on OD and percent viabilities. (g) CV are calculated on percent viabilities.

1) The mean OD of the three Negative Controls (DPBS treated) corresponds to 100% reference viability.

2) For each individual treated tissue (TT) with a test substance, the positive control (PC) and the vehicle control (VC) the relative viabilities are calculated as follows:

OD Treated tissue = OD_{TTraw} – OD blank mean
OD Negative Control (OD_{NC}) = OD_{NCraw} – OD blank mean
OD Positive Control (OD_{PC}) = OD_{PCraw} – OD blank mean

Individual viabilities (%)

%Positive Control1 = [OD_{PC1} / mean OD_{NC}] x 100

%Positive Control2 = [OD_{PC2} / mean OD_{NC}] x 100

%Positive Control3 = [OD_{PC3} / mean OD_{NC}] x 100

%Treated tissue1 = [OD_{TT1} / mean OD_{NC}] x 100

%Treated tissue2 = [OD_{TT2} / mean OD_{NC}] x 100

%Treated tissue3 = [OD_{TT3} / mean OD_{NC}] x 100

Mean viabilities (%)

Mean Positive Control % = (%PC1 + %PC2+ %PC3) / 3

Mean treated tissue % = (%TT1 + %TT2 + %TT3) / 3

3) The mean relative viability is used for classification according to the prediction model (section 8).

7.3 Assay Acceptance Criteria for Negative and Positive Controls

Negative control (NC) acceptance: OD of the negative control (DPBS treated) reflects the viability of the tissues used in the test conditions (after shipping, storing in specific conditions). An absolute OD below the historical established lower boundary of the confidence interval indicates abnormal viability of tissues thus indicating possible difference in sensitivity to chemicals. These tissues should not be used for this application.

We can consider that the NC meets the acceptance, if the mean OD value of the 3 tissues is ≥ 0.8 and ≤ 2.8 and the Standard Deviation value (SD) of the percent viability is ≤ 20.

Positive control (PC) acceptance: OD of the positive control (1% SDS-treated) reflects the sensitivity of the tissues used in the test conditions (after shipping, storing in specific conditions). One positive control in each of the vehicle used should be included in each run (maximum one PC per day) taking into consideration the extraction medium (DPBS or sesame oil) used.

We can consider that the Positive Control meets the acceptance if the mean viability expressed as percent of the NC, is ≤ 50%.

Batch acceptance: all extraction data from one batch are accepted if both the negative and the positive control fulfill the above requirements.

Extraction data acceptance: The inter-batch viability mean is calculated from the three independent assays using intra-batch tissue mean. The 3 intra-batch means must predict the same class of irritation.

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). If 2 or 3 batches give SDs > 20 the assay is not repeated (variability linked to the extract itself).

8. DATA INTERPRETATION – PREDICTION MODEL

Irritant potential of test extracts is predicted by the mean tissue viability of tissues exposed to the test extract. Mean viability is calculated on three batches and three tissues per batch. The irritation potential is predicted if the mean relative viability is below 50% of the negative control.

Criteria for *in vitro* interpretation	Classification
Mean tissue viability is ≤ 50% at any time point	Irritant (I)
Mean tissue viability is > 50% after all exposure times	Non-Irritant (N I)

9. QUALITY ASSURANCE AND ARCHIVING

The study will be conducted as close as possible with the recommendations of good laboratory practice (GLP) or other quality control system (e.g., ISO)**.** All modifications of the study protocol must be noted as amendments by the study director. The reasons and practical impact of each modification will also be mentioned.

Archiving: Computer raw data and all MDS and any modifications or comments are reported and kept in laboratory registers with identification and signatures. Samples used for the study are stocked in the lab in ventilated conditions until the end of the whole study. Equipment calibration and maintenance data (identified and signed) are maintained and stored as specified in the internal procedures for each laboratory.

10. METHOD DOCUMENTATION SHEET (MDS)

MDS allows compliant Quality Control: correct set up, calibration, function of the equipment and quality of preparations. For each epidermis batch and experiment, make hardcopies of the necessary MDS, fill along the experiments the requested information date and sign in the tables of **Annex 1.**
Document the information on the test substances/medical devices in the table of **Annex 2**: Characterization of test substances.

11. TEST METHOD CHECK LIST

Skin Irritation Test for Medical Device Extracts

- **Prepare unknown device extracts in a polar (saline) and non-polar (sesame oil) solvent starting 48 hours prior to tissue arrival. Use for the extraction (37 ± 1) °C for (72 ± 2) hour with continuous agitation/shaking.**
- **Receipt: Transfer epidermis from transport plates medium to maintenance medium**
- **Incubate for 60 minutes (37°C, 5% CO_2, 95% RH in humidified incubator) then transfer epidermis into the fresh medium**
- **Incubate for 18 - 24 hours (37°C, 5% CO_2, 95% RH in humidified incubator).**
- **On the day of the experiment spike the positive control (SDS) into polar (saline) and non-polar (sesame oil) solvents at the concentrations specified.**
- **Apply negative control, positive control, vehicle controls and extracts from the tested materials in a 100 µL volume on the tissue surface.**
- **Incubate at 37°C, 5% CO_2, in a humidified incubator for 18 hours .**
- **Stop treatment by DPBS rinsing.**
- **Transfer tissues to MTT solution.**
- **Medium sampling for interleukin mediators release measurements.**
- **Incubate tissues for 3 hours in MTT (37°C, 5% CO_2, 95% RH in humidified atmosphere).**
- **Transfer and immerse in MTT-100-EXT (isopropanol).**
- **Formazan extraction: 2 hours (room temperature) or overnight (sealed, at room temperature).**
- **Shake and homogenize.**
- **Transfer extracted solution in 96-well plate.**
- **Read OD using a plate spectrophotometer**.

12. REFERENCES

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MatTek Corporation (2012) Protocol for IN VITRO EpiDerm™ SKIN IRRITATION TEST (EPI-200-SIT): For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT). MK-24-007-0023.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunological Methods* **65**, 55-62.

OECD (2015): OECD guideline for the testing of chemicals No 439: In Vitro Skin Irritation: Reconstructured Human Epidermis Test Method. Organisation for Economic Cooperation and Development, Paris, France.

ANNEX 1: METHOD DOCUMENTATION SHEET (MDS)

Assay Number:……

Date: ……

Corresponding XLS data file name: ……

PERFORMED BY: .....SIGNATURES:
.....

TIME PROTOCOL

Receipt of EpiDerm tissues (date, day, hour): ……ID:

Experimental schedule

Procedure	**Date (dd-mm-yy)**	**Set 1**		**Set 2**	
		start	stop	start	stop
Pre-incubation 1 (60 ± 5 min)					
Pre-incubation 2 (18 – 24 hrs)					
Exposure (18 hrs ± 30 min)					
Washing					
MTT test (3hrs ± 5 min)					
Extraction (minimum 2hrs)					
Measurement					

DEVICES VERIFICATION

Incubator verification

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

ID/ Date:

Refrigerator verification

Refrigerator #	Temperature < 5 ± 2°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 fields above:

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

ID/ Date:

Pipette verification (triplicate weightings)

Pipette 3 x H_2O into a small baker 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.

	0.9 mL	2 mL	300 µL	200 µL	100µL	remark
	.....H_2O weight in g.....					
1.						
2.						
3.						
Mean						
SD						

ID/ Date:

KIT COMPONENTS

EpiDerm (EPI-200) Lot no.:	Production date:
Assay medium (EPI-100-NMM) Lot no.:	Expiration date:
MTT concentrate (MTT-100-CON); 2 mL Lot no.:	Expiration date:
MTT diluent (MTT-100-DIL); 8 mL Lot no.:	Production date:
MTT extractant (MTT-100-EXT), 60 mL Lot no.:	Expiration date:
DPBS (TC-DPBS); 125 mL Lot no:	Expiration date:
Position of Ice-packs: (direct contact of the ice-packs 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

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

Specific observations:

SOLUTIONS AND VEHICLES

POSITIVE CONTROLS

SDS 1% (v/v) solution in saline and sesame oil, prepared from 20% solution of SDS :

On a day of experiment, prepare fresh solutions of 1% SDS in saline and sesame oil by mixing 100 µL of 20% SDS with 1.9 mL of vehicle. Vortex thoroughly after preparation and shortly before application to the tissue surface. Expiration is on the same day.

- 20% SDS stock solution reference, batch N°:……
- Saline (0.9% NaCl solution), batch N°:……
- Sesame oil, batch N°:……..
- Preparation date: ……..……..

Saline (0.9 % NaCl) solution preparation:

- NaCl reference, batch N°:……
- Type of sterilisation.....
- Preparation date: ……..……

- Expiration date : ……

(D)PBS solution preparation:

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

- Expiration date : ……

Sesame oil:

- Reference, batch N°:……
- Expiration date : ……

REMARKS TO SINGLE TISSUES

If you will during the assay observe any abnormality, have to substitute tissues, or will deal with any technical problems, fill in following table.

Record the tissue number, substance code and your observation or remark.

Substance code	**Tissue No.**	**Remark**

ID/ Date:

SPECTROPHOTOMETRICAL MEASUREMENT

PLATE CONFIGURATION FOR READING (for transfer to Spreadsheet EpiDerm SIT.xls) :

Record the positions of substances on 96-well plate.
Strictly adhere to the fixed plate design of the SOP given below

	1	2	3	4	5	6	7	8	9	10	11	12	
A	**BLANK**	**BLANK**	**BLANK**	**BLANK**	**BLANK**	**BLANK**							
B	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	Tissue1
C	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	
D	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	Tissue2
E	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	
F	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	Tissue3
G	**NC**	**PC1**	**PC2**	**VC1**	**VC2**	**TA1**	**TA2**	**TA3**	**TA4**	**TA5**	**TA6**	**TA7**	
H													

Note: switch on the reader 10 minutes before reading or as specified in the equipment manual.

Check plate photometer filter
Tick correct (✓) filter setting

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

ID/ Date:

ARCHIVATION

Raw data saved in/as:

Spreadsheet saved in/as:

MDS saved in/as:

ANNEX 2: CHARACTERISATION OF TEST SUBSTANCES

Laboratory:…… Study N°..... Assay N°.....

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:

ANNEX 3: ISO 10993-12 EXTRACTION PROCEDURE

ISO 10993-12:2012
NOTE: In the round robin study 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.

10 Preparation of extracts of samples

10.1 General

If extracts of the 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.

NOTE: For additional guidance on the extraction of samples, see Annex C (of ISO 10993-12:2012).

10.2 Containers for extraction

10.2.1 The extraction shall be performed in clean, chemically inert, closed containers with minimum dead space.

10.2.2 To ensure that the extraction vessels do not adulterate the extract of the test sample, the extraction vessels shall be:

a) borosilicate glass tubes with caps having an inert liner (e.g., polytetrafluoroethylene)

b) other inert extraction vessels, as required for specific materials and/or extraction procedures.

10.3 Extraction conditions and methods

10.3.1 Extraction conditions are based on common practice and are justified on the basis of providing a standardized approach that is, in many ways, an appropriate exaggeration of product use. Extraction shall be conducted under one of the following conditions (see also C.5):

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. For medical devices which are in short-term contact with intact skin or mucosa and which are not implanted, extraction times of less than 24 h, but not less than 4 h, are acceptable (see ISO 10993-5). Extraction temperatures greater than (37 ± 1) °C can adversely impact chemistry and/or stability of the serum and other constituents in the culture medium.

The extraction conditions described above, which have been used to provide a measure of the hazard potential for risk estimation of the device or material, are based on historical precedent. Other conditions that simulate the leachables occurring during clinical use, or that provide an adequate measure of the hazard potential, may be used but shall be described and justified.

Extraction is a complex process influenced by time, temperature, surface-area-to-volume ratio, the extraction vehicle and the phase equilibrium[1]) of the material. The effects of higher temperatures or other conditions on extraction kinetics and the identity of the extraction vehicle(s) should be considered carefully if accelerated or exaggerated extraction is used.

[1]The phase equilibrium of a material during extraction controls the relative amounts of amorphous and crystalline phases present. For the amorphous phase, the glass transition temperature, *Tg,* dictates the polymer chain mobility and the diffusion rate in the phase. Usually, at temperatures higher than *Tg,* the diffusion rate is considerably higher compared with that below *Tg.* The diffusion rate is lowest in the crystalline phase. The extraction conditions should not alter the phase equilibrium of the material. Phase alteration can affect the amount and type of extractables.

For example, two possibilities exist when elevated temperatures are used:

- the energy of the increased temperature may cause increased cross-linking and/or polymerization of the polymer and, therefore, decrease the amount of free monomer that is available to migrate from the polymer;
- the increased temperature could cause degradation products to form that are not typically found in the finished device under conditions of use.

10.3.2 For materials that dissolve or resorb under conditions of use, follow the extraction conditions described in 10.3.1. Perform extraction using the appropriate extraction vehicle and time/temperature conditions to simulate exaggerated exposure wherever possible. Complete dissolution may be appropriate.

10.3.3 The standard surface area can be used to determine the volume of extraction vehicle needed. This area includes the combined area of both sides of the sample and excludes indeterminate surface irregularities. When the surface area cannot be determined due to configuration of the sample, a mass/volume of extracting fluid shall be used. See Table 1.

Other surface-area-to-volume extraction ratios (e.g., those related to evaluation of porous materials, can be used if they simulate the conditions during clinical use or result in a measure of the hazard potential). Materials shall be cut into small pieces before extraction to enhance submersion in the extract media, except when otherwise inappropriate (see 10.3.4). For example, for polymers, pieces approximately 10 mm x 50 mm or 5 mm x

25 mm are appropriate.

Table 1 — Standard surface areas and extract liquid volumes

Thickness mm	Extraction ratio (surface area or mass/volume) ±10 %	Examples of forms of materials
<0,5	6 cm^2/ml	Film, sheet, tubing wall
0,5 to 1,0	3 cm^2/ml	Tubing wall, slab, small moulded items
>1,0	3 cm^2/ml	Larger moulded items
>1,0	1,25 cm^2/ml	Elastomeric closures
Irregularly shaped solid devices	0,2 g/ml	Powder, pellets, foam, non-absorbent moulded items
Irregularly shaped porous devices (low-density materials)	0,1 g/ml	Membranes, textiles
NOTE While there are no standardized methods available at present for testing absorbents and hydrocolloids, a suggested protocol is as follows: — determine the volume of extraction vehicle that each 0,1 g or 1,0 cm^2 of material absorbs; — then, in performing the material extraction, add this additional volume to each 0,1 g or 1,0 cm^2 in an extraction mixture.		

10.3.4 Elastomers, coated materials, composites, laminates, etc. shall be tested intact whenever possible because of potential differences in extraction characteristics between the intact and cut surfaces.

NOTE As a result of manufacturing processes, many elastomers might have surface properties that are different from those of the bulk material.

10.3.5 Extraction using both polar and non-polar extraction vehicles shall be performed. The following are examples of extraction vehicles:

a) polar extraction vehicle: water, physiological saline, culture media without serum;

b) non-polar extraction vehicle: freshly refined vegetable oil (e.g. cottonseed or sesame oil) of the quality defined in various pharmacopoeias;

c) additional extraction vehicles: ethanol/water, ethanol/saline, polyethylene glycol 400 (diluted to a physiological osmotic pressure), dimethyl-sulfoxide and culture media with serum.

NOTE 1 Other extraction vehicles appropriate to the nature and use of the device or to the methods for hazard identification may also be used if their effects on the material and the biological system are known (see Annex D).

NOTE 2 The use of a culture medium with serum is preferred for extraction in testing for cytotoxicity because of its ability to support cellular growth as well as extract both polar and non-polar substances.

10.3.6 Extractions shall be performed with agitation or circulation. When extraction under static conditions is considered to be appropriate, the method shall be justified, specified and reported.

10.3.7 Liquid extracts shall, if possible, be used immediately after preparation to prevent sorption on to the extraction container or other changes in composition. If an extract is stored for longer than 24 hours, then the stability and homogeneity of the extract under the storage conditions shall be verified.

10.3.8 Extract pH shall not be adjusted unless a rationale is provided.

10.3.9 The extract shall not routinely be processed by filtration, centrifugation or other methods to remove suspended particulates. However, if such processing is necessary, the rationale shall be documented.

10.3.10 For hazard identification of polymeric devices, exhaustive extraction conditions shall be considered. The extraction vehicle and conditions of extraction shall be selected on the basis of physicochemical properties of the material and/or predicted low-molecular-weight chemicals that might be extracted.

10.3.11 For materials or devices not expected to dissolve or resorb under conditions of use, any solvents used in the extraction of a polymeric material or device shall not cause dissolution of the polymer formulation. No more than a slight softening of the polymeric material shall occur in the presence of the volatile solvent (e.g. less than 10% dissolution). The solvent shall be removed (prior to use in a bioassay) to the extent that any residues do not adversely affect the biological assay (e.g. cause protein denaturation or skin irritation). For materials or devices expected to dissolve or resorb under conditions of use, see 1 0.3.12.

10.3.12 For solution and soluble materials, the standard extraction methods used for insoluble materials might be inappropriate. The following guidance should be considered in addition to information contained in Table 1.

a) Factors such as test system compatibility, route of administration and extent of dissolution or degradation should be considered in the final preparation for testing. Use an appropriate vehicle and conditions to simulate exaggerated exposure wherever possible. A pre-test can help to determine appropriate conditions.

b) If the material completely dissolves, in a vehicle or diluent that is compatible with the material and the test system, the resulting solution can be evaluated neat, provided the solution properties are also compatible with the test system, e.g. pH, osmolarity, solute concentrations.

c) If the material is an aqueous solution and used in this form, it shall be tested directly and not extracted, provided the solution properties are compatible with the test system [see also a) and b) above].

d) OECD Guidelines for the Testing of Chemicals, or similar chemical testing standards, can be used as guidance in determining maximum concentrations of test substances used for specific test methods.

10.3.13 Where fluids circulate through the device under normal conditions of use, e.g. extra-corporeal devices, extraction via re-circulation may be used. When possible, one or more of

the conditions shall be exaggerated (e.g., temperature, time, volume, flow rate. The rationale for the extraction chosen shall be reported).

10.4 Extraction conditions for hazard identification and risk estimation in the exaggerated use condition (points to consider in relation to Annex D)

10.4.1 Hazards that arise from changes in the manufacturing process or insufficient control of the manufacturing process shall be considered in the design and preparation of samples for test and in the preparation of extracts of those devices, in accordance with ISO 14971. Particular attention shall be given to residues, e.g. trace elements and cleaning and disinfection agents, of those manufacturing processes.

10.4.2 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.

10.4.3 In the case of products that polymerize in situ, the samples to be tested shall represent the intended clinical conditions of use in order to provide information on the potential toxicity of the reacting components in the polymer during the curing process. Test extracts prepared at different times, if appropriate, shall be based on the kinetics of polymerization after mixing the components, including an extract prepared at the expected cure time. Testing of the material after curing shall be justified.

Where extracts are used in the test methods for evaluation of materials that cure in situ, initiation of the extraction shall occur from the point in the cure at which the material is placed in situ.

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.

NOTE Modification of the clinical delivery system might be appropriate so that the designated size or weight of the material is delivered for testing.

11 Records

Documentation of the sample and its preparation shall include, but not be limited to:

a) type and, if known, composition of material, source of material, device, device portion or component; NOTE A written description, drawing, photograph or other methods can achieve all or part of this requirement.

b) lot or batch number, where appropriate;

c) description of processing, cleaning or sterilization treatments, if appropriate;

d) extraction techniques, as appropriate, including documentation of extraction vehicle, extraction ratios, conditions for extraction, means of agitation, as well as any deviations from the conditions specified in this part of ISO 10993, such as filtration of the extract or extraction

media.