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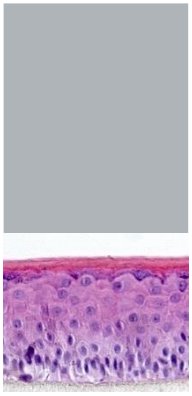
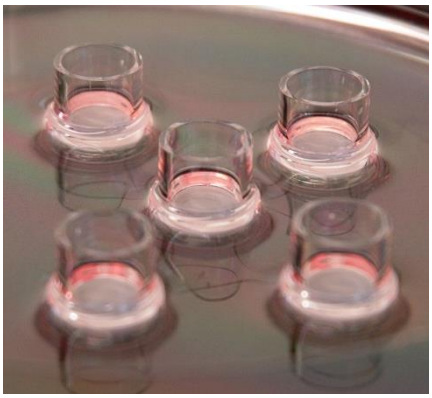
Phenion® OS-REp *in vitro* Skin Irritation Test

Standard Operation Procedure





1 Rationale and background

This Standard Operation Procedure has been developed to conduct an *in vitro* skin irritation test according to the OECD Test Guideline 439 with the Phenion® Open Source Reconstructed Epidermis [OS-REp; Product ID: OS-REp-1; 1].
Phenion has conducted a multicentric validation study with international partners according to the OECD Performance Standards for in vitro skin irritation testing [2] to assess its predictive capacity. The results of the validation study, proving the test method's good predictivity, and the technological background of the OS-REp production are described in detail in 2 consecutive publications [3, 4].

2 Basis of the method

The test is based upon the topical application of test substances on the human reconstituted epidermal models, followed by the determination of cell viability. Cell viability is measured by means of the intracellular enzymatical conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble blue formazan salt, which is then extracted from the tissues. The percentage of viability reduction, compared to untreated control tissues, serves as a measure for the irritating potential of the test substances.

3 Test system

Test system description

The open source reconstructed epidermis model (OS-REp) consists of human primary keratinocytes isolated from juvenile foreskin biopsies. The keratinocytes are cultured onto a polycarbonate membrane, where they proliferate and eventually differentiate into a multilayered epidermal tissue. The epidermis reveals all layers regularly found in healthy

human skin: a well-organized basal layer comprising palisade-shaped cells, followed by spinous and granular layers. The epithelium is covered at its surface by a multilayered stratum corneum.
The epidermal models have a surface area of 0.63 cm^2 and are cultured and shipped in co-culture inserts (Millicell-PCF, 12 mm, pore size 0.4 µm; Millipore Corp.). The whole culture process is based upon the protocol published by Poumay et al. [5], which is slightly modified in a few details.

Quality control
The OS-REp model is produced under standardized conditions in order to assure consistent quality. The barrier function, expressed as the ET50 after a TRITON X-100 treatment, the tissue viability and the histological appearance are routinely checked for every production lot.

Precautions:
The epidermal cells are harvested from human donors knowingly free of HIV and hepatitis infections. Nevertheless, handling procedures for biological materials should be as followed:

- It is recommended to wear gloves during handling with the skin and kit components.
- After use, the epidermal models and all media and materials in contact with it should be disposed according to the recommendations for biological materials.

3.1 Limitations of the procedure

Previous studies have revealed the importance of testing the interference of the substances with the MTT reagent without the presence of living cells. Chemicals with a high reduction potential, e. g. vitamin C, are able to convert the MTT into formazan by themselves, thus possibly leading to false negative results. This property of the test substances is critical if still sufficient amounts of the substances are present at or in the tissues at the time point of the MTT assay.
Therefore, prior to the testing, this aspect has to be clarified by an easy-to-perform testing procedure (see chapter 5.3).

3.2 Brief basic procedure

On day of receipt, the OS-REp models are removed from the package, transferred under a safety cabinet and provided with fresh prewarmed culture medium. The validation study will then be performed not earlier than 24 hours after arrival, when the tissues have recovered from the transport stress. In the case that the tissue models are shipped earlier, they must be cultured under standard conditions until the time point of substance testing.

Prior to the substance testing, all tissue models are transferred into fresh prewarmed culture medium. The models are then topically exposed to the chemicals for 35 minutes each. Three tissues are used per test substance, positive control (PC) and negative control (NC). After the indicated period of time the tissues are thoroughly washed with buffer solution and transferred to fresh prewarmed culture medium. After 42 hours of incubation at 37°C the tissues are transferred to 24-well plates containing 0.2 ml of an MTT solution (1 mg/ml), where they remain for 3 hours. Then the blue formazan dye, which has been deposited inside living cells, is extracted with 2 ml of 2-propanol each for 2 hours at ambient temperature or over night at 4°C. The optical density of the extracted formazan solution is determined in a spectrophotometer at a wavelength between 540 and 600 nm. The relative cell viability is calculated for each tissue as the percentage of the mean negative control tissues. A test substance will be preliminary classified as skin irritant if the viability of the respective tissues is below 50%.

4 Data interpretation procedure (Prediction model)

According to the GHS classification the irritation potential for a test substance is predicted if the mean relative tissue viability of three individual tissues exposed to said substance is reduced below or equal to the 50% threshold of the mean viability of the negative controls.

***in vitro* result**	***in vivo* prediction**
Mean tissue viability ≤ 50% of NC	Irritant
Mean tissue viability > 50% of NC	Non-irritant

5 Materials and methods

5.1 MTT assay components

MTT working solution (1 mg/ml)
5% SDS solution (positive control)
Dulbecco‘s-PBS (negative control)

5.2 Materials

Device/Material	**Purpose**
Laminar flow hood	for safe work under sterile conditions
Humidified incubator (37°C, 5% CO_2, ≥ 95% relative humidity	for incubating tissue prior to and during assays

Vacuum source	for aspirating media and solutions
Laboratory balance	for pipette volume verification
96-well plate spectrophotometer with filter in a wavelength range between 540-600 nm	for reading OD
Stop watch	to be used during application of test substances
Sterile forceps	for handling tissue inserts
37°C water bath	for warming cell culture media
Adjustable pipette/multistep pipette	for pipetting 1.5 ml culture medium
Adjustable pipette/multistep pipette	for pipetting 200 µl MTT solution and formazan extracts
Adjustable pipette/multistep pipette	for pipetting 2 ml of 2-propanol
Positive displacement pipette (25 µl)	for application of semi-solid substances
Dulbeccos's PBS (D-PBS; with Ca^{2+}, Mg^{2+})	use for rinsing tissues
6-well plates	for substance application
24-well plates	for MTT viability assay
2-propanol	for formazan extraction
Parafilm	to seal 24-well plates during formazan extraction
Horizontal Shaker	for formazan extraction
96-well plates	for OD measurement

5.3 Test for direct MTT reduction

As already indicated in chapter 3, some test substances may interfere with the MTT substrate in the absence of viable cells due to their high reduction potentials. In order to identify possible interferences, each of the 20 test substances was checked for its ability to reduce MTT and was found to be negative.

5.4 Grid application

In contrast to previously published protocols we have developed our method without the use of nylon grids. Grids are widely used as an effective means to homogeneously spread liquids over the whole surface of tissue models, thus reducing unwanted capillary effects.
We ourselves have made the observation that some of the reference chemicals react with the nylon grid, which eventually leads to the melting and subsequent fusion of the polymer with the upper tissue layers. Thereafter the grids cannot be properly removed without damaging the tissues. For this reason we apply liquid substances directly onto the tissue model surface without using grids as spreading aids.

Caution:
Some substances are known to reveal physical properties (e. g. surface tension, electrostatical charge, hydrophilicity) which make it difficult to spread them homogeneously over the tissue surface. Please handle them with special care and make sure that the droplet is placed in the center of the tissue. If the liquid does not cover the surface completely in the first instance, take up the droplet with the pipet and release it again onto the surface. Repeat this procedure until the liquid has spread properly.

5.5 Assay quality control

5.5.1 Negative control

The absolute optical density (OD) of the negative control tissues in the MTT assay is an indicator of tissue viability after the culture period and the transport process.

Based on historical data the mean OD at λ = 540-600 nm should be located between 0.6 and 1.8 relative units.
But it also must be taken into account that the MTT reduction merely takes place in the basal and first spinous cell layers of the epidermis (e. g. Cotovio et al., 2005, own unpublished observations). Therefore the OD of the formazan solution does not reflect the overall quality including the thickness and architecture of the epithelium and a well-constructed cornified layer, but hints only towards a viable stratum basale.

5.5.2 Positive control

A 5% aqueous SDS solution is used as a positive control and tested concurrently with the test chemicals.
Viability of positive controls, based on historical data, should be below 10% of the NC.

5.5.3 Standard deviation

Since in each test the skin irritation potential is predicted from the mean viability determined on three single tissues, the variability of tissue replicates should be in an acceptable range. According to the OECD Performance Standards for *in vitro* skin irritation testing [2] the standard deviation must be equal or smaller than 18% (SD ≤ 18%).

5.6 Preparations

5.6.1 MTT solution

Prepare a stock solution of 5 mg/ml MTT in D-PBS (with Ca^{2+}, Mg^{2+}). Immediately prior to use, dilute a sufficient amount (e. g. 4 ml) of the stock solution with PBS at a ratio of 1:5; the resulting working solution (20 ml) has a final concentration of 1 mg/ml MTT.
For 66 epidermal models (for testing10 chemicals + negative control + positive control) you need exactly 13.2 ml of MTT solution. However, because of void volumes in the pipettes you use we recommend to prepare at least 15-20 ml of the reagent solution.

5.6.2 Dulbecco's PBS

Sterile ready-to-use Dulbecco's PBS can be used, which is commercially available at several companies (e. g. GIBCO, article number 14040). If you decide to make your own D-PBS, adjust the pH value up to a physiological value (pH 7.0 – 7.4) and subsequently sterilize the salt solution.

5.7 Test substances

Safety instructions:

In general, all test materials must be treated as if they were irritating and toxic, and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear safety goggles and gloves).
Store all substances according to the recommendations listed in the MSDSs. Respect special storage conditions (special temperature, protection from light, protection from oxidization by nitrogen etc.).

5.7.1 Liquids:

Dispense 25 µl directly atop the tissue surface using an appropriate pipette.

5.7.2 Semisolids:

Dispense 25 µl directly atop the tissue surface using a positive displacement pipette. If necessary, spread substance carefully to match the surface area.

5.7.3 Solids:

Fill a sharp application spoon, volume 25 µl, with fine ground test material. Level the spoon by gently scratching the excess material away with an appropriate aid. Do not apply any pressure onto the powder because due to compaction the amount of substance in the given volume will be increased. Distribute the powder evenly over the surface of the epidermal models. Add 25 µl of sterile PBS in order to wet the surface of the test material and to increase surface contact. If necessary, spread substance carefully to match the surface area. The application time starts after applying the PBS.

6 Experimental procedure (see also flowchart)

6.1 Time prior to testing

Upon arrival at the lab, take out the 24-well plates and remove the lids under the airflow. Take out each insert with sterile forceps, remove remnants of the semi-solid transport medium attached to the outer insert wall, and place the epidermal models in the wells of 6-well plates. Then fill the wells with 1.5 ml prewarmed ALI medium each, and place the models in an incubator (37°C, 5% CO_2, ≥ 95% relative humidity) until the time point of substance testing.

Caution:

Please remove air bubbles trapped underneath the insert membranes after application of fresh culture medium.

6.2 Chemical exposure

Tissue conditions

- Prewarm the culture medium to 37°C in a water bath
- Pipette 1.5 ml of culture medium in each well of sterile 6-well plates
- Transfer 3 epidermal models each from the culture plates to the upper row of the newly filled sterile 6-well plate
- Before test substance application label lids of the 6-well plates properly with the respective test substance code. One plate each is dedicated to the NC and PC, respectively. Apply 25 µl of the undiluted test substance, NC and PC to three single tissues each.
    - If the liquid does not cover the surface completely in the first instance, take up the droplet with the pipette and release it again onto the surface. Repeat this procedure until the liquid has spread properly.
    - When a powder is applied to the surface, the incubation time starts with the addition of 25 µl D-PBS for wetting.
    - In some cases the powder grains reject the buffer droplets due to hydrophobic properties of the substance. Then leave the substances untouched.
    - If the buffer dissolves the powder, the resulting solution can be evenly distributed over the model surface by gentle pipetting.
- Keep the so-treated tissues in the laminar flow at ambient temperature.
- After 35 ± 0.5 minutes of exposure the test substance is carefully aspirated from the tissue surface by using a Pasteur pipette connected to a working vacuum pump.
- All tissues are washed 8 times with sterile D-PBS (with Ca^{2+}, Mg^{2+}): 600 µl of D-PBS are applied into each inserts with a multipette and subsequently the liquid is discarded. This process is repeated 7 times, then carefully aspirate the buffer from inside the insert.

- The inserts are immersed 5 times into a beaker containing 500 ml D-PBS (with Ca^{2+}, Mg^{2+}); after each dipping the liquid must be discarded so that a complete buffer exchange is guaranteed.
    - Exception: the negative control models are not dipped any more into the PBS due to the risk of cross-contamination with remnants of the substances.
- Remaining droplets adhering to the inner and outer insert walls are carefully aspirated.
- Then transfer the tissue inserts to the lower row in the 6-well plates pre-filled with fresh pre-warmed culture medium (see above).

Caution:

- In the case that remnants of the chemical still attaches to the tissue surface, try to remove them with sterile wetted cotton swabs. Record this procedure in the protocol.
- Incubate tissues in an incubator (37°C, 5% CO_2, ≥ 95% relative humidity) for 42 hours.

6.3 MTT assay

- Prior to the performance of the MTT assay, label 3x 24-well plates for the test. An application scheme indicating the position of each individual epidermal model for MTT assay is included in the attached Methods Documentation Sheet. Please keep to this scheme in order to ensure the proper assignment of the samples throughout the whole process.
- Apply 200 µl of the MTT working solution (1 mg/ml in D-PBS) to each well.
- Remove inserts from the 6-well plates, blot the bottoms of the insert, and remove liquid adhering to the outer insert wall with blotting paper.
- Transfer them into the wells of the 24-well plates, prefilled with the MTT working solution.
- Remove air bubbles trapped underneath the insert membranes
- Then place the wells into an incubator (37°C, 5% CO_2, ≥ 95% relative humidity) and incubate the tissues for 3 hours. Record the starting time of incubation.
- After the 3 hours incubation time remove the inserts from the wells and remove liquid adhering to the outer inserts wall with blotting paper.

- Transfer the inserts into the wells of 3 fresh 24-well plates and fill them with 2.0 ml of 2-propanol. Due to its volume some 2-propanol will flow into the inserts and thus immerse the tissues from atop. Keep to the application scheme.
- Seal the 24-well plates with parafilm and transfer them into a refrigerator (4°C) overnight or place them for 2 hours on a shaker at ambient temperature (approx. 250 rpm; take care that the liquids do not spill from one well to another if the shaker velocity is chosen to high). Then take a pair of tweezers and prick the insert membrane with the tip, so that the fluid inside the inserts is mixed with the fluid in the wells. Alternatively you can also take a sharp cannula instead of the tweezers.
- If the 24-well plates were kept in the refrigerator overnight, subsequently place them on the shaker (approx. 250 rpm) for another 30 minutes in order to obtain a homogeneous formazan solution.
- From each tissue sample transfer 2 x 200 µl of the formazan extraction solution to the wells of translucent 96-well flat-bottom microtiter plates according to a defined plate design (included in the Methods Documentation Sheet). Use 2-propanol as blanks. Read the OD in a multiplate spectrophotometer (ELISA reader) at a given wavelength between 540 and 600 nm, without using a reference filter.

Note:

In contrast to normal photometers, in multiplate readers pipetting errors influence the OD by varying the pathway length of the light through the solution. Therefore it is important to measure 2 aliquots from each tissue extract in order to recognize possible errors.
If the ODs from both aliquots of a single tissue extract markedly deviate from each other in the way that a clear read-out is not possible, fill a new 96-well plate with all samples and repeat the measurement. Ensure that the formazan extraction solutions are homogeneously mixed.
For the same reason it is important to use flat-bottom wells in order to ensure identical pathway lengths at every measuring point.

7 Data documentation and calculations

The data generated by the multiplate spectrophotometer are fed into an EXCEL spreadsheet for further processing.

- Calculate the mean OD of the blanks (2-propanol; OD_{blank})
- Subtract the mean OD_{blank} from the OD data of all other samples
- For each sample calculate the relative tissue viability in comparison to the NC using the following formulae:

Relative viability TS (%) = [OD_{TS} / mean of OD_{NC}] x 100

Relative viability NC (%) = [OD_{NC} / mean of OD_{NC}] x 100

Relative viability PC (%) = [OD_{PC} / mean of OD_{NC}] x 100

TS: testing substance

NC: negative control

PC: positive control

- For each TC, NC and PC calculate the mean relative viability of the three individual tissue; these data are the basis for the subsequent substance classification.

- Calculate the standard deviation (SD) of the means of the 3 tissues; the SD must fulfill the criteria for test acceptance mentioned in section 5.5.3.

The acceptance criteria for the negative and positive controls (NC and PC) are listed in section 5.5.

The acceptance criteria for a valid run and a valid run sequence, respectively, for each tested substance and the whole set of reference chemicals are defined in the OECD Performance Standards for in vitro skin irritation [8].

8 References

[1] OECD Guidelines for the testing of chemicals: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method. 439, 2015

[2] OECD. (2015). Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Reconstructed Human Epidermis (RhE) Test Methods for Skin Irritation in Relation to TG 439. Environment, health and Safety Publications, Series on Testing and Assessment (No. 220). Organisation for Economic Cooperation and Development, Paris.

[3] Mewes KR, Fischer A, Zöller NN, Laubach V, Bernd A, Jacobs A, van Rompay A, Liebsch M, Pirow R, Petersohn D.: Catch-up validation study of an in vitro skin irritation test method based on an open source reconstructed epidermis (phase I). Toxicol In Vitro, 2016, 36:238-253

[4] Groeber F, Schober L, Schmid FF, Traube A, Kolbus-Hernandez S, Daton K, Hoffmann S, Petersohn D, Schäfer-Korting M, Walles H, Mewes KR.: Catch-up validation study of an in vitro skin irritation test method based on an open source reconstructed epidermis (phase II). Toxicol In Vitro, 2016, 36:254-261

[5] Poumay, Y., Dupont, F., Marcoux, S., Leclercq-Smekens, M., Hérin, M., Coquette, A.: A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies. Arch Dermatol Res, 2004, 296:203–211

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Flowchart: OS-REp Skin Irritation Test

Transfer tissues into culture medium (6 well plate; 1.5 ml medium)

↓

Apply 25 µl TS, NC or PC on 3 tissues each

↓

Expose at ambient temperature for 35 minutes

↓

After 35 minutes, stop exposure by thoroughly washing with PBS

↓

Transfer tissues into new wells with fresh culture medium

↓

Incubate for 42 hours (37°C, 5% CO_2)

↓

Blot tissues and transfer them into MTT medium (200 µl)

↓

Incubate for 3 hours (37°C, 5% CO_2)

↓

Blot tissues and transfer them into new wells

↓

Immerse the tissues with 2.0 ml 2-propanol

↓

Extract formazan at 4°C in the dark over night or 2 hours at ambient temperature

↓

Equilibrate extracts from beneath and atop the tissues

↓

Read OD in a microplate photospectrometer at λ = 540-600 nm