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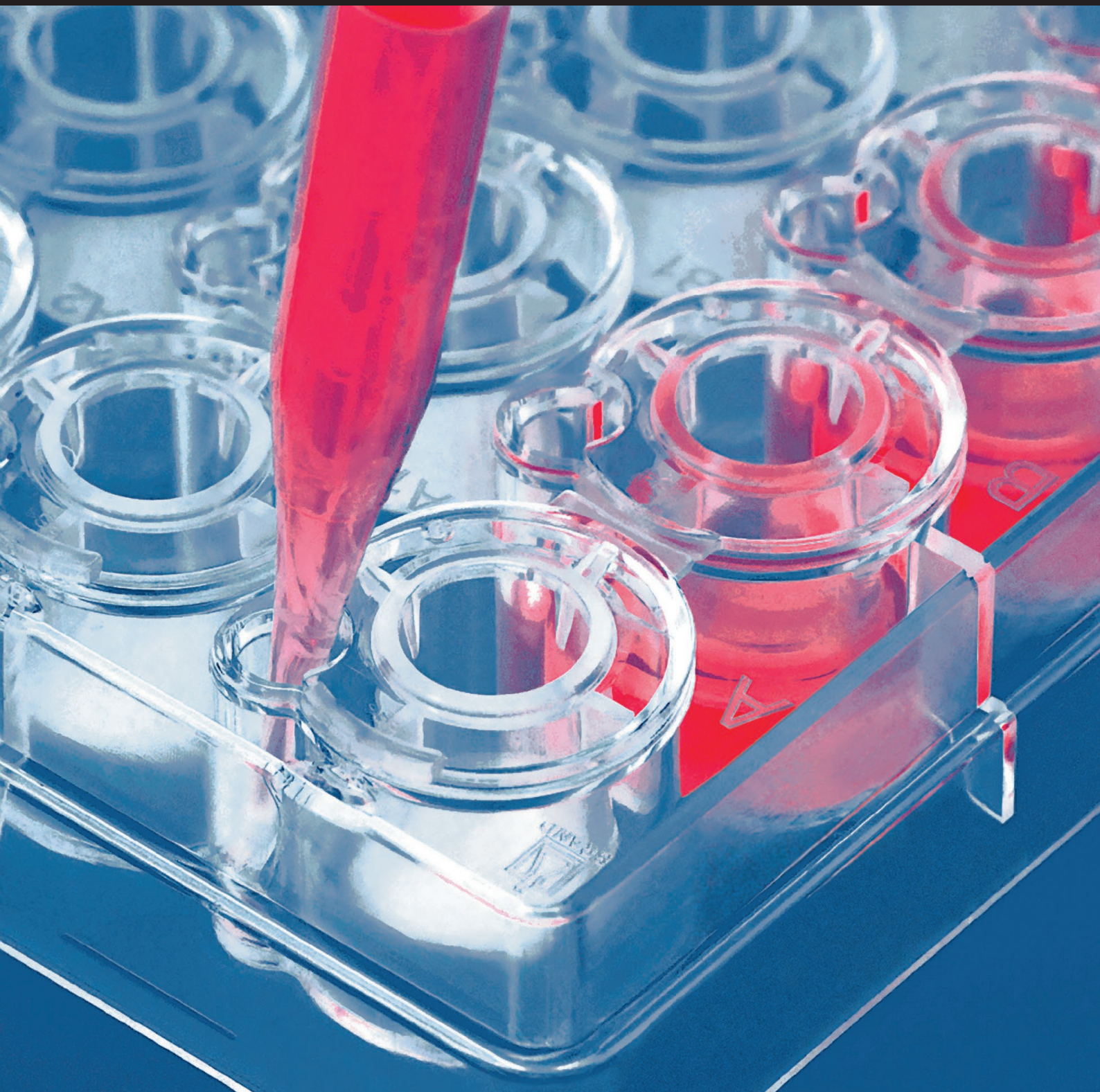
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BRAND

Reconstruction of full thickness skin equivalents using BRAND*plates*® Insert System

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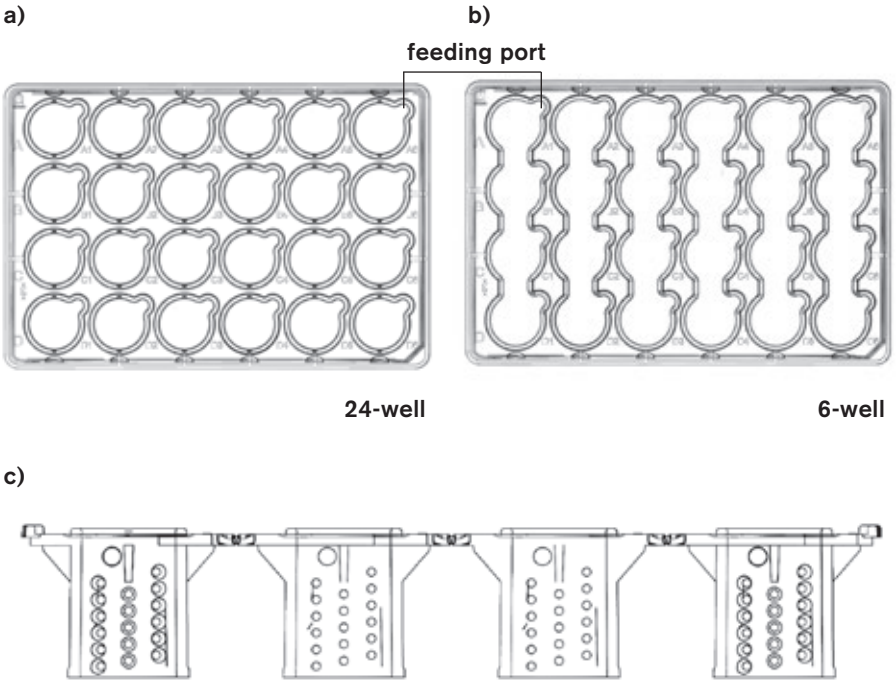
Reconstruction of full thickness skin using BRAND*plates*® Insert System

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Introduction

In the past decade new cell and tissue culture technologies have been generated to follow the European legislative 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 day by day. In spite of every progress made in terms of media compositions and supplements, setup and handling of organotypic cultures still needs a lot of time and expertise.



Up to now, increased hands-on time and human-induced variations in culture processes can cut the success in high throughput reconstruction of human tissue. To deplete 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 BRAND*plates*® Insert System. This 24-well platform is especially customized 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 BRAND*plates*® Insert Strips consist of 4 inserts in a row (Figure c) and are hold in a fixed position at any time of automated handling.

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, pag. 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- und 6-well BRAND*plates*®.

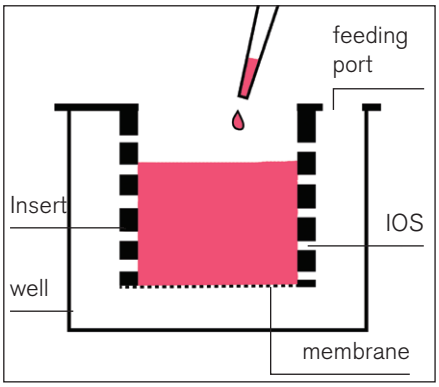
Insert:

- Divided BRAND*plates*® Insert Strips for subsequent analysis.

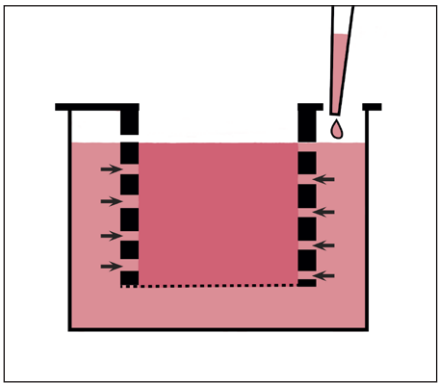
Volumes needed for different culture phases

	24-well	6-well
Insert (e.g., coating, cell seeding)	50 - 400 µl	50 - 400 µl
Well: submerged culture	1.6 - 2 ml	8 - 10 ml
Well: air-liquid interface (wetted membrane)	0.8 ml	3.5 ml

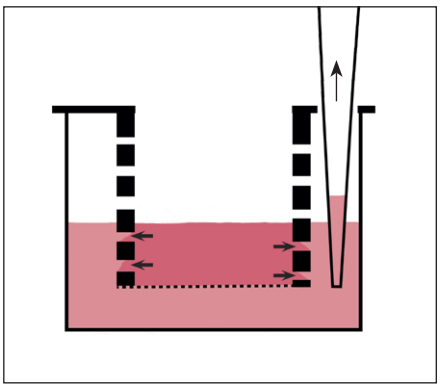
1) Coating, cell seeding



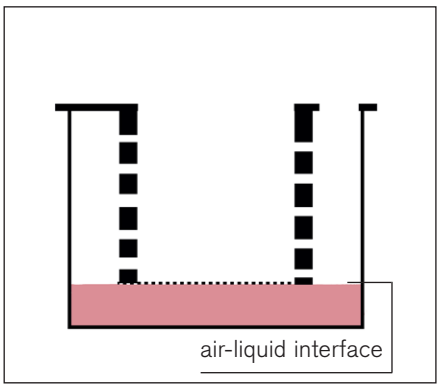
2) Medium application for submerge culture



3) Medium change



4) Establish air-liquid interface



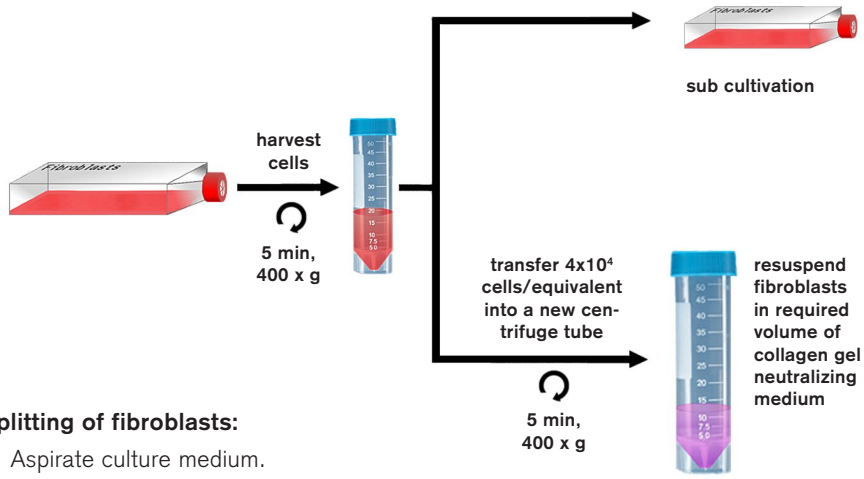
BRAND*plates*® Inserts are available with the Inlet Opening System (patent pending, Figure c, pag. 3) which is dedicated to support the automated *in vitro* reconstruction of human skin. This peerless feature interconnects the medium of wells and inserts, giving the opportunity to establish the air-liquid 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 BRAND*plates*® Insert System.

I. Preparation of dermal components for full thickness skin equivalents

Protocol according to Promocell

1. Passaging of primary fibroblasts

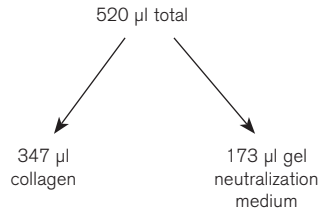


Splitting of fibroblasts:

1. Aspirate culture medium.
2. Wash culture with prewarmed PBS.
3. Add 0.25 % trypsin/EDTA, incubate for 5 min at 37°C, 5 % CO_2.
   - 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.

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

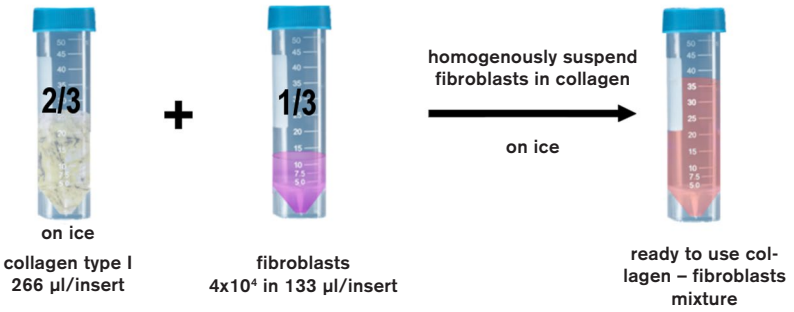
Advice: calculate an extra volume of 30 % to compensate for pipetting errors!
> 4.0 x 10^4 fibroblasts/400 µl
> 5.2 x 10^4 fibroblasts/520 µl



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

2. Preparation of Collagen type I fibroblast mixture

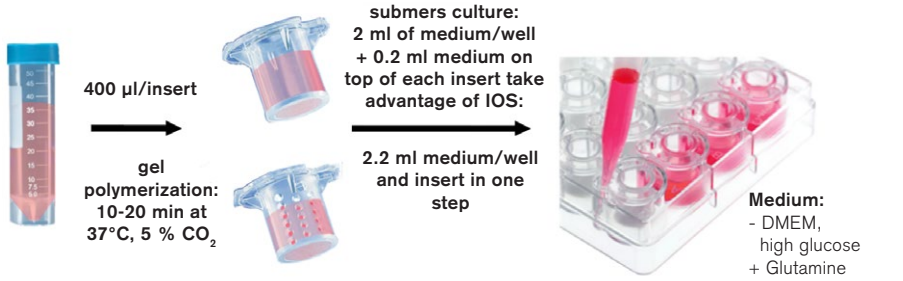


> Recommended collagen type I concentration: 6 mg/ml.

> 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!

3. Establishing fibroblast – collagen submerse culture



Use of BRAND*plates*® Inserts with Inlet Opening System (IOS)

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

Volume for submers culture in BRAND*plates*®:

> 24 well: 2 ml
> 6 well: 10 ml

II. Co-culture of collagen embedded fibroblasts and keratinocytes

0 - 7 DIV

> Cultivate dermal equivalents for 5-7 DIV.
> Change medium every 2 days.
> Stepwise reduce FCS-concentration from 10 % to 5 %.

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 CO_2 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.

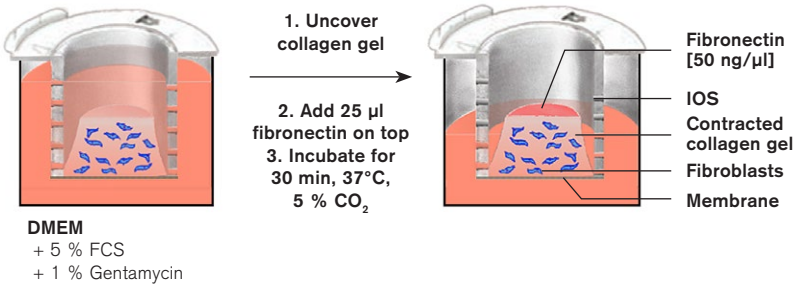
KGM: Keratinocyte Growth Medium

> KBM final concentration

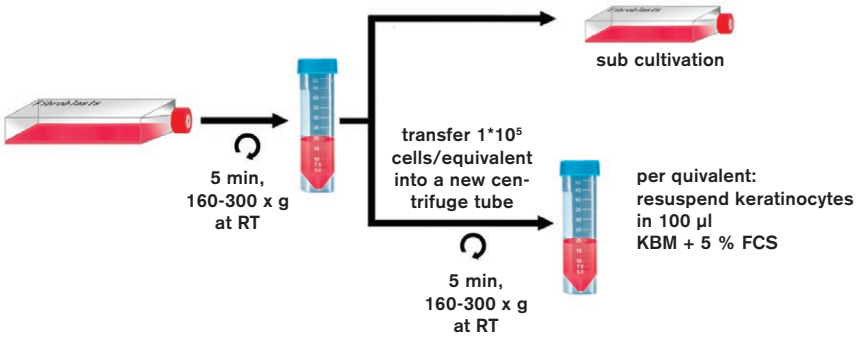
+ Bovine Pituitary Extract	0.4 % v/v
+ Insulin (recombinant human)	5.0 µg/ml
+ Hydrocortisone	330 ng/ml
+ Epidermal Growth Factor (recombinant human)	125 ng/ml
+ Epinephrine	390 ng/ml
+ Transferrin, holo (human)	10 µg/ml
+ $CaCl_2$	0.06 mM
+ 1 % Gentamycin	

(accord. to KGM2, PromoCell, Heidelberg, Germany)

4. Coating dermal equivalents with fibronectin



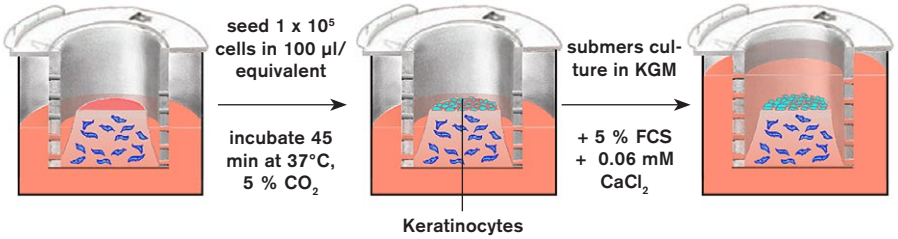
5. Passaging of primary keratinocytes



Splitting of keratinocytes:

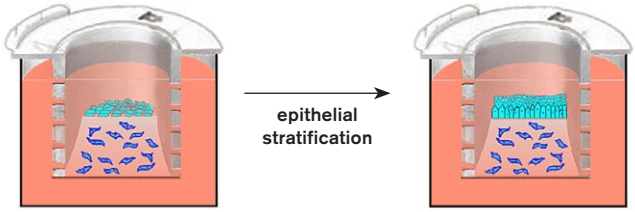
1. Remove culture medium.
2. Wash culture with prewarmed PBS/EDTA.
3. Add PBS/EDTA and incubate 10 min at 37°C, 5 % CO_2.
4. Add 0.025 % trypsin/EDTA, incubate 5 min at 37°C, 5 % CO_2.
   - T 25 flask: 2 ml
   - T 75 flask: 4 ml
   - T175 flask: 8 ml
5. Stop trypsin digestion by KBM + 10 % FCS.
6. Transfer keratinocytes into a centrifuge tube.

6. Seeding of keratinocytes on top of dermal equivalents



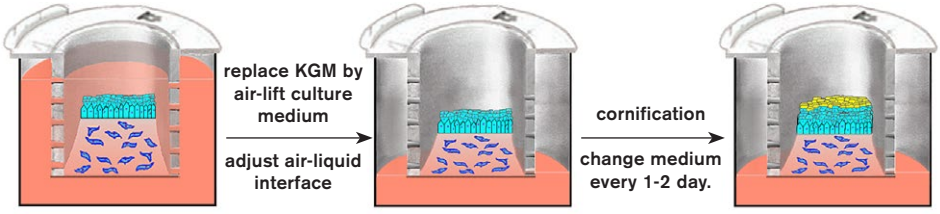
III. Air-lift culture

7. Keratinocyte proliferation and stratification



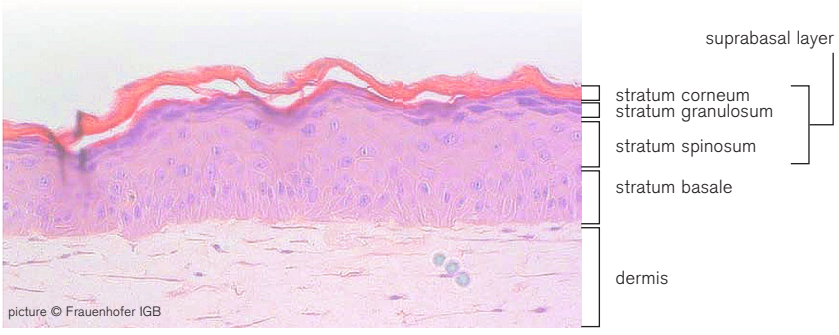
Medium: KGM
+ 5 % FCS
+ 0.06 mM $CaCl_2$

Medium: KGM
+ 2 % FCS
+ 0.06 mM $CaCl_2$



Medium:
KGM no FCS
+ 0.06 mM $CaCl_2$

8. Histological characterization of full thickness skin equivalents



haematoxylin/eosin staining

8 - 19 DIV

Air-lift-medium:

> KGM supplemented with
+ Recombinant human insulin
+ Hydrocortisone
+ Transferrin, holo (human)
+ Epinephrine
+ 1.88 mM $CaCl_2$

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 croneum. Involucrin is part of cornified envelope of corneocytes.

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

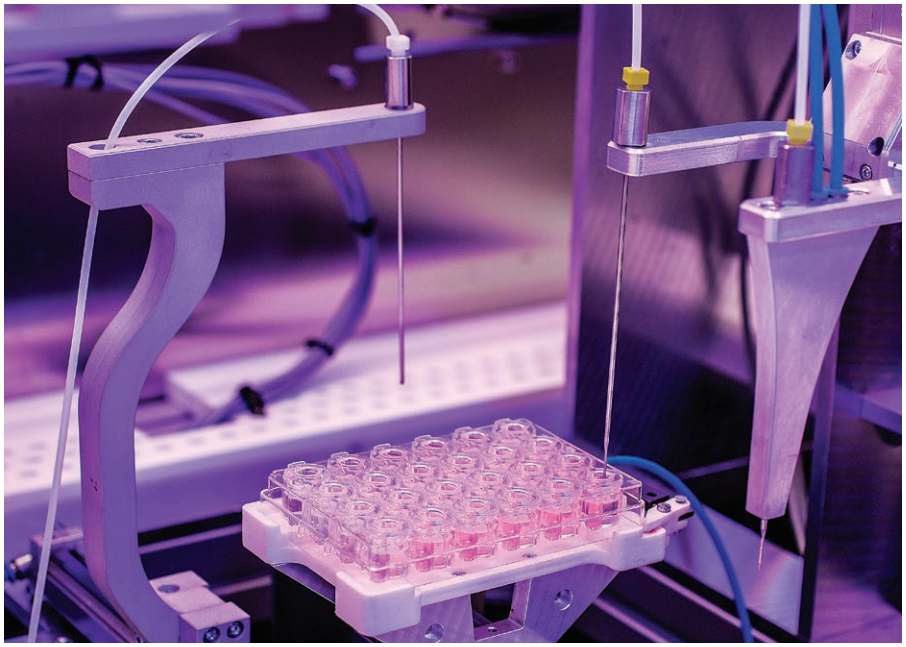
Loricrin:
expressed in stratum granulosum and deposited beneath the plasma membrane. Cross-linked to other proteins like involucrin, repetin, S100 proteins by transglutaminase-1, loricrin is part of the cornified envelop (Steinert PM et al., 1995).

Summary

The use of the BRAND*plates*® 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 inserts during the entire culture process. The geometry of inserts and plates define the so called feeding port. This extra cavity guarantees 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 BRAND*plates*® Insert System the only 24-well culture insert- platform which can be implemented totally into a robot handled cell culture.

The described properties of the 6-well plate create even more benefit when combined with BRAND*plates*® Inserts showing the Inlet Opening System (IOS) (patent pending). The medium of wells and inserts is interconnected by the IOS, so that it is possible to control medium level in both compartments simultaneously resulting in just 6 instead of 48 pipetting steps during medium exchange and when an air-liquid interface needs to be established.

The fusion of four inserts into one insert strip provides also remarkable advantages. Whenever inserts have to be moved, it is easy to grip one strip and transfer the four inserts in one move, e.g. transferring inserts from well to well during fixation, dehydration, washing or staining steps.



Ordering Data

BRAND*plates*® Insert Strips

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

Description	Pore size µm	Pack of	PC membrane Cat. No.	PET membrane Cat. No.
smooth-walled	8.0	12 (individually wrapped)	**7828 60**	**7828 70**
with Inlet Opening System	8.0	12 (individually wrapped)	**7828 61**	**7828 71**

24-well and 6-well multiwell plates

With lid. Pack of 10 pieces (individually wrapped).

Surface	Wells	Working volume (ml)	Grpwth surface (mm²)	Cat. No.
pureGrade™ S	24	3.1	210	**7828 80**
cellGrade™ plus	24	3.1	210	**7828 90**
pureGrade™ S	6	8 - 10	855	**7828 81**
cellGrade™ plus	6	8 - 10	855	**7828 91**

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