Protocol

epiCS®

Skin Sensitisation and Potency Test

(SSPT)

Training Kit

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A. Protocol Introduction

This kit is designed to carry out a first run of the epiCS Skin Sensitisation and Potency Test method (**SSPT** - method). The protocol is based on a protocol which was originally developed by Dr Sue Gibbs (NL) and Dr Emanuela Corsini (Italy) and CellSystems GmbH.

For the full SOP of the SSPT method, please consult our homepage: www.SkinInVitro.com.

epiCS® SSPT method

The epiCS SSPT method is used to identify contact allergens and to rank them according to their potency.

ABBREVIATIONS & DEFINITIONS

MTT: 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide

NC: negative control

OD: optical density

PC: positive control

ref.: reference

RH: relative humidity

RT: room temperature

SD: standard deviation

B. Technical Description

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:

a. Wear gloves during handling of kit components

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

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

Please, contact CellSystems if you have any questions or concerns.

MATERIALS AND PREPARATIONS

Standard epiCS SSPT Kit Components

18 epiCS (tissues)	*18 inserts with epidermis tissues (origin human keratinocytes) on agarose in sealed 24-well plate.*
MBL IL-18 ELISA kit, 96 wells	*MBL ELISA kit #7620 (MBL, Japan)*
6-well plates, 5x	*For assay / culture*
24-well plate, 1x	*For MTT-Assay*
96-well plate, 1x	*For OD of MTT*
AOO: Acetone / Olive Oil (4+1), 2x 4 ml	*Solvent / solvent*
Salicylic Acid 2 ml (200 mg/ml in AOO)	*Skin irritant (non-sensitiser)*
DNCB solution 2 ml (10 mg/ml in AOO)	*Skin sensitiser*
epiCS Culture Medium, 50 ml	*For tissue culture*
MSDS	
MTT (25 mg)	*MTT reagent*
Paper Filter (sterile); 25 pieces	*For substance exposure*
PBS Ca/Mg-free, 25 ml	*For diluting MTT reagent prior to use in the MTT assay*

Expiration and Kit Storage

store liquids as indicated on the product labels

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

Additional 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*
96-well plate spectrophotometer	*For reading OD*
Plate shaker	*For extraction of formazan*

Timers	*To be used during application of test materials*
Sterile, forceps or tweezers	*For handling tissue inserts*
37±1°C water bath	*For warming Media and MTT solution*
Adjustable pipette	*For pipetting MTT isopropanol*

Consumables:

Sterile paper towel	For blotting of cell culture inserts
Adhesive tape or Parafilm	Covering plates during formazan extraction
Isopropanol	*For extraction of the formazan crystals*

PREPARATIONS

Media and Endpoint Assay Solutions:

MTT solution (prepare freshly on day of testing)

Add 25 ml of PBS to the MTT reagent into the MTT containing flask (this gives a 1 mg/ml solution) thoroughly mix this working solution. Keep the MTT working solution in the dark and warm to 37°C prior to usage (use within 2 hrs).

Safety precaution: MTT is toxic (Risk phrases: R26, R68, R22, R36, R37, R38).

Wear protective gloves during manipulation with MTT solution.

ELISA kit

Please follow the instructions of the MBL ELISA kit #7620 (MBL, Japan) with the following exception:

The analysis of the collected culture medium for the IL-18 content should be done **without** dilution (1 insert/well in a six well plate, 1 ml medium for 24h). Only, if OD values are out of the photometer's linearity range dilute the samples before using the ELISA

Test substance:

Follow safety instructions for respective substances.

Solvent

AOO (4+1) is the solvent used in this kit. It is prepared by combining 4 parts acetone with 1 part of olive oil.

It is used to dilute the skin sensitiser and the skin irritant (non-sensitiser) in this kit.

Skin Sensitiser

Dinitrochlorbenzol (DNCB) is the skin sensitiser used in this kit. It needs to be dissolved in AOO (s. Annex 1).

Skin Irritant

Salicylic Acid is the irritant that is a non-sensitiser. It needs to be dissolved in AOO (s. Annex 1)

Negative Controls (NC)

1. Unexposed epiCS - no paper filter and no solvent is applied to epiCS.

2. Solvent treated epiCS - paper filter + AOO.

The calculation of x-fold IL-18 release is done in relation to the solvent control (AOO).

METHOD

ROUTINE CULTURE PROCEDURE:

Day prior to testing:

Place the epiCS Culture Medium into the refrigerator (2-8°C).

Tissue conditioning:

1. *Do not pre-warm epiCS Culture Medium!*
2. Pipette 1 ml of the cool epiCS Culture Medium into the wells of sterile 6- well plates. Start to use the plate design as described in Annex 2.
3. Remove the shipped multi well plate from the package. Under sterile condition 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.
4. Avoid air bubble formation underneath the tissue culture inserts.

5. 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.
6. Incubate the plates at least for 4 hrs or overnight 37±1°C, 5±1% CO_2, 90±10% RH).
7. Store remaining epiCS Medium at 2-8°C.
8. Prepare and sterilise all devices which will be used in the assay.

TEST MATERIAL EXPOSURE PROCEDURES:

Dissolving substances

The sensitising and the non-sensitising substances are diluted with the solvent AOO as described in Annex 1.

Preparation of substance test concentrations

It is known that AOO dissolves some plastic lab devices. It is therefore recommended to use polypropylene tubes to prepare the substance solutions dissolved in AOO and to impregnate the paper filters for the substance exposure on devices composed of glass (e.g. glass slides or tissue culture dishes made of glass).

Take into account that acetone is a volatile liquid. Therefore, open and close tubes with AOO (+/- substance) rather quickly to avoid too much evaporation of AOO.

Do not use small volumes < 300 µl. If you do so this will increase error margins.

SUBSTANCE EXPOSURE

Only unexposed epiCS and solvent treated epiCS are tested in duplicates.

epiCS exposed to the test substances are tested in single tissues.

Preparation of testing substance concentrations

1. Substances must be handled with care, as they may be harmful.
2. Substances must be freshly prepared before epiCS exposure (to avoid oxidation of the substances, it is recommended to immediately close the tubes firmly and wrap Parafilm around the tubes).
3. The use of gloves, lab-coat and mask is recommended.

Handling the solvent AOO

It is known that AOO dissolves some plastic lab devices. It is therefore recommended to use polypropylene tubes for substance dilutions in AOO. Impregnate the paper filter disks for the substance exposure on glass devices (e.g. glass slides, tissue culture dishes made of glass).

1. Remove old medium from the wells with the inserts and replace it with 1 ml of pre-warmed epiCS Culture Medium (37°C).
2. Place sterile filter paper disks (8 mm) in a 100 mm tissue culture dish using sterile tweezers (one filter paper disk per epiCS culture). Label the upper side of each filter by marking the filter with a sharp point of a pencil.
3. Impregnate filter paper discs with 25 µl of the testing samples (substance dilutions and solvents (take along singles or duplicates for each testing condition)).
4. Hold the filter paper disk with tweezers and gently tap them at least 8 times vertically to the plate until the excess of solution slides out from the filter. Be sure to close tissue culture dish immediately to prevent evaporation of solvent in the flow.
5. After impregnating filters, take the epiCS cultures out of the incubator.
6. Quickly (to avoid evaporation of solvent) apply impregnated filters topically to the epiCS surface according to the plate layout described in Annex 2. Place the filter with the pencil labeled side upwards on the cultures.
7. Incubate exposed cultures for 24 hours (37°C, 5% CO_2, 95% humidity).
8. After 24 hours incubation, take exposed cultures out of the incubator and gently remove the filter paper disks with tweezers.
9. Perform MTT assay on epiCS to assess cell viability after substance exposure.
10. Transfer the culture medium to 1.5 ml tubes for IL-18 assessment. Store at -20°C or for longer storage at -80°C until ELISA testing.

ENDPOINT MEASUREMENT(S):

MTT assay is used to determine the MTT EC_{50} value (EC_{50} = effective substance concentration required to reduce cell viability to 50% of the maximum value compared to solvent exposed cultures). IL-18 release is used to characterize the allergenic potential of the tested substance and to give extra potency data.

MTT assay:

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes. This results in its accumulation within viable cells. Cells will be lysed by the addition of isopropanol resulting in the liberation of the crystals, which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a scanning spectrophotometer. As MTT is a colorimetric reaction, a scanning spectrophotometer is necessary to quantify the generated colour correlated to cell viability. For incubation steps, an incubator 37 °C, 5% CO_2,

95% humidity is needed. The MTT assay therefore measures changes in metabolic activity of the cultures, which in turn correlates to changes in viability of the cultures

To perform the MTT assay, follow the steps below:

1. The MTT analysis is performed in a 24-well plate.
2. Use the 1 mg/ml MTT solution as described above.
3. Pipette 300 µl per well of the MTT solution in each well of a 24 well plate.
4. Remove carefully the paper filter discs from epiCS test cultures with tweezers and discard them.
5. Pick up the epiCS test cultures with tweezers and tap the cultures gently on a paper to dry the bottom of the culture. Place the cultures into the 24-well plate previously filled with the MTT solution.
6. Once samples are in contact with MTT solution, incubate for 3 hours in the dark at 37 °C, 5% CO_2, 95% humidity. Make sure that no bubbles are present between the bottom of the cultures and the MTT solution in the well. If so, tap the plate gently to remove the bubbles present underneath the cultures.
7. It is strongly recommended to take photographs of the 24-wells plate after the incubation of the cultures with the MTT solution, before the incubation with the isopropanol, in order to document the results visually, in case any abnormalities may arise from this point on.
8. After the 3 hours incubation, pipette 2 ml per well of isopropanol in a new 24-well plate.
9. Pick up the epiCS cultures from the plates with MTT solution. Tap the cultures gently on a paper until the bottom of the culture is dry. Place the cultures into the 24-well plate previously filled with 2 ml isopropanol. Make sure that no bubbles are present between the bottom of the cultures and the isopropanol in the well. If so, tap the plate gently to remove the bubbles present underneath the cultures. Cover the plate with Parafilm to avoid isopropanol evaporation.
10. Incubate the plates with cultures in isopropanol overnight, covered with Parafilm and protected from light or with gently shaking on a plate shaker (~ 100 rpm) for 2 hours at room temperature.
11. After the extraction period is complete, pierce the inserts with an injection needle and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Before transferring the extract to 96-well plates pipette up and down 3 x until solution is homogenous.
12. Per each tissue transfer 2 × 200 µl aliquots of the blue formazan extracting solution into a 96-well flat bottom microtiter plate. Use isopropanol as blank.
13. Read plates using a spectrophotometer at 540-570 nm wavelength without using a reference filter. Save the plate until step 15 is completed.
14. Calculate cell viability:

    Absorbance of the wells from the solvent exposed cultures is taken as 100% cell viability. The higher the toxicity of a particular condition, the lower the absorbance value will be. Controls, which are viable have a purple coloured supernatant. The more toxic the condition, the more yellowish the supernatant is and the lower the absorbance value is.

e.g.: Absorbance from control = 1.432

Absorbance from a testing condition = 0.258

1.432 --- 100% viability

0.258 --- x% viability → cell viability at this specific condition = 18.02%

15. If the absorbance of any of the samples is above the linear range of your spectrophotometer samples need to be further diluted and measured again. Proceed as follows: for all samples within a given experiment transfer 100 µl (1:2 dilution) to a new plate and add 100 µl isopropanol. Repeat step 13 and 14 above.

DATA ANALYSIS

MTT:

An estimation of the EC_{50} values can be calculated as follows:

EC50	
conc.	**viability**
CV<50 **(A)**	CV<50 **(B)**
CV>50 **(C)**	CV>50 **(D)**
EC50 value	50
slope	#WAARDE!
y intercept	#WAARDE!

Explanantion	
CV<50 **(A)**	fill in the chemical conc. (in mM, mg/mL or %) with a rel. viability <50%
CV<50 **(B)**	fill in the rel. cell viability (in %) for the chemical conc. with a rel. cell viability <50%
CV>50 **(C)**	fill in the chemical conc. (in mM, mg/mL or %) with a rel. viability >50%
CV>50 **(D)**	fill in the rel. cell viability (in %) for the chemical conc. with a rel. cell viability >50%
50	this is the 50% relative cell viability
EC50 value	this it the EC50 value (in mM, mg/mL or %) that induces a rel. cell viability of 50%; the following formula is used to calculate the EC50 value: **= (50 - y intercept) / slope**
slope	**= (B - D) / (A - C)**
y intercept	**= B - (slope * A)**

Chemical X	
mg/mL	**viability**
50,00	28,90
25,00	84,80
40,56	50
slope	-2,24
y intercept	140,70

EC50 value	40,56 mg/mL

Determining the IL-18 release by ELISA

IL-18 release is assessed by ELISA. Standards are run in duplicates, samples (0.1 ml) in singles.

Results are expressed in pg/ml.

Please follow the instructions of the MBL ELISA kit.

IL-18 Data Analysis

For assessment of the sensitising potential (YES/ NO): The relative amount of IL-18 protein present in supernatants given in pg/ml. The result is expressed as fold change, calculated by the following equation:

$$\text{IL-18 SI} = \frac{\text{IL-18 pg/ml in substance-treated epiCS}}{\text{IL-18 pg/ml in solvent-treated epiCS}}$$

If the fold increase in intracellular IL-18 is ≥ 5.0 when cell viability ≥ 5% and ≤ 40% (EC_{5-40}) in at least one of the concentrations tested the substance is classified as contact sensitiser (R43).

If the fold increase in intracellular IL-18 is < 5.0 when cell viability ≥ 5% and ≤ 40% (EC_{5-40}) in all concentrations tested the substance is classified as non contact sensitiser.

PREDICTION MODEL

A substance is classified as contact allergen if it induces ≥ 5 fold increase in IL-18 release (ELISA) compared to solvent treated epiCS at cell viability ≥ 5% and ≤ 40% (EC_{5-40}) in at least one of the concentrations tested. For sensitising potency, the EC_{50} value and IL-18 (SI-2) value (secondary parameter) is determined.

Read out:

For sensitising potential: ≥ 5 fold increase in IL-18 release (ELISA) compared to solvant treated epiCS at cell viability ≥ 5% and ≤ 40% (EC_{5-40}).

For potency assessment:

Primary parameter: Cytotoxicity (MTT assay) expressed as EC_{50} value (EC_{50} = effective substance concentration required to reduce epiCS metabolic activity - corresponding to cell viability - to 50% of the maximum value compared to solvent exposed cultures).

ANNEXES

Annex 1

Dissolving substances in the solvent acetone:olive oil 4+1 (AOO)

→ In the dose response 7 x 2-fold serial dilutions (1x, 2x, 4x, 8x, 16x etc) are tested for both substances.

Steps

1. Dilutions are made in AOO as indicated in the table below starting with the Master Stock concentration
2. Impregnate the filter paper discs with the substance dilutions and expose and analyse the cultures according to the protocol described.

Based upon the results from the dose response experiments an EC_{50} value for each substance might be determined.

Dilution to be tested	µl of previous dilution	µl of AOO (4+1)	DNCB [mg/ml]	Salicylic Acid [mg/ml]
1x dilution	Master Stock concentration in 100% AOO	--	10	200
2x dilution	300 uL (1x dilution)	300 uL	5	100
4x dilution	300 uL (2x dilution)	300 uL	2.5	50
8x dilution	300 uL (4x dilution)	300 uL	1,25	25
16x dilution	300 uL (8x dilution)	300 uL	0.63	12.5
32x dilution	300 uL (16x dilution)	300 uL	0.31	6.25
64x dilution	300 uL (32x dilution)	300 uL	0.16	3.13

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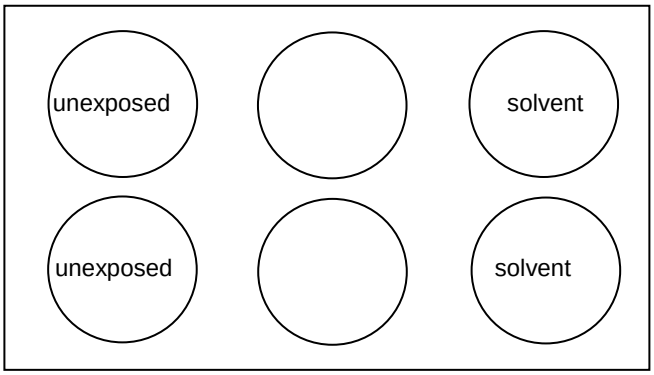
Annex 2

Plate Design

Control plate:



Test chemical dilution plates: