Reconstruction of full thickness skin equivalents using BRANDplates® Insert System
Cleanroom production

BRAND disposable items for the life sciences are produced using the most advanced cleanroom techniques in one of the world's largest cleanrooms for laboratory disposable items.

The ongoing cleanroom monitoring includes continuous measurements of air particulates, positive air pressure, air exchange rate, room temperature, the relative humidity, among other things. This ensures that the actual parameters can immediately be checked against the nominal values. Deviations are detected immediately, and suitable countermeasures can be taken before the limit values are exceeded.

The high-precision control of environmental conditions provides a very high degree of stability in the corresponding parameters, especially the room temperature. This uniformity, together with quality testing of the final product by batch, guarantees the consistently high quality of life science products from BRAND.

For the production of disposables, Class 8, 7 and 5 manufacturing environments are available. Compliance of our Class 8 cleanroom with ISO 14664-1 is validated and certified by external, independent auditors.
Reconstruction of full thickness skin using BRANDplates® Insert System

Author: Lena Schober, Andrea Traube; Fraunhofer Fraunhofer-Institut für Produktionstechnik und Automatisierung IPA, Nobelstraße 12, 70569 Stuttgart, Germany

Introduction

In the past decade new cell and tissue culture technologies have been generated to comply with the European legislation that restricts animal experiments to a minimum. Particularly, the improvement of culture conditions for reconstructed human full thickness skin and epidermis equivalents based on cell culture inserts, lead to a successful commercialization of these tissue models, e.g. EpiDerm™ and EpiDerm™ FT (MatTek, Ashland, MA), EpiSkin™ and EpiSkin™ FTM (SkinEthic, Lyon, France) or Phenion FT (Henkel, Düsseldorf, Germany). Today, with the help of artificial human tissues, pharmaceutical and cosmetic industry carry out tolerance, toxicology and irritation studies daily. In spite of every progress made in terms of media compositions and supplements, setup and handling of organotypic cultures still requires a lot of time and expertise.

Hands-on time and human-induced variations in culture processes can negatively impact success in high throughput reconstruction of human tissue. To reduce sources of unintended process fluctuations and to ensure high quality and reproducibility of in vitro tissues, the Fraunhofer Society and BRAND GMBH + CO KG collaborated in the development of the BRANDplates® Insert System. This 24-well platform is specially designed to meet all requirements for a totally automated handling of insert-based tissue cultures. The carrier plates are designed in a 24-well or in a modified 6-well shape according to requirements of ANSI/SLAS standards 1 and 4 (Figure a) and b)). The corresponding BRANDplates® Insert Strips consist of 4 inserts in a row (Figure c) and are held in a fixed position at any time of automated handling.
BRANDplates® Inserts are available with the Inlet Opening System (IOS) (patent pending, Figure c, page 3) which is dedicated to support the automated in vitro reconstruction of human skin. This peerless feature interconnects the medium of wells and inserts, giving the opportunity to establish the air-liquid interface without entering the inserts with pipette tips. In addition to this increase in safety for cultures, the IOS reduces the number of pipetting steps needed to change medium within the two compartments.

This user manual describes in short the reconstruction of full thickness skin equivalents and provides tips for the handling of BRANDplates® Insert System.

6-well plate:
- Use just one or two inserts per well to extend medium change interval.
- For up to 4 inserts, medium in the well can be changed in one step (Figure b, page 3).

Inlet Opening System (IOS)
- No leaking during cell seeding or initial coating.
- Simultaneous change of medium in the well and insert.
- Setup of air-liquid-interface in one step.
- Compatible with 24- and 6-well BRANDplates®.

Insert:
- Divided BRANDplates® Insert Strips for subsequent analysis.

**Volumes needed for different culture phases**

<table>
<thead>
<tr>
<th></th>
<th>24-well</th>
<th>6-well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert (e.g. coating, cell seeding)</td>
<td>50-400 µl</td>
<td>50-400 µl</td>
</tr>
<tr>
<td>Well: submerged culture</td>
<td>1.6-2 ml</td>
<td>8-10 ml</td>
</tr>
<tr>
<td>Well: air-liquid interface (wetted membrane)</td>
<td>0.8 ml</td>
<td>3.5 ml</td>
</tr>
</tbody>
</table>
I. Preparation of dermal components for full thickness skin equivalents

1. Passaging of primary fibroblasts

Splitting of fibroblasts:
1. Aspirate culture medium.
2. Wash culture with prewarmed PBS.
3. Add 0.25 % trypsin/EDTA, incubate for 5 min at 37° C, 5 % CO₂.
   - T 25 flask: 2 ml
   - T 75 flask: 4 ml
   - T175 flask: 8 ml
4. Stop trypsin digestion by DMEM + 10 % FCS.
5. Transfer fibroblasts into a centrifuge tube.

2. Preparation of Collagen type I fibroblast mixture

3. Establishing fibroblast – collagen submers culture

Protocol according to Promocell

> 400 µl collagen - fibroblasts mix is needed per dermal equivalent.

Advice: calculate an extra volume of 30 % to compensate for pipetting errors!
> 4.0 x 10⁴ fibroblasts/400 µl
> 5.2 x 10⁴ fibroblasts/520 µl

520 µl total

347 µl collagen
173 µl gel neutralizing medium

Gel neutralizing medium contains:
1. 25 µl/ml Chondroitin-4-sulfate (gel cross linker)
2. 25 µl/ml Chondroitin-6-sulfate (gel cross linker)
3. 2 x DMEM
4. 90 mM HEPES
5. 3 % FCS

> Recommended collagen type I concentration: 6 mg/ml.
> Preparation of collagen mixture on ice impede risk of premature gelation.
> Use chilled pipets while mixing collagen.
> Avoid air bubbles!
> Collagen type I is usually dissolved in acetic acid (0.1 M, pH 3) and needs to be neutralized by titration of NaOH before adding cells.
> Alternatively use medium containing HEPES and/or sodium dicarbonate.
> Use inserts with membranes of 8 µm pore size!

Use of BRANDplates® Inserts with Inlet Opening System (IOS)

> Prevents culture damage during medium application/changes.
> Reduced number of pipetting steps needed for medium application/changes.
> Provides lateral nutrient supply for collagen embedded fibroblasts.

Volume for submers culture in BRANDplates®:
> 24 well: 2 ml
> 6 well: 10 ml
II. Co-culture of collagen embedded fibroblasts and keratinocytes

4. Coating dermal equivalents with fibronectin

1. Uncover collagen gel
2. Add 25 µl fibronectin on top
3. Incubate for 30 min, 37° C, 5 % CO₂

DMEM + 5 % FCS + 1 % Gentamycin

4. Coating dermal equivalents with fibronectin

0 - 7 DIV (Days in vitro)
> Cultivate dermal equivalents for 5-7 DIV.
> Change medium every 2 days.
> Stepwise reduce FCS-concentration from 10 % to 5 %.

5. Passaging of primary keratinocytes

1. Uncover collagen gel
2. Add 25 µl fibronectin on top
3. Incubate for 30 min, 37° C, 5 % CO₂

transfer 1*10⁵ cells/equivalent into a new centrifuge tube

per equivalent: resuspend keratinocytes in 100 µl KBM + 5 % FCS

Submers culture in KGM

5 min, 160-300 x g at RT

5 min, 160-300 x g at RT

Fibronectin [50 µg/µl]

IOS

Contracted collagen gel

Fibroblasts

Membrane

Splitting of keratinocytes:
1. Remove culture medium.
2. Wash culture with prewarmed PBS/EDTA.
3. Add PBS/EDTA and incubate 10 min at 37° C, 5 % CO₂
4. Add 0.025 % trypsin/EDTA, incubate 5 min at 37° C, 5 % CO₂
   - T 25 flask: 2 ml
   - T 75 flask: 4 ml
   - T175 flask: 8 ml
5. Stop trypsin digestion by KBM + 10 % FCS.
6. Transfer keratinocytes into a centrifuge tube.

6. Seeding of keratinocytes on top of dermal equivalents

seed 1 x 10⁵ cells in 100 µl equivalent

incubate 45 min at 37° C, 5 % CO₂

Keratinocytes

submers culture in KGM

+ 5 % FCS + 0.06 mM CaCl₂

DMEM
+ 5 % FCS
+ 1 % Gentamycin

During fibronectin incubation

> KBM: keratinocyte basal medium

When trypsinization is inefficient:
> Add recommended volume of trypsin/EDTA, incubate for 2-3 min at 37° C.
> Carefully remove solution from the culture, add trypsin/EDTA again and incubate for another 5 min at 37° C.
> Keratinocytes may react sensible to trypsin. Do not expose keratinocytes for more than 10 min to trypsin.
> Check under a microscope whether cells start to detach!
> Max. activity of trypsin is given at 37° C and between pH 7.6-7.8.
> Make sure that culture flasks are tightly sealed to avoid CO₂ dependent pH decrease in the incubator.

After fibronectin coating

> Co-culture fibroblasts and keratinocytes for 6 to 7 DIV. Change medium every 2 days.
> Stepwise reduce FCS-concentration from 5 % to 0.

KGM: Keratinocyte Growth Medium
> KBM final concentration
  + Bovine Pituitary Extract 0.4 % v/v
  + Insulin (recombinant human) 5.0 µg/ml
  + Hydrocortisone 330 ng/ml
  + Epidermal Growth Factor (recombinant human) 125 ng/ml
  + Epinephrine 390 ng/ml
  + Transferrin, holo (human) 10 µg/ml
  + CaCl₂ 0.06 mM
  + 1 % Gentamycin
(accord. to KG M2, PromoCell, Heidelberg, Germany)
III. Air-lift culture

7. Keratinocyte proliferation and stratification

- Medium: KGM + 5% FCS + 0.06 mM CaCl₂
- Medium: KGM + 2% FCS + 0.06 mM CaCl₂
- Medium: KGM no FCS + 0.06 mM CaCl₂

8. Histological characterization of full thickness skin equivalents

- Haematoxylin/eosin staining

Well known markers for keratinocyte proliferation and differentiation:

- Keratin 5 (K5)/ Keratin 14 (K14): building heterodimers, subunits of a common intermediate filament, proliferation marker, normally expressed by keratinocytes of the stratum basale (Poumay et al., 2004)

- Keratin1 (K1)/ Keratin 10 (K10): building heterodimers, subunits of a common intermediate filament, early differentiation marker, expressed by keratinocytes of stratum spinosum (Poumay et al., 2004).

- Involucrin: localized in stratum spinosum and stratum corneum. Involucrin is part of the cornified envelope of corneocytes.

- Filaggrin: a terminal differentiation marker in stratum corneum, present as profilagrin in stratum granulosum, important for skin barrier function (Sandiland A et al., 2009)

- Loricrin: expressed in stratum granulosum and deposited beneath the plasma membrane. Cross-linked to other proteins like involucrin, repetin, S100 proteins by transglutaminase-1, loricrin is part of the cornified envelope (Steinert PM et al., 1995).
Summary

The use of the BRANDplates® Insert System has various advantages when compared to common cell culture inserts. The special 6-well plate utilizes a unique conjoined 24-well design to optimize centering of the well insert during the entire culture process. The geometry of inserts and plates define the so called feeding port. This extra cavity enables access to the well without shifting or rotating the inserts and disturbing the culture. The defined location of inserts and feeding ports helps to determine the position of applicators or aspirators integrated in automated processes. These attributes make the BRANDplates® Insert System the only 6- and 24-well culture insert platform which can be implemented totally into a robot handled cell culture.

Ordering Data

**BRANDplates® Insert Strips**

Insert Strips, smooth-walled or with inlet channels (Inlet Opening System*). PS. cellGrade™ plus surface, sterile. Strips of 4 inserts (divisible).

<table>
<thead>
<tr>
<th>Description</th>
<th>Pore size μm</th>
<th>Pack of</th>
<th>PC membrane Cat. No.</th>
<th>PET membrane Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>smooth-walled</td>
<td>0.8</td>
<td>12 (individually wrapped)</td>
<td>782860</td>
<td>782870</td>
</tr>
<tr>
<td>with Inlet Opening System</td>
<td>0.8</td>
<td>12 (individually wrapped)</td>
<td>782861</td>
<td>782871</td>
</tr>
</tbody>
</table>

**BRANDplates® Insert System**

6-well plates filled with 6 insert strips, PS. cellGrade™ plus surface, sterile. Insert strips, smooth-walled or with inlet channels (Inlet Opening System*). With lid with condensation rings.

<table>
<thead>
<tr>
<th>Description</th>
<th>Pore size μm</th>
<th>Pack of plates with lid</th>
<th>PC membrane Cat. No.</th>
<th>PET membrane Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>smooth-walled</td>
<td>0.8</td>
<td>5 (30 insert strips)</td>
<td>782862</td>
<td>782872</td>
</tr>
<tr>
<td>with Inlet Opening System</td>
<td>0.8</td>
<td>5 (30 insert strips)</td>
<td>782863</td>
<td>782873</td>
</tr>
</tbody>
</table>

* patent pending
** additional Insert Plates, Strips, and System products available. For more information, www.brandtech.com

BRANDplates®, BIO-CERT® and BRAND® are trademarks of BRAND GMBH + CO KG, Germany. BrandTech® is trademark of BrandTech® Scientific. Other reproduced brands are the property of the respective owner.

Our technical literature is intended to inform and advise our customers. However, the validity of general empirical values, and of results obtained under test conditions, for specific applications depends on many factors beyond our control. Please appreciate, therefore, that no claims can be derived from our advice. The user is responsible for checking the appropriateness of the product for any particular application.

Subject to technical modification without notice. Errors excepted. 0214

For product information in the USA and Canada, contact BrandTech® Scientific, Inc. at 888-522-2726, www.brandtech.com