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PrediSkin

Standard Operating Procedure

Version 1.0

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PURPOSE AND APPLICABILITY

Definitions

EUROSAFE has developed an ex *vivo* assay consisting of the evaluation of cutaneous cytotoxicity, using discs of freshly excised human skin, named “PREDISKIN. This method estimates the risk of cutaneous irritation before clinical tests on human volunteers.

This PREDISKIN model provides a prediction of the risk of acute skin irritation relating to cell death (measured using MTT metabolism) when a single application is performed. Other toxic pathways which induce significant cutaneous irritation, such as sensitization, sub-acute inflammation (low level of cell death), or irritation due to reiterated applications, are not assessed in this protocol.

In summary, PREDISKIN is able to assess, using fresh human skin discs, the potential cutaneous irritation of a cosmetic product related to:

- its toxic effect on skin cells,
- its condition of exposure.

Health Safety and Environment

It is recommended to wear a clean lab coat and clean powder-free protective gloves during the entire procedure to avoid contamination.

Contamination from one sample to another can be avoided by the use of disposable equipment.

METHODOLOGY

The principle of the method is based on the evaluation of the toxicity of a test item (and/or its metabolites) measured using the MTT assay. The MTT assay measures the residual mitochondrial related metabolic activity of the skin cells, after a 20-hour exposure with the test item. The described procedures in this protocol are based on the following documents:
[1] the appendix 1 B.40 "Cutaneous corrosion" of the Norm 2000/33/EC of the commission dated from April 25th, 2000
[2] OECD guideline for the testing of chemicals – N°439 – *In vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method, adopted on July 26th, 2013.

Negative and positive reference controls are tested under the same conditions as the test item. Demineralized water is typically used as the negative control (a hydrophilic vehicle); however, an alternative negative control Paraffin oil (lipophilic vehicle) can be used when the test item demonstrates a hydrophobic tendency. The positive reference control recommended in this study is **Sodium Dodecyl Sulfate (SDS),** which is a well-known surfactant. Its cytotoxic membranolytic effect is directly related to its dose-level. The dose-dependent toxicity of SDS after a single application on human skin discs has been established previously in multiple independent experiments. The historical data for SDS include the concentration required to induce 50% loss of viability (IC50) and the slope of the SDS dose-toxicity curve + Standard Error.

Reagents

Reagents	Role	Example list of suppliers
Demineralized water	Hydrophilic vehicle to be used as a negative control	BIOPREDIC INTERNATIONAL
Paraffin oil	Lipophilic vehicle to be used as a negative control	SIGMA (18512)
Culture medium	Maintenance of the skin disks	BIOPREDIC INTERNATIONAL (MIL080 + ADD309)
PBS [1X]	Rinsing solution	D. DUTSCHER (L0615)
MTT	Colouring dye	SIGMA (M2128)
Isopropyl alcohol	Solvent	SIGMA (34863)
Sodium Dodecyl Sulfate (SDS) 50 mg/mL (in demineralized water) **	Positive control	SIGMA (L3771)

**This solution can be stored at 4 ° C for 2 months.

Consumables

- 24 well plates (ex : BPI, PLA128) ;
- 96 well plates (ex : BPI, PLA129) ;
- Petri dish 35 mm ; (ex : BPI, BOI071) ;
- Filter paper disks 8 mm diameter (ex : BPI, DIS002) ;
- Parafilm;
- Tubes eppendorf, 1,5 mL;

- Absorbent paper;
- Aluminum folio

Equipment

Equipment	Manufacturer	Reference
Incubator/Oven without CO_2 and hygrometry > 60%	MEMMERT	100
Water bath	JULABO	ED (v.2)
Spectrophotometer	BMG LABTECH	FLUOstar Optima
Cold enclosure at +4 °C	SAMSUNG	RL 34 SCSW
Chronometer	ELECTRONIC CALCULAGRAPH	419CA
Hygrometer	MOINEAU INSTRUMENTS	9222AT/111LM
Pipettes	EPPENDORF	Research plus
Microman	GILSON	M100
Plates shaker	STUART	SSM5
Balance	SARTORIUS	R 160 D
Sorbonne	AIRT 3000 / ERLAB	SBA 120 / Filtair 824
Vertical air flow cabinet	TECHGEN	BSC-EN 1.6

Test material preparation

The test item will be tested pure or diluted, according to the cosmetic product category.

For a rinse-off product: In order to reproduce expected "normal conditions of use", a concentration denoted "in normal conditions of use" will be chosen for the different categories of product:

Shampoo	Shower gel	Exfoliant gel	Soap	Bubble bath
10%	10%	10%	10%	3%

Note: Depending on the customer, different concentrations can be selected.

For a non-rinse-off product: The product will be tested directly (mom-diluted). For example: care products (cream, lotion etc.), beauty masks, make-up products (mascara, foundation etc.), perfumes.
For an ingredient: The tested concentration will be specified by the sponsor.

Concentration(s) tested will be specified in the study specific supplement. Dilutions will be prepared according to a weight/weight (w/w) relationship, using a hydrophilic (demineralized water) or lipophilic (paraffin oil) vehicle, according to the hydrophilic or lipophilic property of the test item.

Notes:

- *If necessary, the solubility of the test item will be improved by stirring (e.g. vortex mixing) and/or sonication and/or heating.*
- *If the test item is solid, it will be ground using a pestle and mortar.*
- *The homogeneity and the stability of the test item in the experimental conditions will be determined:*
    - *Homogeneity will be obtained by stirring just before application.*
    - *If the test item is diluted, stability will be maintained by its extemporaneous preparation*

PROCEDURE

Note: Precautions during the test: Do not shake or tilt the dishes containing the skin explants.

Treatment of explants by single application

1. Each skin disk should have approximately 750 µL media (6cm dish) or 200 µL media (24h well plate) . Add media if there are losses during the transportation.
2. Before any sampling, homogenize the test products well.
3. Add **25 µL or 25 mg** of test sample on filter paper disks 8mm.
    a. using a P100 pipette for liquid products.
    b. using an M100 pipette for thick or viscous products.
4. The test product is applied to the filter paper disc while the latter is held using forceps, and thereafter applied directly to the implant, (product against the epidermis) thus ensuring semi-occlusion
    a. If the product is solid: finely cut a coverslip with a diameter of approximately 8 mm and place it directly on the implant, thus ensuring semi-occlusion.
    b. If the product is a powder: weigh a quantity of 25 mg in a 1.5 mL Eppendorf tube and deposit the powder by inversion on a paper disc previously moistened with 25 µL of demineralized water, which is applied directly to the 'explant.
    c. If the product contains volatile ingredients (eg: alcohol, perfume, etc.), evaporate it by shaking the filter paper for 15s, then after application to the explant, leave the Petri dish open under the hood for 60 min. Afterwards add 250 µL of complementary culture medium to compensate for the loss of medium due to evaporation, making sure to respect the time of 60 min for each dish.
5. Before closing the Petri dishes, make sure that the culture medium permeates the filter paper.
6. The skin disks are incubated at +32°C ± 1 ° C for **20h ± 2h** in the incubator without CO2 with a pan filled with water to maintain humidity > 60%.
7. Check with a hygrometer and record the measurement.
8. For maximum humidity, position the Petri dishes in the center of the oven tray.

Measurement of viability after single application: MTT test

Note: The MTT is sensitive to light, to be handled under a hood, light off.

1. Take a 24-well plate and place **freshly prepared** 500 µL of the MTT solution (2 mg / mL in 1X PBS) in each well.
2. Test the parallel wells for absence of interference of the MTT with the test product (s): place a small quantity of product in the bottom of the well (approximately 10 µL or 10 mg) then add 500 µL of the MTT solution (2 mg / mL) without trying to homogenize. The aim is to test

whether the test product visually stains in the presence of MTT.

3. Incubate for 20 h ± 2 h.
4. Take each skin disk with forceps and rinse with 1ml of 1X PBS (maintained at room temperature) in order to remove product residues. If it is difficult to remove the product during the test, perform 1 to 2 additional rinses and indicate, if the case arises, the wells where the explants are not properly rinsed.
5. Afterwards sponge on absorbent paper. *Note: In order to avoid any cross contamination, the explants are "drained" on different areas of the absorbent paper.*
6. Then place each skin explant per well containing the MTT solution (epidermis oriented towards the bottom of the MW well) and incubate the plates as well as the interference control plate for 4 h, at +32 °C ± 1 ° C in the CO2-free oven with a box filled with water to maintain humidity> 60%.
7. After 4 hours of incubation:
    a. Observe the wells of the MTT interference control and note the observations: absence or presence of purple coloration.
    b. For the wells with the skin disks, remove the MTT solution using a pipette
    c. Distribute 2 mL of isopropyl alcohol in each well.
    d. Place an unstretched parafilm film on each plate, replace the cover and place the plates for at least 17 h at + 4 ° C
8. The next day:
    a. Remove the skin disks using forceps, exerting pressure to release the isopropyl alcohol as much as possible, then transfer the explants to a container reserved for biological waste.
    b. Homogenize the solutions, take 2 x 200µL per well then transfer them to a 96-well plate (s), additionally provide on one of the 2 * 200µL plates for the blanks
    c. Reading the optical density of the 96-well plate (s) at 590 nm against isopropyl alcohol blanks.
    d. For OD > 3.0, provide a 1/2 dilution in isopropyl alcohol and take a reading against new isopropyl alcohol blanks (2 * 200 * µL).

CALCULATIONS

1. The average of the percent viability is then calculated for each experimental condition.
2. After reading with a spectrophotometer, an OD value per well is obtained.
3. Calculate the average of the two ODs obtained for each well, then subtract the average of the ODs of the blanks.
4. The percentage of cell viability is then calculated, for each treated well, relative to the corresponding control group (hydrophilic or lipophilic) according to the formula:

$$\% \text{ viability} = \text{Mean OD treated wells} \times 100 / \text{Mean OD control wells}$$

The average percent cell viability and the standard deviation are calculated for each experimental condition.

- If the standard deviation> 15%, one of the 3 values can be deleted insofar as it is not homogeneous with respect to the other two. In this case, the standard deviation will not be calculated.

Decision criteria for deleting a value

Calculation of the relative difference between each viability, i.e. 3 results:

- If relative difference> 25 for only 1 result, no value removed because considered homogeneous (case n ° 1).

- If relative difference> 25 for 2 results, possibility of removing the value responsible for the difference (case n ° 2).

Example:

Case n ° 1: Viability: 40 - 61 - 25 or 42 ± 18

Relative difference: 61-40 = 21, 61-25 = 36 and 40-25 = 15

1 difference> 25, the 3 values are considered homogeneous between them

Case n ° 2: Viability: 70 - 104 - 119 or 97 ± 25

Relative deviation: 104-70 = 34, 119-104 = 15 and 119-70 = 49

2 deviations> 25, 70 is the value responsible for the deviation

DATA MANAGEMENT

All data will be managed via Edelweiss Data. Additionally, a digital workflow for automated data transfer and analysis will be put in place.

REFERENCES

[1] Appendix 1 B.40 "Cutaneous corrosion" of the Reg. 2000/33/EC of the commission dated from April 25th, 2000.

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