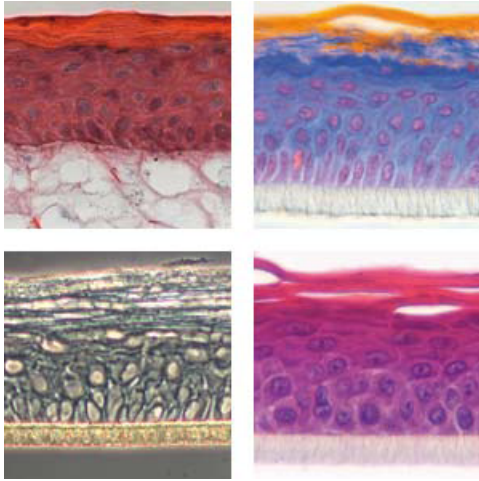


CellSystems®  
Biotechnologie Vertrieb GmbH



# Manual AST2000 Advanced 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](mailto: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 substances.

To satisfy these requirements CellSystems® provides two alternative *in vitro* skin models: the full-thickness model "Advanced Skin Test 2000 (AST2000), consisting of epidermal cell layers supported by a dermis equivalent underneath, and the epidermis model "Epidermal Skin Test 1000 (EST1000). These 3D all human skin models are ideal tools for pharmaceutical and chemical compound testing. Toxic or irritating effects of test substances can be determined by multiple end-point analysis including viability, histology and cytokine release.

AST2000 comprises reconstructed human epidermis with an underlying dermis equivalent and a functional basal lamina. The *in vitro* maturation of the epidermal part is stimulated by a culture at air-liquid interphase ("airlift culture") under defined media conditions. This physiological architecture allows the examination of the complex crosstalk between the differentiating keratinocytes and the supporting fibroblasts. AST2000 is cultured in inserts (0.6 cm<sup>2</sup>) with a polycarbonate membrane.

## 2. Kit Contents

- Human skin equivalents in 24-well plate, embedded in transport medium
- 50 ml Maintenance Medium
- 25 ml Assay Medium
- 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<sub>2</sub>)
- Pipettes (sterile)
- Pair of tweezers (sterile)
- Water bath 37°C

## 4. Instructions

### *Preparation on Receipt*

Immediately upon receipt check if the kit is complete and undamaged. Read the instructions completely and carefully 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 1500 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 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 obliquely the insert into the culture dish.
- Incubate the culture dishes at least 6 to 8 h - or overnight - at 37 °C, 5 % CO<sub>2</sub>, 95 % humidity before performing first experiments.
- After this adaptation phase, your test substances can be applied onto the stratum corneum or added to the medium.
- Cultivate the skin equivalents in the incubator (37 °C, 5 % CO<sub>2</sub>, 95 % humidity).
- In case you intend to cultivate the skin equivalents for more than 24 h, a daily medium change is required by aspirating the medium and replacing it by 1500 µl new maintenance medium (37°C) for each well.
- Store the medium at 4 °C.

## 5. Determination of Cell Viability by Standard MTT-Assay

One major parameter to evaluate effects of a given compound to the skin is the viability of the treated skin equivalents before and after exposure to the test substances.

For statistical relevance 3 skin equivalents should be used for each time point and compound. As a control PBS is spread onto one skin equivalent per time point.

Inserts should be incubated for the desired periods of time depending on the suggested effect. To spread the compound equally on the surface of the **AST2000** sometimes it might be necessary to cover the inserts with a piece of gauze. After the indicated time periods inserts 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 water insoluble purple formazan crystals. The addition of a detergent solubilizes the crystals. The amount of colour produced is directly proportional to the number of viable cells and the absorbance is quantified with an absorbance plate reader at a wavelength between 540 nm and 570 nm. The relation of the absorbance of a tested insert to the absorbance of a control insert defines the rate of cell survival.

A final classification of the toxic effects of the test substance becomes possible by comparing treated and untreated skin equivalents with those incubated with a reference substance.

## 6. Protocol for Substance Testing

### *6.1 Required Materials*

- Class II biological safety cabinet
- Incubator (37 °C, 5 % CO<sub>2</sub>)
- Water bath 37 °C
- 96-well plate reader spectrophotometer
- Pair of tweezers (sterile)
- Micro-Pipettes (sterile)
- Pipette tips
- Pipettes, 10 ml, sterile
- 24-well culture plate
- 96-well culture plate
- Centrifugation tubes, 15 ml
- Centrifugation tubes, 50 ml
- 1 x PBS (Phosphate buffered saline) , sterile
- MTT (Sigma) 1 mg/ml in maintenance medium (pre-warmed to 37 °C)
- Isopropanol

### *6.2 Application of Test Sample (under sterile conditions)*

- Prepare a solution of the test sample.
- Apply 10 µl of the sample topically to the centre of the skin equivalent by using a sterile pipette.  
For each incubation period there are at least 3 inserts required.
- One additional equivalent is required per incubation time as control. Spread 15 µl of 1 x PBS instead of test substance.
- Place all skin equivalents in an incubator at 37 °C, 5 % CO<sub>2</sub> for the desired exposure time.
- Use sterile forceps to pick up each insert and remove the test sample by washing each skin equivalent 3 times in at least 250 ml sterile 1 x PBS. Ensure that PBS is removed completely from the skin equivalents and inserts after washing.
- Transfer the inserts into a 24-well plate.

### *6.3 Addition of MTT*

- Prepare a 1.0 mg/ml solution of MTT in Assay Medium and add 1.3 to 1.6 ml (depending on the 24-well plate) of the MTT medium into each well (the skin equivalents must be submerged completely).
- Return the 24-well plate to the incubator for 3 hrs (37 °C, 5 % CO<sub>2</sub>, 95 % humidity).

### *6.4 Extraction of Formazan Crystals*

- Carefully detach the skin equivalent from the inner wall of the insert with forceps and transfer it to a 1.5 ml snap cap test tube.
- Add 500 µl of isopropanol to each tube ensuring that the sample is submerged. Cap the tubes tightly.
- Secure a rack with test tubes on a vortex platform at stage 3 for 1 h at RT.
- Centrifuge the caps for 5 min at 13.000 rpm.
- Dilute the sample extract 1:3 to 1:5 in isopropanol.
- At the end of the extraction period, dispense 200 µl of each sample extract to an appropriate well of a 96-well flat bottom plate.
- Use a 96-well plate reader spectrophotometer to measure the absorbance between 540 nm and 570 nm using isopropanol as blank.
- Viability is calculated as follows:

$$\text{Viability (\%)} = (\text{absorbance test substance} / \text{absorbance control}) \times 100$$

## 7. Preparation of Cryo-cuts

### *7.1 Required Materials*

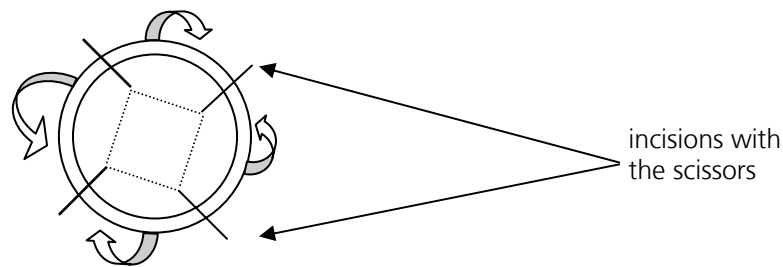
- 24-well plate
- Pointed, bent pair of tweezers
- Fixation solution: 8% formaldehyde in 200 mM HEPES – buffer pH 7.3
- Tissue freezing medium "OCT Compound" (R. Jung GmbH, Nussloch, Germany)
- Aluminium platelets
- Liquid nitrogen
- Slides (Super Frost Plus)
- Cryo-microtome
- Fine pipette 1000 µl
-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

### *7.2 Fixation*

- Place the inserts in a 24-well plate.
- Cover carefully the inserts with 1.5 ml fixation solution.
- Incubate 2 h 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 1 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.

### 7.3 Embedding

- Make angled incisions (4 x ca. 3 mm) to the edges of aluminium platelets (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.

### 7.4 Freezing

- Fill a suitable container (e.g. Dewar) with liquid nitrogen.
- Grip the aluminium dishes with a pair of tweezers at one edge and hold it into the gas 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  $-20\text{ }^{\circ}\text{C}$ .

### 7.5 Cutting

- Displace the embedded tissues from the aluminium dishes.
- Cut at  $-30\text{ }^{\circ}\text{C}$  with a cryo-microtome (5 – 10  $\mu\text{m}$  thickness).
- Collect cuts on coated slides ("Super Frost Plus") and dry.
- Wash for 1 minute with 1x PBS.
- Wash for 1 minute in  $\text{H}_2\text{O}_{\text{dest.}}$  and dry.  
Now, the cryo-cuts are ready for histological staining.

## 8. Paraffin Embedding

We highly recommend using CellSystems® Embedding Kit, Cat. No. CS-0011, consisting of 140 ml embedding wax and 6 stainless steel dishes.

### *8.1 Required Materials*

- Heating cabinet (adjustable to 37°C)
- Micro-pipettes
- Pasteur pipettes
- Pointed, bent tweezers
- 24-well plate
- Fixation solution: 4 % formaldehyde in 200 mM HEPES, pH 7.3
- 1 x PBS (Phosphate buffered saline)
- Ethanol

### *8.2 Fixation and Dehydration*

- Place inserts in a 24-well plate.
- Carefully add 1.3 to 1.6 ml (depending on the 24-well plate) of the fixation solution into each well (the skin equivalent must be submerged completely).
- Incubate 2 h at room temperature.
- Wash 4 times for 10 minutes by aspirating the solution carefully with a Pasteur pipette and by adding 1.3 to 1.6 ml (depending on the 24-well plate) PBS into each well (the skin equivalent must be submerged completely).
- Detach the skin equivalent carefully from the rim of the insert and lift it from the bottom using a pointed and bent pair of tweezers.
- Place the skin equivalents in appropriate dishes and add sequentially 15 %, 30 %, 50 %, 70 % ethanol in distilled water for 1 h each. Carefully remove the fluid each time not to cause any shrinkage by drying the skin equivalents.
- Finally, dehydrate twice for 30 minutes in ethanol<sub>abs</sub>.

### *8.3 Infiltration*

- Melt the Embedding Medium in the heating cabinet or in a water bath at 37 °C.
- Make 3 different infiltration solutions by mixing the Embedding Medium with ethanol at 37 °C completely:  
Solution I:           1 part Embedding Medium, 3 parts ethanol<sub>abs.</sub>  
Solution II:           1 part Embedding Medium, 1 part ethanol<sub>abs.</sub>  
Solution III:          3 parts Embedding Medium, 1 part ethanol<sub>abs.</sub>
- Remove the fluid of the last dehydration step and incubate with 2 ml of Solution I, II and III sequentially for 4 h at 37 °C each.
- Replace 2 times with 2 ml of undiluted Embedding Medium and incubate for 2 h at 37 °C each.

### *8.4 Embedding*

- Pipette 2 ml wax each in stainless steel embedding dishes (15 x 15 x 5 mm).
- Place the inserts in the centre of the dish and cover with another 2 ml of wax.
- Initial hardening of the preparation is performed at room temperature for 1 h then let it completely harden overnight at 4 °C.
- Cut the preparation at the microtome (5 - 10 µm). "Catch" the cuts on coated slides (Poly-L-Lysin, Aminoethoxysilan) and let them start to melt at 37 °C on a stretch bench.
- Wash the warm melted cut for 10 minutes in ethanol to remove the wax, then wash in distilled water and let dry.

### **Note**

Time indications are minimum times. Incubation of the infiltration steps are also possible overnight.

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