Standard Operating Procedure (Version 1.4)

epiCS® Skin Sensitisation and Potency Test (epiCS® SSPT)

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

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

SkinInVitro was formerly known as "CellSystems Biotechnologie Vertrieb GmbH"

Henkel AG & Co. KGaA
Henkelstr. 67 . 40589 Düsseldorf . Germany
www.phenion.com . info@phenion.com

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A Protocol Introduction

1. ABSTRACT

The content of the protocol is based on a SOP kindly provided by Prof. Dr. Sue Gibbs (The Netherlands) and Prof. Dr. Emanuela Corsini (Italy) "Identification and ranking potency of a contact sensitizer using the *in vitro* skin equivalent epiCS". The protocol is established and approved by SkinInVitro GmbH (formerly known as CellSystems Biotechnologie Vertrieb GmbH).

epiCS® Skin Sensitization and Potency Test (SSPT)

The epiCS® SSPT method is used to identify contact allergens and to rank them according to their potency (extreme, strong, moderate and weak sensitizing potency) with the aid of the human skin equivalent epiCS (SkinInVitro GmbH) and the release of interleukin-18 (IL-18) into the culture medium. The test system is available as a kit, comprising reconstructed epidermis, culture media and culture plates. The IL-18 ELISA kit is not included.
Please, contact the SkinInVitro GmbH experts before you start this test, to help you establish it at your site.

1.1 Changes to the SOP:

Since the publication of the former version of this SOP (Version 7.2) new developments made this new version (Version 7.3) necessary.

The most important changes are:

a) CellSystems Biotechnologie Vertrieb GmbH changed name in „SkinInvitro GmbH“as of April 1, 2019

2. OBJECTIVES & APPLICATIONS

2.1 Type of Testing:

Replacement, as standalone test or within an Integrated Testing Strategy

2.2 Level of Toxicity Assessment:

Hazard identification, toxic potential, contact allergen identification and potency determination

2.3 Purpose of Testing:

Classification and labelling, safety. Determine the allergenic potential and potency (strength) of low molecular weight substances.

2.4 Context of Use:

Currently no validated *in vitro* method exists to replace animal testing for the identification of skin sensitizing substances. Several cell-based methods are under development and might be required to be used in a test battery to fully replace animal testing. To date, the mouse LLNA is the preferred and accepted method for assessing skin sensitization of most substances.

2.5 Applicability Domain:

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

3. BASIS OF THE METHOD

Skin sensitisation is an immune mediated reaction to low molecular weight substances, involving several cell types which actively participate in contact allergy within the human skin (Cavani 2007). Keratinocytes, the major cell type in human epidermis, play a role in all phases of allergic contact dermatitis, from the early initiation phase with penetration through the stratum corneum and the secretion of inflammatory cytokines which are required for Langerhans' cells migration and T-cell trafficking, leading to the development of allergic contact dermatitis. In vivo, the local lymph node assay (LLNA) is used to study the induction phase of skin sensitisation and provides quantitative data suitable for dose-response assessment (OECD TG 429, 2010).

This protocol of in vitro skin sensitisation test using epiCS may also enable to identify contact allergens and to rank them according to their sensitising potency (extreme, strong, moderate and weak). The advantage of the assay is the possibility to identify in the same test the allergenic potential of a substance and its potency. While topically applied, it mimics human exposure and overcomes all drawbacks of traditional submerged culture, including substance solubility and stability in culture medium.

The test consists of a topical exposure of test substances to a human reconstituted epidermis model followed by a cell viability test to determine the EC_{50} value. Substances can be diluted in the solvent that shows best solubility. Neat mixtures and finished products might be tested for their allergenic potential without dilution prior to experimentation.

Cell viability is measured by dehydrogenase conversion of MTT *[(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyl tetrazolium bromide*], present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues. The reduction of viability of tissues exposed to substances in comparison to negative controls (treated with solvent) is used to determine to calculate EC_{50}.

Interleukin-18 (IL-18) is constitutively secreted by keratinocytes and may be induced by test substances to different degrees. The IL-18 production level induced by a test substance is one parameter of the prediction model for this assay.

4. EXPERIMENTAL DESCRIPTION

4.1 Endpoint & Endpoint Detection:

Cytotoxicity is measured by MTT assay and the release of IL-18 into the culture medium by ELISA.
MTT Assay: Cell viability is used as endpoint and measured by dehydrogenase conversion of MTT [*(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide*], present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues. The reduction of viability of tissues exposed to substances in comparison to negative controls (treated with solvent) is used to calculate EC_{50}.
IL-18 ELISA: A commercially available IL-18 ELISA kit (#7620, MBL International Corporation, Japan) can be used to determine the secretion of the cytokine into the culture medium within 24 hours.

4.2 Endpoint Value:

Results from the dose response experiments are expressed as fold increase in IL-18 release in order to determine the allergenic potential. A substance is classified as contact allergen if it induces ≥ 5-fold increase in IL-18 release (ELISA) compared to solvent treated control at cell viability ≥ 5 % and ≤ 40 % (EC_{5-40}) in at least one of the concentrations tested.
For sensitiser potency, the EC_{50} value and IL-18 (SI-2) value is determined. The EC_{50} value and IL-18 (SI2) of the unknown substance is correlated to the values obtained for a standard test panel of substances (dos Santos et al., 2011).

4.3 Test System:

epiCS [SkinInVitro GmbH, Troisdorf, Germany (formerly known as CellSystems Biotechnologie Vertrieb 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 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, and 6-well plates.

4.4 Basic Procedure:

On day of receipt, epiCS are conditioned by incubation for release of transport-stress related compounds and debris at least 4 h or overnight. After pre-incubation, tissues are supplied with new pre-warmed culture medium. Tissues are topically exposed to the test substances impregnated in 8 mm diameter paper filters (designed for the use with 8 mm Finn Chambers). The paper filters were loaded with 100 µl for water soluble compounds or 25 µl for ethanol or AOO soluble compounds on a glass plate under sterile conditions, excess liquid removed, and applied on to the tissues surface for 24 h. Hereafter, cytotoxicity is measured by MTT assay and the release of IL-18 into culture medium

by ELISA. Results from the dose response experiments are expressed as fold increase in IL-18 release in order to determine the allergenic potential.
MTT assay: Transfer the tissues to 24 well plates containing 300 µl MTT medium (MTT 1 mg/ml). After a 3 h MTT incubation the blue formazan salt formed by cellular mitochondria is extracted for 2 h (RT) or overnight sealed (2-8°C) with 2 ml isopropanol per tissue and the optical density of the extracted formazan is determined in a spectrophotometer at 540-570 nm. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues.
Correction for MTT reducers: Please follow the test for interference of substances with MTT as described in the epiCS Skin Irritation INVITTOX Protocol (see Annex 1).
IL-18 ELISA: Please follow the instructions of the IL-18 ELISA kit (#7620, MBL International Corporation, Japan).

5. DATA ANALYSIS / PREDICTION MODEL

The test protocol allows predicting the skin sensitisation potential of unknown test substances according to the following prediction model:
Allergenic potential:
≥ 5-fold increase in IL-18 release (ELISA) compared to solvent treated epiCS at cell viability ≥ 5 % and ≤ 40 % (EC_{5-40}).
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 tissues).
Secondary parameter: IL-18 Substance concentration resulting in: ≥ 2-fold increase in IL-18 release (ELISA) compared to solvent treated tissues (SI-2).

6. TEST COMPOUNDS & RESULTS SUMMARY

The assay is performed according to the SOP in order to determine the EC_{50} value of a substance of (un)known identity, solubility and molecular mass, and the release of IL-18 associated with its treatment. First the maximum solubility of the substance is identified by dissolving the compound in both AOO and in 1% DMSO in epiCS Culture Medium. Dilutions are made in the following order: 200 mg/ml, 150 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.20 mg/ml, 0.10 mg/ml until a clear solution is reached. The solvent with the highest dissolving capacity (AOO (4+1) or 1% DMSO) is chosen. Substances are tested in a dose response starting with the highest soluble concentration (preferably max. 200 mg/ml) and decreasing with 2-fold serial dilutions until 0.1 mg/ml (or the closest lower concentration) is reached. Whereas substance dose responses are tested in single fold in each independent experiment, control conditions (unexposed, solvent(s) and positive control), should preferably be tested in duplicate per independent experiment.

During the development of this assay 11 coded sensitisers and 9 coded non-sensitisers (11 *in vivo* contact allergens and 9 *in vivo* irritant substances) were tested and the results published in Gibbs et al (2013). Five sensitisers* and 5 non-sensitisers** of this panel were taken from the reference substances list mentioned in the OECD TG 429 "Skin Sensitisation: Local Lymph Node Assay". A ring trial in three different laboratories is performed with a small subset of substances to evaluate the prediction model (see above) followed by an independent statistical analysis in 2014.

* Sensitisers from the reference substances list (OECD TG 429):
DNCB
Isoeugenol
2-Mercaptobenzothiazole
Eugenol
Citral

** Non-sensitisers from the reference substances list (OECD TG 429):
Sodium Lauryl Sulfate
Lactic Acid
Methyl Salicylate
Salicylic Acid
Chlorobenzene

6.1 Modifications of the Method:

None

7. DISCUSSION

The epiCS Sensitisation Test is an easy to perform *in vitro* test which only needs minimal training. Besides a standard cell culture and substances laboratory no special equipment is required.
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 may serve as a complete replacement of the *in vivo* LLNA test in mice. In view of stimulated discussions on Integrated Testing Strategies to replace the LLNA assay the epiCS Skin Sensitisation Test might support those testing strategies and thereby complement the assay portfolio to reach this goal.
Limitations: The solvents that have been successfully tested on epiCS are water, PBS, acetone: olive oil (AOO) (4 parts acetones: 1-part olive oil), absolute ethanol and DMSO (maximum 1%). Water insoluble substances can be tested although care should be taken that solvents are not used at irritant concentrations. The substance needs to be in solution in order to be tested.

8. STATUS

8.1 In Development:

The epiCS SSPT method was published by Gibbs et al. (2013).

8.2 Known Laboratory Use:

epiCS is used in industry and academia for research, efficacy testing and regulatory toxicology testing [epiCS skin irritation testing (TG 439,2019 and DBALM 212_P_epiCS Skin Irritation Test) skin corrosivity testing (TG 431 and DBALM 213_P_epiCS Skin Corrosion Test).

8.3 Participation in Validation Studies:

This protocol was refined by CellSystems Biotechnologie Vertrieb GmbH (now known as SkinInVitro GmbH) and might be part of a blind trial multicentre validation study. Modifications of the protocol are possible.

8.4 Regulatory Acceptance:

To date there is no regulatory accepted *in vitro* skin sensitisation test method available.

9. PROPRIETARY ISSUES

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

10. 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
UN-GHS:	United Nations Globally Harmonized System.
DNCB:	Dinitrochlorbenzol

A Procedure Details, Latest Version: October 2019

epiCS® Skin Sensitisation and Potency Test SSPT

Contact Person
M.Sc. Bernadette Thiede
SkinInVitro GmbH
Langeler Ring 5
53842 Troisdorf
Germany

bernadette.thiede@skininvitro.com
telephone: +49 (0) 2241-25515-0
Fax: +49 (0) 2241-25515-30

1. Health and Safety Issues

1.1 General Precautions:

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

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

Wear gloves during handling with the skin and kit components

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

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

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

1.2 MSDS Information:

- Non-coded test chemicals should be handled following chemical safety datasheet.
- DNCB (P: 261-273-280-301+310-302+350-305+351+338 H: 301+331-310-315-317-318-373-410)

  Acetone (P: 210-240-305+351+338-403+233 H: 225-319-336)

  Ethanol (P-11 H: (2-)7-16)

Isopropanol (P11, P36, P67 –H225, H319, H336)

MTT (P68, P36/38 –H315, H319, H335, H341)

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

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

2. Materials and preparations

2.1 Test system:

2.2 Standard epiCS Kit Components:

Sealed 24-well plate (epiCS)	*Contains up to 24 inserts with epidermis tissues (origin human keratinocytes) on agarose*
6-well plates (sterile)	*For assay / culture*
epiCS Culture Medium	*For tissue culture*

2.3 Expiration and Kit Storage:

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

Note: *Examine all kit components for integrity. If there is a concern call 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 % CO2, 90±10 % relative humidity (RH))	*For incubating tissues prior to and during assays*
Laboratory balance	*For pipette verification and checking substance weight*
Vacuum source/trap (optional)	*For aspirating media and solutions*
96-well plate spectrophotometer	*For reading OD*
6-well plate and 24-well plate	*For culture and MTT assay*
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*

2.6 Consumables:

Sterile paper towel	For blotting of cell culture inserts
Adhesive tape or Parafilm	Covering plates during formazan extraction
96-well plates	*For reading OD*
6-well plate and 24-well plate	*For culture and MTT assay*
Finn Chamber filter paper discs 8 mm (Smart Practice; Ref. AL5112GN); website with local distributors: http://finnchambers.com/en/Distributors.aspx	*For substance application*

2.7 Media, Regents, Sers, Others

PBS Ca/Mg-free (sterile)	*For MTT-Assay*
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*
DMSO, Acetone, Olive oil, Ethanol	*For solvent preparation*

3. PREPARATIONS

3.1 Media and Endpoint Assay Solutions:

MTT solution (prepare freshly on day of testing)

Per 24 tissues, dissolve 12.5 mg MTT (Sigma ref. M5655) in 12.5 ml PBS and thoroughly vortex this working solution. Keep the MTT solution in the dark and warm to 37°C prior to usage (use within 2 h).

Alternatively, a stock solution might be used as follows:

- Prepare MTT stock solution in PBS (5 mg/ml) and store aliquots at -20°C until use.
- For 50 ml stock solution, weigh 250 mg of MTT and add 50 ml PBS.
- PBS should NOT contain Ca^{2+} (calcium) or Mg^{2+} (magnesium).
- MTT working solution is 1 mg/ml diluted in PBS (2.5 ml stock solution plus 10 ml PBS).

Safety precaution: MTT is toxic. Consider the hazard (H) and precautionary (P) statement and consult the safety data sheet.

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, samples should be diluted.

Prepare the standard curve at a concentration range from 0 to 500 pg/ml ideally with equidistant concentrations.

3.2 Test Compounds:

Follow safety instructions for respective compounds.

The use of solvent for application onto the tissues differs depending on substance solubility; see chapter “test compounds and results summary”.

3.3 Positive Control Solution(s):

Dinitrochlorbenzol (DNCB) dissolved in AOO should serve as a positive control. AOO is prepared by combining 4 parts acetones with 1 part of olive oil. The DNCB concentration that will serve as PC needs to be determined in previous experiments. This can be done with the use of the SSPT training kit (CS-1018-SSPT) or when testing in the range of 0.156 to 10 mg/ml).

3.4 Negative Control:

Unexposed epiCS (no paper filter and no solvent) and solvent treated epiCS (with paper filter) serve as a negative control.

B Method

Experimental System Procurement

SkinInVitro GmbH
Langeler Ring 5
53842 Troisdorf
Germany
Phone: **+49 (0) 2241-25515-0**
Fax: **+49 (0) 2241-25515-26**
E-mail: **info@skininvitro.com**

1. Routine Procedures:

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

Contact person:

M.Sc Bernadette Thiede

SkinInVitro GmbH

phone: +49 (0) 2241-25515-0

email: bernadette.thiede@skininvitro.com

2. Document all information about supplied material in the MDS.
3. Place the DPBS into the refrigerator (2-8°C) and the vial containing the MTT concentrate in the freezer (-20±5°C).
4. Prepare 6-well plates for all epiCS tissues. Pipette 1 ml cold epiCS Culture Medium into each well.
5. Remove the shipped multiwell-plate containing the epiCS tissues from the inner shipping box and strip off the tape. Open the 24-well plate under a sterile airflow. Carefully take out each insert containing the epidermal tissue, rapidly remove any remaining agarose that might adheres to the outer side of the insert by gentle blotting onto a sterile tissue paper, and immediately place it in a well of the prepared 6-well plate. Act quickly as the epidermal cultures dry out rapidly when not in contact with medium. Make sure that no air bubbles are formed underneath the insert.
6. Place the 6-well plates containing the tissues in a humidified (37±1°C, 5±1% CO_2, 95% RH) incubator overnight.

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

2. Test Material Exposure Procedures:

Performing substance exposure experiments involves several steps. Each step is addressed individually.

2.1 Preparation of solvents:

Six different solvents have been tested topically on epiCS.

- AOO (4+1) - 8 ml acetone + 2 ml olive oil
- AOO (1+19) - 0.5 ml acetone + 9.5 ml olive oil
- 1% DMSO - 50 µl DMSO + 4950 µl epiCS culture medium
- PBS – 100% PBS
- Water – sterile dH_2O
- Absolute ethanol

2.2 Dissolving substances:

When dissolving substances in the appropriate solvent, the substances themselves contribute to the total volume of a substance solution by having “their own volume” in the substance solution. This should be considered when preparing the substance solution. Therefore, the following applies when handling liquid, solid and viscous substances:

Example liquid substances:

Assumption: 1 µl of liquid substance equals 1 µl in volume in the substance solution. Maximum test concentration of the liquid substance is 200 mg/ml, the solvent is AOO (4+1).

- e.g. you take 75 µl liquid substance → this weighs 65 mg
- to obtain the max. test concentration of 200 mg/ml you need to dissolve the 65 mg in a total volume of 325 µl AOO (calculation (65*1000)/200 = 325 µl AOO)
- you need to add 325 µl – 75 µl = 250 µl AOO to the 75 µl liquid substance.

Example solid and viscous substances:

Assumption: 1 mg solid or viscous substance equals 1 µl in volume in the substance solution.

Max. test concentration of the solid or viscous substance is 200 mg/ml, the solvent is AOO (4+1).

- e.g. you take 92 mg solid or viscous substance → these 92 mg equal 92 µl
- to obtain the max. test concentration of 200 mg/ml you need to dissolve the 92 mg in a total volume of 460 µl AOO (calculation → (92*1000)/200 = 460 µl AOO)
- you need to add 460 µl – 92 µl = 368 µl AOO to the 92 mg solid or viscous substance.

Be aware: When a liquid compound is viscous, handle it as a solid compound. This is more accurate.

Determination of Master Stock Concentration: Maximum solubility of the substance

The maximum solubility of the substance is identified by dissolving the compound in one of the above-mentioned solvents. Wherever possible AOO (4+1) should be the preferred solvent of choice. Dilutions are made in the following order: 200 mg/ml,150 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.20, 0.10 mg/ml until a clear solution is reached. The solvent with the highest dissolving capacity is chosen for the substance application.

This highest test concentration is approved if it does **not** result in

i) Two layers

ii) A milky suspension

iii) Visible precipitation of the substance (after 10 min waiting time at RT)

If any of these effects occur, then substance concentrations must be lowered in the solvent(s) until these effects do not occur anymore. That will be then the highest possible test concentration in the exposure experiments.

General remarks

- Be aware that the volume of substances is considered when dissolving the substances
- Substances must be handled with care as they may be harmful
- Be aware that substances may be quite vaporous. Work in appropriate ventilated environment (e.g. acid cabinet or flow cabinet with external air outlet)
- Also make sure that laboratory equipment used during the substance exposure experiment and the analysis after the exposure is properly cleaned and waste is properly removed
    - it is therefore recommended to perform the analysis after the 24 h exposure as well in the laminar flow
- Substances must be freshly made just before application
- The use of gloves, lab-coat and mask is recommended

- For liquid substances, all crystals should be dissolved before pipetting, by leaving the liquid substances at RT for up to 1 hour after removal from cooling (if cooling conditions are required for the storage of the substance).

2.3 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 impregnate the paper filters for the substance exposure on devices composed of glass (e.g. glass slides or tissue culture dishes made of glass).

If AOO is the solvent of choice for the substance to be tested, consider that acetone is a volatile liquid. Therefore, open and close tubes with AOO (+/- a substance) rather quickly to avoid too much evaporation of the AOO. Do not use small volumes < 300 µl. If you do so this will increase error margins.

- Determination of interference of substance with MTT assay (an alternative method is to follow what is described in the SOP epiCS Skin Irritation Test method, see Annex 1)
- Some substances may interfere with the MTT assay, which is used to determine the EC_{50} value and therefore needed to be excluded from the epiCS potency assay – these substances fall outside of the applicability domain of the assay. In order to determine whether a substance interferes with the MTT assay, the highest soluble substance concentration is incubated with the MTT solution in the absence of EE. If the MTT assay results in a colour change then the substance must be excluded from further analysis.
- If necessary, dilute in isopropanol if the OD is above the limits of the plate reader (e.g. outside the linear range of the plate reader).

Cross reactivity of a substance is determined by the following procedure:
50 µl of the maximum dose of the substance (e.g. 200 mg/ml) is pipetted onto an 8 mm filter paper disk
Treat the filter as if it were an epiCS and perform MTT assay using the filter according to section ENDPOINT MEASUREMENT.

The test substance should not change the colour of the isopropanol into any other colour. It may not increase OD values more than 2 times the mean OD of background controls. If so, this leads to exclusion of the test substance due to interference with the MTT assay.

<u>Preparation of dose response experiments to determine the EC_{50} value</u>
In order to determine an EC_{50} value, substances are tested using two independent epiCS batches in a dose response, starting with the highest soluble concentration (preferably max. 200 mg/ml) and decreasing in 2-fold dilutions until 0.1 mg/ml (or 1st lower concentration hereof) is reached.

Example 1: 200 mg/ml, 150 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml, 0.20 mg/ml, 0.10 mg/ml

Example 2; 150 mg/ml, 75 mg/ml, 37.5 mg/ml, 18.75mg/ml, 9.38 mg/ml, 4.69 mg/ml, 2.34 mg/ml, 1.17 mg/ml, 0.58 mg/ml, 0.29 mg/ml, 0.15 mg/ml, 0.07 mg/ml
Whereas substance dose responses are tested in single fold in each independent experiment, control conditions (unexposed, solvent(s) and positive control), should preferably be tested in duplicate per independent experiment.

Control conditions and quality criteria for the control conditions
Below a description is given for the different control conditions that are taken along in each experiment. The number of different solvents tested depends on the number of solvents needed for the different substances to be tested within one experiment.

Unexposed epiCS cultures
Unexposed epiCS are cultures that do not receive any treatment during the substance exposure experiment. No filter paper disc, no substance solution and no medium are topically applied to these cultures. In the substance exposure experiments they are taken along as “naked” cultures.

Solvent exposed epiCS cultures
Different solvents, applied to paper filters, have been tested topically on the epiCS: water, PBS, absolute ethanol, 1% DMSO, AOO (4+1) and AOO (1+19)

Quality Criteria for the control conditions
Solvent exposure should not result in more than 30% decrease in relative cell viability compared to unexposed cultures. If the decrease in relative cell viability is > 30% for the solvent exposed cultures, then the epiCS batch does not fulfil the quality criteria required for this assay and the experiment should therefore be excluded from further analysis.

2.4 Substance exposure:

Unexposed and solvent are only tested in duplicate per batch epiCS, while substances are tested in single.

Preparation of solvents:

- AOO (4+1) - 8 ml acetone + 2 ml olive oil
- AOO (1+19) - 0.5 ml acetone + 9.5 ml olive oil
- 1% DMSO - 50 µl DMSO + 4950 µl epiCS Culture Medium
- PBS – 100% PBS
- Water – sterile dH_2O
- Absolute ethanol

Preparation of testing substance concentrations

- Be aware that the weight / volume of substances is considered when dissolving the substances to obtain the Master Stock solution (maximum soluble concentration ≤ 200 mg/ml).
- Substances must be handled with care, as they may be harmful.
- Substances must be freshly prepared before epiCS exposure (to avoid oxidation of the substances, it's recommended to immediately close the tubes firmly and wrap Parafilm around the tubes).
- The use of gloves, lab-coat and mask is recommended.
- For liquid substances, all crystals should be dissolved before pipetting, by leaving the liquid substances at room temperature for up to 1 h after removal from cooling (if cooling conditions are required for the storage of the substance).

Handling the solvent AOO

It is known that AOO dissolves some plastic lab devices. It is therefore recommended to use polypropylene tubes to make the substance solutions dissolved in AOO. And 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 pre-warmed (37°C) epiCS Culture Medium.
2. Place pre-sterilized filter paper disks (8 mm) in a 100 mm tissue culture dish using a sterile pincet (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 duplicates for each testing condition)).
4. Hold the filter paper disk with a pincet and gently tap them minimal 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 stratum corneum according to the plate layout described in Annex 3. Place the filter with the pencil labeled side upwards on the cultures.
7. Incubate exposed cultures for 24 h (37°C, 5%CO_2, 95% humidity).
8. After 24 h of incubation, take exposed cultures out of the incubator and gently remove the filter paper disks with a pincet.

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.

3. Endpoint Measurement:

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 (IL-18 SI-2 value).

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 a detergent (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. Prepare MTT stock solution in PBS (5 mg/ml): For 50ml stock solution, weigh 250 mg MTT and add 50 ml PBS. Be aware that the PBS that will be used does NOT contain Ca^{2+} (calcium) or Mg^{2+} (magnesium). The stock solution can be frozen in aliquots until use. Use MTT working solution at a concentration of 1 mg/ml diluted with PBS.
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 a pincet and discard them.
5. Pick up the epiCS test cultures with a pincet 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 h 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, but before the incubation with the isopropanol, in order to document the results also visually, in case any abnormalities may arise from this point on.
8. After the 3 h 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 h 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 14 is completed.
14. Calculate cell viability:
    In the template files (if provided) in the Excel sheets, formulas and graphs have been incorporated in order to calculate the metabolic activity for each condition.

    Absorbance of the wells from the solvent exposed cultures is taken as 100% cell viability. The higher the toxicity of a 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 = 0.958
    Absorbance from a testing condition = 0.258
    0.958 --- 100 % viability
    0.258 --- x % viability → cell viability at this specific condition = 26.93%

15. If the absorbance of any of the samples is above 2.0, then samples need to be further diluted and read again with the spectrophotometer. Proceed as follows: for ALL of the

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.

4. ACCEPTANCE CRITERIA

4.1 Control conditions and quality criteria for the control conditions:

Below a description is given for the different control conditions that are taken along in each experiment. The number of different solvents tested depends on the number of solvents needed for the different substances to be tested within one experiment.

4.2 Unexposed epiCS cultures:

Unexposed epiCS are cultures that do not receive any treatment during the substance exposure experiment. No filter paper disc, no substance solution and no medium is topically applied to these cultures.

4.3 Solvent exposed epiCS cultures:

Six different solvents, applied to paper filters, have been tested topically on epiCS: water, PBS, absolute ethanol, 1% DMSO, AOO (4+1) and AOO (1+19).

4.4 Quality Criteria for the control conditions:

Solvent exposure should not result in more than 30% decrease in relative cell viability compared to unexposed cultures. If the decrease in relative cell viability is > 30% for the solvent exposed cultures, then the epiCS batch does not fulfil the Quality Criteria required for this assay and the experiment should therefore be excluded from further analysis.

5. DATA ANALYSIS

MTT:

An estimation of the EC50 values can be calculated also 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

5.1 Expression of IL-18 rationale

IL-18, formerly known as IFN-γ-inducing factor (IGIF), which belongs to the IL-1 cytokine family, is a potent inducer of IFN-γ by activated T cells (Okamura et al., 1995). IL-12 and IL-18 play important roles in the development of T helper type I (Th1) cells and are synergistic in the induction of IFN-γ by T-cells (Okamura et al., 1995). IL-18 has been demonstrated to favour Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-8 and IFN-γ, and to play a key proximal role in the induction of allergic contact dermatitis (Cumberbatch et al., 2001). Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999). Antonopoulos et al., (2008) have recently demonstrated that IL-18 is a key proximal mediator of LC migration and contact hypersensitivity, acting upstream of IL-1β and TNF-α, suggestive of a central role in regulation of cutaneous immune response.

IL-18 release is assessed by ELISA. Standards are run in duplicate, samples (0.1 ml) in single. Results are expressed as pg/ml. The commercially available IL-18 ELISA kit from MBL should be used (#7620, MBL, Japan). Please follow the instructions of the MBL ELISA kit.

IL-18 Data Analysis

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

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

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.
For a given substance, the same classification must be obtained in both independent experiments. The 5.0-fold increase is indented for at least one of the concentrations tested.
For potency assessment (IL-18 SI-2): the relative amount of IL-18 protein present in supernatants given in pg/ml is determined. The SI-2 is determined using the same spread sheet design as shown above.

6. 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 sensitiser potency, the EC_{50} value and IL-18 (SI-2) value is determined.

Read out:
For allergenic potential: ≥ 5-fold increase in IL-18 release (ELISA) compared to solvent 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).
Secondary parameter: IL-18 Substance concentration resulting in: ≥ 2-fold increase in IL-18 release (ELISA) compared to solvent treated epiCS (SI-2).

C ANNEXES

1. ANNEX 1

1.1 Interference Tests*:*

Test for interference of substances with MTT as described in the epiCS Skin Irritation INVITTOX Protocol - here adapted to Skin Sensitisation.

Test for interference of substances with MTT endpoint and correction procedures

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

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

Step 1:

Add 25 µl (liquid) of the test substance into 0.3 ml of deionised water. Perform the test in a transparent, preferably glass test tube since plastic test tubes may react with the test articles during the incubation time. Incubate the mixture in the incubator (37±1°C, 5±1 % CO_2, 90±10 % RH) for 60 min. At the end of the exposure time, shake the mixture and evaluate the presence and intensity of the staining (if any). If the solution changes colour significantly, the test substance is presumed to have the potential to stain the tissue. A functional check on viable tissues should be performed (Step 2).

Step 2:

To check the tissue-binding of a coloured test article (or a substance that changes into a coloured substance) the following test should be performed with - we recommend - 3 tissue replicates concurrently tested with each and every test performed with the coloured substance):Expose three tissues with impregnated filter papers. . In parallel, expose a tissue to PBS (negative control). Follow all procedures as described in this protocol in the Method Section except incubate the tissue for 2h in PBS without MTT (37±1°C, 5±1 % CO_2, 90±10 % RH) instead of incubating in PBS containing MTT. After the 2 h incubation, rinse the tissues and extract the tissues using 2.0 ml of isopropanol and measure the optical density (OD) at 540-570 nm.

Data correction procedure

If the extract from tissues treated by coloured substance (or substance detected in step 1) has an OD between 5 % and 30 % of the negative control tissue (treated with PBS), the substance should be concurrently tested with each and every test performed with the coloured material using the procedure described above. The OD (unaffected by interference with the coloured test materials) is calculated using following formula:

OD = **OD** coloured tissue (MTT assay) – **OD** coloured tissue (no MTT assay)

Note: If the extract from tissues treated by coloured substance (or substance detected in Step 1) has an OD <5% of the PBS treated control tissue and the tissue viability (determined in MTT assay) is not close to the classification cut-off (50 %), correction of the results is not necessary.

If the OD of extract from the tissue treated by coloured substance (or substance detected in Step 1) is > 30 % of the PBS treated control tissue, additional steps and expert judgment must be performed to determine if the test substance must be considered as incompatible with the test.

Step 3:

All test materials (including those already evaluated in Step 1 and Step 2) should be further evaluated for their potential to interfere with MTT assay. To test if a material directly reduces MTT, add 25 µl test substance to 1 ml PBS containing MTT and incubate in the incubator (37±1°C, 5±1 % CO_2, 90±10 % RH) for 60±2 min. As negative control uses 1 ml PBS containing MTT. If the MTT solution turns blue/purple, the test substance reduces MTT and additional functional check (Step 4) must be performed.

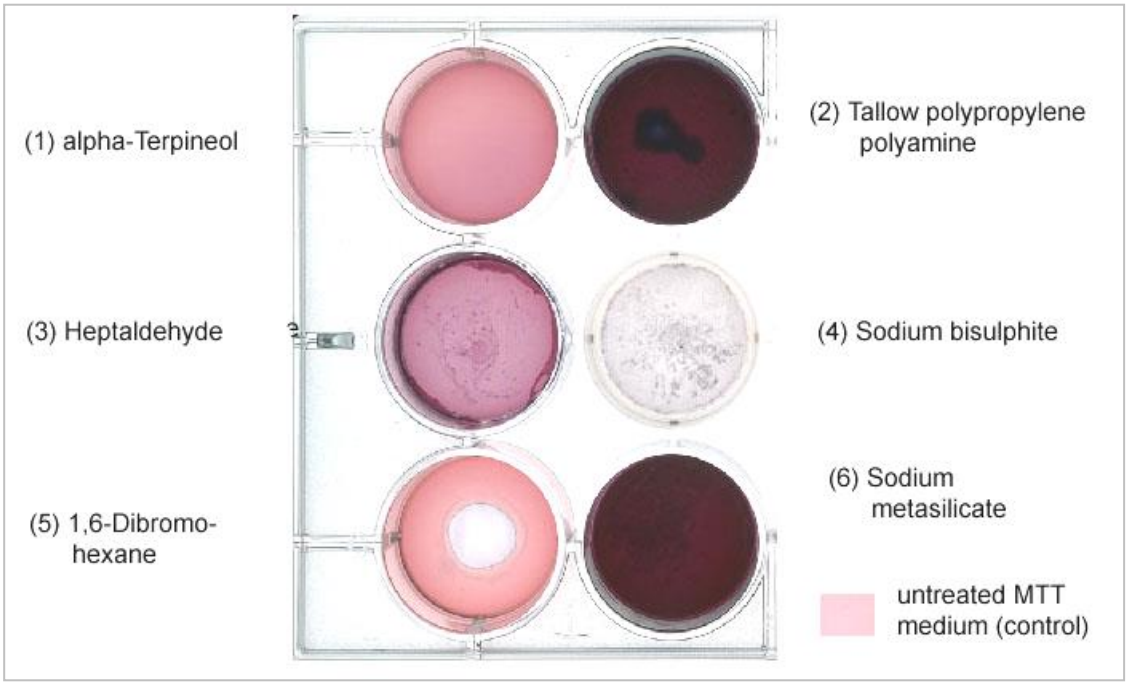

Figure 1: Example of test for direct MTT reduction ability (Step 3). Test substances (2) (3) and (6) have directly reduced MTT. In these cases, Step 4 must be performed.

Step 4:

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

Prepare freeze-killed tissues:

a) transfer epiCS in 24-well plates without cell culture medium

b) freeze tissue at -20°C (or -80°C) for at least 12 h (3 tissues / MTT-interacting test substance)

c) thaw tissues and repeat step b) twice

d) thaw tissue 1 h (±10 min) before use

e) keep at RT

Correction of non specific MTT reduction should be performed with 3 tissue replicates in a single run, independently of the number of independent tests performed with the direct MTT-reducing substance:

Each MTT reducing substance is applied to three freeze-killed tissue replicates. In addition, three freeze killed tissues are left untreated (Note: The untreated killed controls will show a small amount of MTT reduction due to residual reducing enzymes within the killed tissue). The entire assay protocol is performed with the frozen tissues in parallel to the viable epiCS tissues. Data are then corrected as follows:

Data correction procedure

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

= OD tvt – OD kt

OD kt = (mean OD tkt – mean OD ukt)

tvt = treated viable tissue

kt = killed tissues

tkt = treated killed tissue

ukt = untreated killed tissue (NC treated tissue)

If the interference by the test substance is greater than 30 % of the negative control value, additional steps must be considered or the test substance may be considered incompatible with this test system

If the interference by the test substance is ≤ 30 % of the negative control value, the net OD of the test substance treated killed control may be subtracted from the mean OD of the test substance treated viable tissues to obtain the true amount of MTT reduction that reflects metabolic conversion only.

2. ANNEX 2

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

Dose Response for AOO soluble substances (100% final AOO)

→ In the dose response 12x 2-fold serial dilutions (1x, 2x, 4x, 8x, 16x etc) are tested for each substance.

Steps to be taken

1. Make AOO (4+1): 8 ml acetone + 2 ml olive oil
2. Dilutions are made in AOO as indicated in the table below starting with the Master Stock concentration
3. 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)
1x dilution	Master Stock concentration in 100% AOO (e.g. 200 mg/ml)	--
2x dilution	300 µl (1x dilution)	300 µl
4x dilution	300 µl (2x dilution)	300 µl
8x dilution	300 µl (4x dilution)	300 µl
16x dilution	300 µl (8x dilution)	300 µl
32x dilution	300 µl (16x dilution)	300 µl
64x dilution	300 µl (32x dilution)	300 µl
128x dilution	300 µl (64x dilution)	300 µl
256x dilution	300 µl (128x dilution)	300 µl
512x dilution	300 µl (256x dilution)	300 µl
1024x dilution	300 µl (512x dilution)	300 µl
2048x dilution	300 µl (1024x dilution)	300 µl

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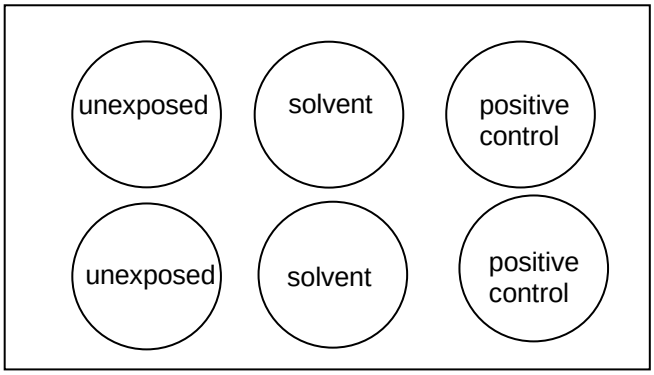
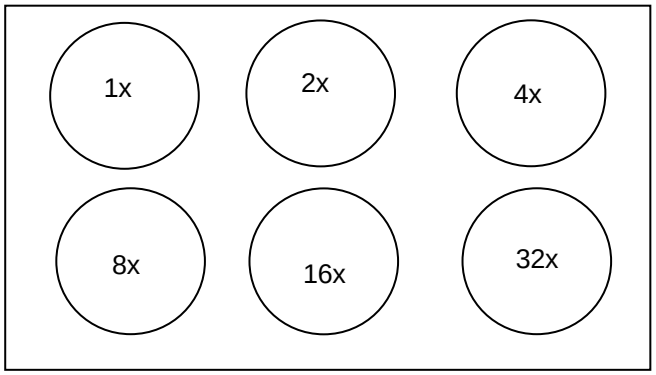
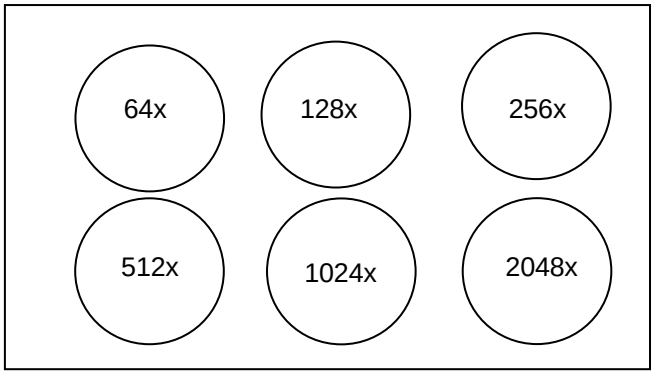
3. ANNEX 3

Control plate



Test chemical dilution plates





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