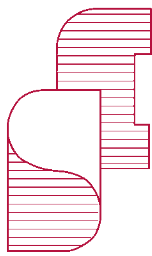

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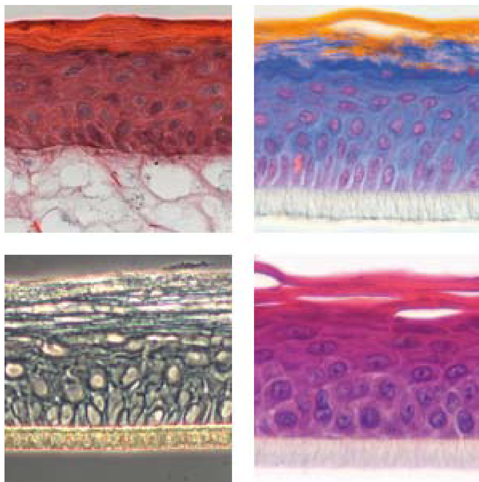
Biotechnologie Vertrieb GmbH



Manual
EST1000
Epidermal Skin Test

Epidermal Skin Test is now available in the United States exclusively through Lifeline Cell Technology. For more information, or to place a U.S. order, please call 1.877.845.7787 or email info@lifelinecelltech.com

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1. Introduction

Due to legal restrictions for animal testing, three dimensional *in vitro* models are increasingly used to measure effects of skin-active materials.

To satisfy these requirements CellSystems® provides the epidermal skin model "Epidermal Skin Test 1000" (EST1000). This reconstructed epidermis is an ideal tool for pharmaceutical and chemical substance testing. Toxic or irritating effects of test materials can be determined by multiple end-point analysis including viability, histology and cytokine release.

Epidermal Skin Test EST1000 is validated for the classification of compounds concerning skin corrosion according to the OECD test guideline 431 for testing of chemicals: "In Vitro Skin Corrosion: Human Skin Model Test". The European Centre for the Validation of Alternative Methods (ECVAM) has accepted this method to be used for distinguishing between corrosive and non-corrosive chemicals.

ECVAM has published the ESAC statement on the scientific validity of EST1000 method for skin corrosion testing (June, 12th, 2009). It can be used for reliably predicting the corrosive potential of chemical substances.

EST1000 consists of normal human epidermal keratinocytes from a single neonatal donor. A submerse culture of the keratinocytes is followed by a culture at air-liquid interphase. This "airlift culture" under defined media conditions stimulates the differentiation of the cells and the generation of the physiological epidermal layers. The standardized production process of EST1000 in CellSystems® ISO 9001:2008 certified laboratories in Germany ensures a highly reproducible epidermis model.

The cellular structure of EST1000 resembles that of natural human epidermis showing a basement membrane, proliferating keratinocytes and a stratum corneum with an intact barrier function. Furthermore, it shows an excellent *in vitro/in vivo* comparability and allows a high reproducibility of results. This efficient and economic alternative to animal testing becomes the system of choice due to instant availability and convenient use.

The skin model is cultured in inserts (0.6 cm^2) with a polycarbonate membrane. The epidermis model is supplied in convenient 24-well formats to facilitate topical application of test materials.

2. Kit Contents

- Human skin equivalents in 24 well plate, embedded in transport medium
- Maintenance medium (volume depending on no. of skin models)
- MTT-assay medium (volume depending on no. of skin models)
- Six-well-plates (no. depending on no. of skin models)
- Detailed certificate

3. Additional Required Materials

- Class II biological safety cabinet
- Incubator (37 °C, 5 % CO_2, 95 % humidity)
- Water bath (37 °C)
- Plate shaker
- 96-well plate reader spectrophotometer
- Micro-Pipettor (sterile)
- Pipette tips (sterile)
- Pair of tweezers (sterile)
- Squeeze bottle (e.g. Nalgene, Ref. FEP16-500)
- 1000 ml beaker
- 24-well culture plates
- 96-well culture plate
- 1 x PBS (Phosphate buffered saline), sterile
- MTT (e.g. Sigma) 1 mg/ml in MTT-assay Medium (pre-warmed to 37 °C)
- Isopropanol
- Positive displacement pipette for semisolid materials
- Mortar with pestle for solid materials
- Sharp spoon for solid materials (e.g. Aesculap, Ref. FK 623, MEDKA Medizinprodukte GmbH, Berlin, Germany)
- Sterile meshes (for skin irritation) (e.g. CellSystems, Ref. CS-1150)
- Needle

4. Instructions

Preparation on receipt

Immediately upon receipt check the kit for completeness and potential transport damages.
Carefully read the entire instruction before handling the skin equivalents as described below:

- The culture dishes with the cooled skin equivalents are in the inner transport box.
- Set up the culture dishes (6-well) and pipette 1000 µl cold maintenance medium (4 °C to 8 °C) into each well.
- Remove the Parafilm™ from the transport plate with the skin equivalents and open the culture dish under sterile conditions.
- Lift the inserts with a sterile pair of tweezers and transfer them into the sterile 6-well plate filled with maintenance medium. Make sure not to transfer any agarose.
- Avoid bubbles between the insert and the bottom of the culture dish by setting the insert at an angle into the culture dish.
- Incubate the culture dishes at least 2 hours at 37 °C, 5 % CO_2, and 95 % humidity before performing first experiments. It is also possible to incubate overnight. In this case change medium before applying the test materials.
- After this adaptation-phase, your test materials can be applied onto the stratum corneum or added to the medium.
- Cultivate the skin models in the incubator (37 °C, 5 % CO_2, 95 % humidity).
- In case you intend to cultivate the skin equivalents for more than 24 hours, daily medium change is required by aspirating the medium and replacing it by 1000 µl new maintenance medium (37°C) for each well.
- Store the medium at 4 °C.

5. Corrosion Testing
(According to OECD TG 431)

*Epidermal Skin Test **EST**1000 is validated for the classification of compounds concerning skin corrosion according to the OECD test guideline 431 for testing of chemicals: "In Vitro Skin Corrosion: Human Skin Model Test".*

*The European Centre for the Validation of Alternative Methods (ECVAM) has published the ESAC statement on the scientific validity of **EST**1000 method for skin corrosion testing (June, 12th, 2009). It can be used for reliably predicting the corrosive potential of chemical substances.*

One major parameter to evaluate the skin corrosive potential of a given material is the viability of the treated skin equivalents before and after exposure to the test materials. A substance is classified as corrosive if the cell viability is reduced below 50 % after 3 min of exposure. Even if the viability after 3 min exceeds 50 % a substance is corrosive when the viability is reduced below 15 % after 1 hour.

For statistical relevance three skin models should be used for each time point and substance. As a control PBS is spread onto one skin model per time point. Additionally, one skin model for every single substance to be measured should be sacrificed completely by freezing at -20 °C.

According to the definition of skin corrosion, skin models should be incubated with the substance for 3 min and 1 h. To spread the substance equally on the surface of the EST1000 sometimes it might be necessary to cover the skin models with a piece of gauze (Nylon Meshes, e.g. CellSystems, Ref. CS-1150). After the indicated time skin models are rinsed thoroughly.

Finally, cell viability is measured by performing a standard MTT assay. Metabolically active cells reduce the yellow tetrazolium salt MTT (3-[4,5–dimethylthiazol-2-yl]– 2,5–diphenyltetrazolium bromide) to insoluble purple formazan crystals. The addition of solvent dissolves the crystals and the change in colour can be quantified by standard absorbance measurement. The relation to the absorbance value of the control skin model defines the rate of cell survival.

A final classification of the skin corrosive potential of the test material becomes possible by comparing treated and untreated skin equivalents with those incubated with a reference material.

6. Irritation Testing
(According to ECVAM´s Performance Standards)

Currently EST1000 undergoes a blind-trial multicenter validation study for in vitro skin irritation testing.

One major parameter to evaluate the skin irritative potential of a given material is the viability of the treated skin equivalents before and after exposure to the test materials. A substance is classified as irritant if the cell viability is reduced below 50 % after 20 min of exposure and 42 hrs of subsequent incubation after removing test material.

For statistical relevance 3 skin models should be used for each time point and substance. As a negative control PBS and as positive control 5 % SDS diluted in water are used.

According to ECVAM´s "Performance standards for applying human skin models to *in vitro* skin irritation testing", skin models should be incubated with the substance for 20 min. To spread the substance equally on the surface of the EST1000 in some cases it is necessary to cover the skin models with a piece of gauze ("Mesh"). After the indicated time skin models are rinsed thoroughly, placed in a new 6-well plate with fresh maintenance medium and subsequent incubation for 42 hrs at 37 °C, 5% CO_2 and 95 % humidity.

Finally, cell viability is measured by performing a standard MTT assay. Metabolically active cells reduce the yellow tetrazolium salt MTT (3-[4,5–dimethylthiazol-2-yl]– 2,5–diphenyltetrazolium bromide) to insoluble purple formazan crystals. The addition of solvent dissolves the crystals and the change in colour can be quantified by standard absorbance measurement. The ratio to the absorbance value of the control skin model defines the rate of cell survival.

A final classification of the substance for skin irritative potential is possible by comparing treated skin equivalents with those incubated with the negative control (PBS).

7. Phototoxicity Testing

The core test for evaluating the skin phototoxicity potential of a substance is the measurement of a UV/ visible absorption spectrum to identify absorption at relevant wavelength, followed by an in vitro assay for phototoxicity, the Balb 3T3 neutral red uptake phototoxicity test (OECD 432). However, this test has its limitations, as non-hydrosoluble chemicals can be tested only at low concentrations due to their lack of aqueous solubility. Also many complex mixtures or formulations cannot be tested. Consequently, it does not take into account the bioavailability of test chemicals topically applied to skin and in many cases e.g. after having a phototoxic effect in this test system, such information is required.

To overcome these limitations, the use of reconstructed skin models is an interesting alternative and a useful follow-up test according to INVITTOX protocol 121.

8. Protocols

8.1. Application of test sample (under sterile conditions)

8.1.1 Corrosion Testing

- Liquids: Apply 50 µl of the sample topically to the centre of the skin equivalent by using a sterile micro-pipettor.

- Semisolids: Dispense 50 µL directly on top of the tissue using a positive displacement pipette. Cover the entire surface of the model.

- Solids: Crush and grind the substance with a mortar and pestle whenever this improves the consistency. Fill a sharp spoon with 25 mg fine ground substance. Level the spoon by gently removing excess material with an appropriate aid, avoiding compression ("packing") of the material. Add 25 µl PBS to increase contact between the tissue surface and the substance. If necessary increase the volume of PBS. For materials with a waxy consistence try to form a flat "cookie like" piece of about 8 mm diameter and place it on top of the tissue; wet with 15 µl H_2O.

- For each incubation period there are at least 3 skin models required.

- As a control, a set of additional equivalents is required per incubation time point. Spread 50 µl of 1 x PBS each instead of the test material.

- Incubate all skin models at RT for the desired exposure time.

8.1.2. Irritation Testing

- Liquids: Apply 30 µl of the sample topically to the centre of the skin equivalent by using a sterile micro-pipette. Cover with a sterile mesh.

- Semisolids: Dispense 30 µl directly on top of the tissue using a positive displacement pipette. Cover the entire surface of the model and cover with a sterile mesh.

- Solids: Crush and grind the substance with a mortar and pestle into fine ground substance. Fill a sharp spoon with at least 30 mg fine ground substance. Level the spoon by gently removing excess material with an appropriate aid, avoiding compression ("packing") of the material.
  Apply 50 µl PBS to the surface of the skin model before applying the substance on top. This increases contact between substance and the skin model surface.
  Apply the powder to the pre-wetted surface of the skin model. Ensure that the whole surface is covered with the powder.
  For materials with a waxy consistence try to form a flat "cookie like" piece of about 8 mm diameter and place it on top of the tissue; wet with 50 µl H_2O. Cover with a sterile mesh.

- For each material period there are at least three skin models required.

- As controls a set of additional equivalents is required. Spread 30 µl of 1 x PBS each instead of the test material.

- After 20 min incubation at RT, rinse the skin models thoroughly:
  Take off the mesh from the skin model. In order to remove the test material, rinse the tissue thoroughly by using a squeeze water bottle (recommended: Nalgene, FEP16-500) with 1x PBS (20 times), followed by rinsing the skin model by waving it in a beaker filled with 1 L 1x PBS.
  Ensure that the top and bottom are void of test substance.
  Repeat 20 times washing with PBS by using the squeeze bottle.
  Remove excess PBS by gently shaking the insert and blotting the bottom with a piece of paper towel.

- Place each insert into a new 6-well plate with fresh maintenance medium (1000µl).

- Place all inserts in an incubator at 37 °C, 5 % CO_2, 95 % humidity for 42 hrs.

- A change of the medium after 24 hrs is necessary.

8.1.3 Phototoxicity Testing

For statistical reasons, the assay has to be carried out in triplicates. The assay comprises testing of substances, which are applied to skin models followed by UV-irradiation (n=3) and the same substance applied onto skin models which will not be UV-irradiated (n=3). Controls are carried out in the same way. This means, for each test sample and for each control 6 skin models are required.

- Test Sample: Apply 30 µl of the test substance topically to the centre of the skin model by using a sterile micro-pipette.

- Controls: 1. Positive control: Apply 30 µl of the positive control (e.g. chlorpromazine 0.01 %) to the centre of the skin model.

  2. Negative control: Apply 30 µl of the negative control (1 x PBS) to the centre of the skin model.

- Incubate all skin models for 24 hrs at 37 °C, 5 % CO_2, 95 % humidity.

<u>Irradiated skin models</u>

Irradiate 3 skin models (one triplicate of test substance or control) with 5 J/cm^2 UV.

<u>Non-irradiated skin models</u>

In parallel store in the dark at room temperature the corresponding 3 skin models (serving as non-irradiated control tissues, one triplicate)

- .To remove the test substance from the surface of the skin model, rinse the tissue thoroughly by using a squeeze water bottle (recommended: Nalgene, FEP16-500) with 1 x PBS for at least twenty times, followed by rinsing the skin model by waving it in a beaker filled with 1 Litre 1 x PBS.
  Ensure that the skin models are void of test substance.

- Remove excess PBS by gently shaking the insert and blotting the bottom with a piece of paper towel.

- Incubate all skin models at 37°C, 5 % CO_2 and 95 % humidity for 18 hrs.

- Perform a standard MTT-assay to determine the viability of the models.

8.2 Addition of MTT

8.2.1 Corrosion

- Prepare a 1.0 mg/ml solution of MTT in MTT-assay medium and add 300 µl of the MTT medium to each well of a new 24-well plate.

- Prepare a second 24-well plate as a “holding plate” with 300 µl Maintenance Medium in each well.

- Pick up each insert with tweezers. For removing the test material, rinse the tissue gently with 1x PBS (20 times) using a squeeze bottle. Remove excess PBS by gently shaking the insert and blotting the bottom with a piece of paper towel.

- Place the insert into the prepared holding plate.

- Pick up each insert and remove excess medium by blotting the bottom on a paper towel.

- Transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- When every treated tissue has been rinsed transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- Incubate the 24-well plates for 3 hrs (37 °C, 5 % CO_2, 95 % humidity).

8.2.2 Irritation

- Prepare a 1.0 mg/ml solution of MTT in MTT-assay medium and add 300 µl of the MTT medium to each well of a new 24-well plate.

- Pick up each insert and remove excess medium by blotting the bottom on a paper towel.

- Transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- When every treated tissue has been rinsed transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- Incubate the 24-well plates for 3 hrs (37 °C, 5 % CO_2, 95 % humidity).

8.2.3 Phototoxicity

- Prepare a 1.0 mg/ml solution of MTT in MTT-assay medium and add 300 µl of the MTT medium to each well of a new 24-well plate.

- Pick up each insert and remove excess medium by blotting the bottom on a paper towel.

- Transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- When every treated tissue has been rinsed transfer each insert quickly into one well of the prepared 24-well plate with the MTT-assay reagent.

- Incubate the 24-well plates for 3 hrs (37 °C, 5 % CO_2, 95 % humidity).

8.3 Extraction of the formazan crystals

- Pick up the inserts with tweezers.

- Remove excess MTT-assay reagent by blotting the bottom with a paper towel.

- Transfer the inserts to a new 24-well plate.

- Add 2 ml of isopropanol directly to each insert. The insert in the well should be submerged completely.

- Wrap the plate in parafilm

- Shake the plate for 2 hours at RT on a vertical shaker or store at 2 - 8 °C overnight.

- Pierce / puncture the membrane of each insert with an injection needle (~gauge 20, ~ 0.9 mm diameter) and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded.

- Carefully shake on a vertical shaker for about 10 min.

- Dispense duplicates of 200 µl of each sample dilution to an appropriate well of a 96-well flat bottom plate.

- Using a 96-well plate reader spectrophotometer read the absorbance at 540 – 570 nm using isopropanol as a blank.

- If necessary dilute the sample extract 1:3 to 1:5 in isopropanol and repeat the photometric measurement.

- Viability is calculated as follows:

  Viability (%) = (absorbance test material / absorbance control) x 100

9. Preparation of cryo-cuts

9.1 Required material

- 24-well plate
- Pointed, bent pair of tweezers
- Fixation solution: 8 % formaldehyde in 200 mM HEPES – buffer pH 7.3
- Tissue freezing medium "OCT Compound" (R. Jung GmbH, Nussloch, Germany)
- Aluminium discs
- Liquid nitrogen
- Coated Slides ("Super Frost / Plus")
- Cryo-microtome
- Fine pipette 1000 µl
- Pasteur pipette
- Sharp scalpel

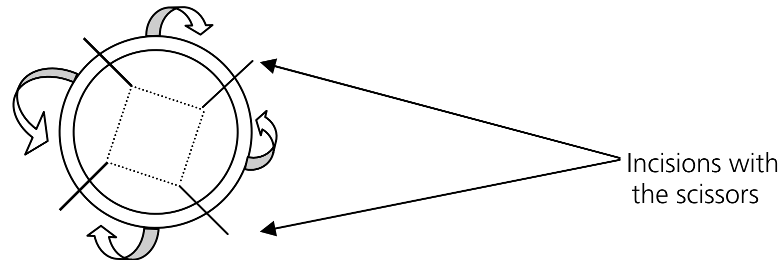
9.2 Fixation

- Place the inserts into a 24-well plate.
- Cover carefully the inserts with 1.5 ml fixation solution.
- Incubate at least 30 min at room temperature.
- Aspirate the fixation solution from top of the tissue using a Pasteur pipette.
  Take care not to touch and injure the epidermis.
- Wash 3 times for 5 minutes with 2 ml of PBS each. Remaining fixation solution is washed away by this treatment.
- Cut the membrane from the bottom of the insert using a sharp scalpel.

9.3 Embedding

- Make angled incisions (4 x ca. 3 mm) to the edges of aluminium discs (2 cm in diameter) with a pair of scissors.

- Bend the sides upwards and press the rims firmly together. This way a small dish is created for embedding of the tissue piece.



Bend the cut edges upwards

- Fill these dishes to one third with “OCT Compound”, place the skin equivalents on top, carefully adjust them so they lay straight and horizontal in the dish, fill up with “OCT Compound” until totally covered.

9.4 Freezing

- Fill a suitable container (Styrofoam, plastic safe for freezing, dewar) with liquid nitrogen.

- Grip the aluminium dishes with a pair of tweezers at one edge and hold it into the gaseous phase of the liquid nitrogen until the embedding medium looks homogenously white. This leads to slow freezing and therefore forming of ice crystals in the tissue is reduced.

- The preparation is now completely frozen and can be stored at – 80 °C.

9.5 Cutting

- Displace the embedded tissues from the aluminium dishes.

- Cut at – 30 °C with a cryo-microtome to 5 – 10 µm slides.

- Collect cuts on coated slides (“Super Frost / Plus”) and dry.

- Wash for 1 minute with 1x PBS.

- Wash for 1 minute in H_2O dest. and dry. Now, the cryo-cuts are ready for histological staining.