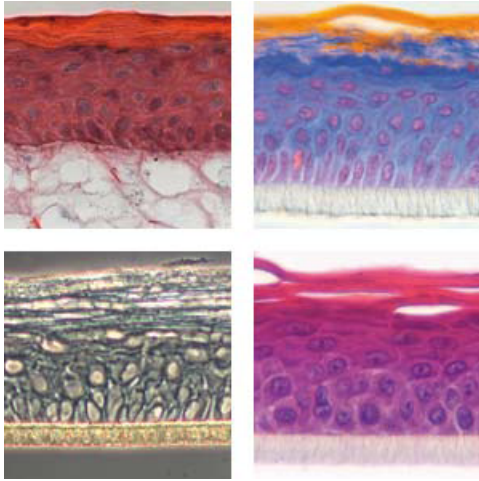


CellSystems®
Biotechnologie Vertrieb GmbH



Applications In Vitro Skin Models **EST1000/AST2000**



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

This application guide gives an overview of the use of two different reconstructed skin models from CellSystems®; **EST1000** (Epidermal Skin Test) and **AST2000** (Advanced Skin Test).

Epidermal Skin Test 1000 (**EST1000**) is a reconstructed epidermal model made from primary human keratinocytes from one neonatal donor. It comprises a fully differentiated epidermis with viable and cornified cell layers and is used as a 3D *in vitro* model for both validated toxicological applications and research applications.

Advanced Skin Test 2000 (**AST2000**) is a full thickness model. It comprises a dermal equivalent with embedded fibroblasts as a basis and an epidermal layer of keratinocytes on top. This model is mainly used in research applications but will also be used in some toxicological applications.

2. EST1000 in regulated *in vitro* toxicology testing

2.1 Skin Corrosion according to OECD TG 431

Corrosion is defined as irreversible destruction of tissue in response to exposition to chemically active substances, like organic and inorganic acids and bases. The security requirements for packing and handling these potentially dangerous substances make it necessary to test those substances for their corrosive effects. Toxicologically working research institutes use different *in vivo* test assays to define potential risks, generally based on methods of Draize et al. [6]. In an effort to replace animal testing by *in vitro* test methods OECD Test Guideline 431 (*In vitro* Skin corrosion: Human Skin Model Test) regulates the use of *in vitro* reconstructed, human skin models as approved test methods for distinguishing between corrosive and non-corrosive chemicals.

EST1000 (Epidermal Skin Test 1000) shows an amazing similarity to morphology and immunohistochemistry of normal human *in vivo* skin. Additionally, results of two blinded trial multicentre validation studies demonstrated that **EST1000** meets all OECD requirements for *in vitro* skin models.

EST1000 is an accepted *in vitro* assay model for skin corrosion testing. It is validated for the classification of compounds concerning skin corrosion according to the OECD test guideline 431 for the 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

Additional literature concerning "*in vitro* skin corrosion" can be found in the addendum: [1; 2; 4; 5; 20]

2.2 Skin Irritation according to OECD TG in draft

Skin Irritation is the visible, pathological alteration of tissue after single or repeated contact to reactive substances. Irritation, in comparison to corrosion, is defined by a less severe effect. Only in cases of strong irritation necrotic tissue lesions are caused. Therefore, effects caused by irritating substances are usually reversible pathologic processes. Despite intensive research the pathophysiological mechanisms being activated as a consequence of contact to irritating substances have not been fully understood yet. This is due to the complex interactions of skin cells with exogenous effects.

The barrier function of the skin can be changed through:

- Denaturation of skin proteins
- Removal of skin lipids
- Inhibition of cell proliferation
- Release of different biochemical mediators (cytokines, growth factors, chemokines and proteolytically active biomolecules)

As a consequence the permeability of the skin is markedly increased. Characteristically this causes a drastic increase in percutaneous absorption of the skin and increasing Transepidermal Water Loss (TEWL).

Because of the complexity of these biological processes the establishment of alternative test methods for risk evaluation for potentially irritating substances is extremely difficult. During a first validation study it has been demonstrated that human skin models were able to characterise potentially irritative substances *in vitro*. As a consequence ECVAM published the "Performance Standards for Applying Human Skin Models To *In Vitro* Skin Irritation Testing" in May 2007 [4]. An OECD test guideline for *in vitro* skin irritation is currently under development. **EST1000** is in prevalidation phase. On the basis of the newly published performance standards **EST1000** will undergo a blinded multicentre study in the context of a new OECD test guideline (in draft).

Additional literature on „*in vitro* skin irritation“ can be found in the addendum:
[6; 7; 8; 9, 15; 16; 17]

3. Non-regulated applications of EST1000 and AST2000

3.1 Photo Reaction (Phototoxicity)

Phototoxic substances develop a toxic effect on cells only in combination with high energetic radiation (i.e. UV-A of sun light). The testing for phototoxic potential of substances with the murine fibroblast cell line 3T3 is regulated by OECD test guide line 432 (3T3-NRU Phototoxicity Test). According to the guideline the test substances are dissolved in cell culture medium. Thereafter, the cells are irradiated with distinct doses of high energetic radiation and incubated under usual cell culture conditions. Finally, the viability of the irradiated fibroblasts is determined by the Neutral Red Uptake (NRU) assay. For this end point it is irrelevant which of the following biochemical mechanisms lead to the phototoxic effect of the substance.

- UV induced binding of the substances to cellular proteins and UV induced change of membrane characteristics
- Radiation induced substance decay into toxic derivatives
- Substance mediated formation of toxic end products by cellular metabolism under the influence of high energetic radiation

The 3T3-NRU-phototoxicity assay has important limitations:

- The test substances have to be dissolved in the cell culture medium. Insoluble substances can not be tested.
- Using a monolayer cell culture model precludes testing the influence on the physiological barrier.
- Topical application of test substances and therefore testing of formulations like creams and ointments is not possible.

By testing of substances with potentially phototoxic effects using CellSystems® skin models **AST2000** and **EST1000** it has been demonstrated that the number of substances of different nature can be increased by the use of three-dimensional skin models. Even insoluble compounds or formulations can be tested on their phototoxic effects by topical application. Additionally, the influence of the physiological barrier has been taken into account as well. In the end, particular experiments have shown differences between topical and systemic application of substances.

Table 1:

Use of 3D *in vitro* reconstructed human skin models for the evaluation of potentially phototoxic substances.

<i>Property</i>	<i>AST2000 EST1000</i>	<i>3T3-NRU⁽¹⁾</i>
<i>Validated test protocol</i>	NO	OECD TG 432
<i>Structure</i>	Three-dimensional architecture	Single cell monolayer
<i>Physiological barrier</i>	YES	NO
<i>Nature of test substance</i>	Topical application: all kinds of substances Systemic application: soluble substances	Limited to soluble substances
<i>Substance application topically and systemically</i>	YES	Only systemic application possible
<i>Influence of absorption of radiation by the medium</i>	Topical Application: NO Systemic Application: YES	YES

⁽¹⁾3T3 Fibroblast Neutral Red Uptake

Additional literature concerning "*in vitro* phototoxicity" can be found in the addendum: [11; 12, 13, 14]

3.2 Skin Sensitization

The most difficult task for toxicological research seems to be the search for reliable *in vitro* test methods for evaluation of potentially allergic effects of substances. Establishing *in vitro* experiments to test skin-sensitizing effects generally fail, because of the complex mechanisms leading to local immune reactions. From the immunological point of view this is based on the fact that besides epidermal keratinocytes and dermal fibroblasts additional immune competent cell types are involved in these mechanisms.

Table 2:

Cell types and their roles in the local antigen-specific immune reaction of the skin

<i>Cells of the Skin Immune System</i>	<i>Immunological function</i>
<i>Keratinocytes</i>	Initial processes for activation of dendritic cells (Langerhans Cells)
<i>Fibroblasts</i>	Interaction with keratinocytes and activated Langerhans cells support and lead the Langerhans cell migration
<i>Langerhans Cells</i>	Antigen uptake, processing and presentation in the local skin draining lymph node
<i>Cells of the local skin draining lymph nodes (T-Cells TH₁/TH₂)</i>	Induction of local antigen-specific immune responses and formation of an immunological memory
<i>Effector Cells (TH₁, Macrophages etc.)</i>	Allergen removal and regulation of the immune reaction

Several different research groups have treated freshly isolated PBMC's (Peripheral Blood Mononuclear Cells) with subtoxic doses of potentially sensitizing substances. They have shown activation increase by higher expression of typical surface marker proteins. Additionally, a characteristic expression pattern of the pre-inflammatory cytokines IL-1 alpha and IL-8 has been shown in experiments with reconstructed human epidermis models particularly after topical application of sensitizing substances. These results show that both keratinocytes and dendritic cells play a key role in the induction of antigen specific local immune reactions.

Until now it was not possible to provide a commercially available *in vitro* reconstructed skin model to the toxicological research community which includes functional dendritic cells. Recently, we were able to demonstrate in several studies using **AST2000** that the exposure to sensitizing substances in subtoxic doses leads to a release of IL-1 alpha, IL-6, IL-8 and immune modulating mediators particularly because of the crosstalk between epidermal keratinocytes and dermal fibroblasts. This is especially true for mediators of chemokine nature which besides their chemotactic properties also do have regulatory functions in development of local immune reactions.

In all relevant parameters suitable for characterization of potentially sensitizing substances which have been measured so far with our human, organotypical *in vitro* full-thickness and epidermis models are summarised.

Table 3:

Release of immunologically relevant factors in studies using Advanced Skin Test 2000 (AST2000) and Epidermal Skin Test 1000 (EST1000):

<i>Parameter</i>	<i>AST2000 In vitro reconstructed human full-thickness skin model</i>	<i>EST1000 In vitro reconstructed human epidermis model</i>
<i>IL⁽¹⁾-1 alpha</i>	+++	+++
<i>IL-8</i>	+++	+++
<i>IL-6</i>	+++	n.d.
<i>GM-CSF⁽²⁾</i>	++	n.d.
<i>PGE2⁽³⁾</i>	++	---
<i>Chemokine MCP-1⁽⁴⁾</i>	+++	n.d.
<i>Chemokine IP-10⁽⁵⁾</i>	++	n.d.

⁽¹⁾Interleukine

⁽²⁾Granulocyte Macrophage-Colony Stimulating Faktor

⁽³⁾Prostaglandine E2

⁽⁴⁾Monocyte Chemoattractant Protein 1

⁽⁵⁾Interferon-gamma Inducible Protein 10

+++ very well detectable

++ well detectable

--- not detectable

n.d. not determined

Additional literature on „in vitro skin sensitization“ can be found in the addendum:
[15; 16; 17; 18; 19]

4. Summary

There is a wide range of applications for both models, the **AST2000** and the **EST1000**. Depending on the questions the one or the other model can be used. The following tables 5 and 6 give you an overview about the parameters that can be measured in the respective model and a range of applications of the models. The CellSystems® team is looking forward to discuss with you the ideal model for your project and to answer your questions.

Table 5: Detectable parameters using **AST2000** and **EST1000**

<i>Parameter</i>	<i>AST2000 human full- thickness model</i>	<i>EST1000 human epidermis model</i>	<i>Literature</i>
<i>Physiological barrier</i>	++	+++	[9;13;17;18;19]
<i>Cell viability (MTT)</i>	+++	+++	[9;13;17;18;19]
<i>Morphology comparison: in vivo/in vitro</i>	+++	+++	[9;13;16;17;18;19]
<i>Physiological lipid distribution</i>	++	+++	
<i>Spectrum of epidermal markers</i>	+++	+++	[9;13;16;17;18;19]
<i>IL⁽¹⁾-1 alpha</i>	+++	+++	[9;18]
<i>IL-8</i>	++	++	[9;16;17;18]
<i>IL-6</i>	++	n.d.	[16;18]
<i>GM-CSF⁽²⁾</i>	++	n.d.	[9]
<i>PGE2⁽³⁾</i>	+++	---	[9]
<i>Interaction dermis/epidermis</i>	+++	---	[9;16;18]
<i>Recovery experiments (MMPs⁽⁴⁾)</i>	++	(+)*	[9;16;18]
<i>Spectrum of cytokines</i>	+++	(+)**	[16;18]
<i>Spectrum of chemokines</i>	+++	(+)**	[16;18]

⁽¹⁾Interleukine

⁽²⁾Granulocyte Macrophage-Colony Stimulating Faktor

⁽³⁾Prostaglandine E2

⁽⁴⁾Matrixmetalloproteinases

+++ very well detectable

++ well detectable

--- non detectable

(+)* limitations, but no MMPs

(+)** limited because no interaction with dermal fibroblasts possible

n.d. not determined

Table 6:
Applications of AST2000 and EST1000

<i>Application</i>	<i>AST2000 In vitro reconstructed human full-thickness skin model</i>	<i>EST1000 In vitro reconstructed human epidermis model</i>
<i>In vitro corrosion</i>		X
<i>In vitro irritation</i>		X
<i>Phototoxicity</i>	X	X
<i>Sensitization</i>	X	
<i>Proliferation and differentiation studies</i>	X	X
<i>Drug metabolism</i>	X	X
<i>Percutane absorbtion / penetration</i>		X
<i>Wound ealing / recovery</i>	X	
<i>Genotoxicity</i>	X	X
<i>Reconstituted target tissue for toxicogenomics & proteomics</i>	X	X
<i>Basic research (studies on mechanisms)</i>	X	X

(X) Application

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