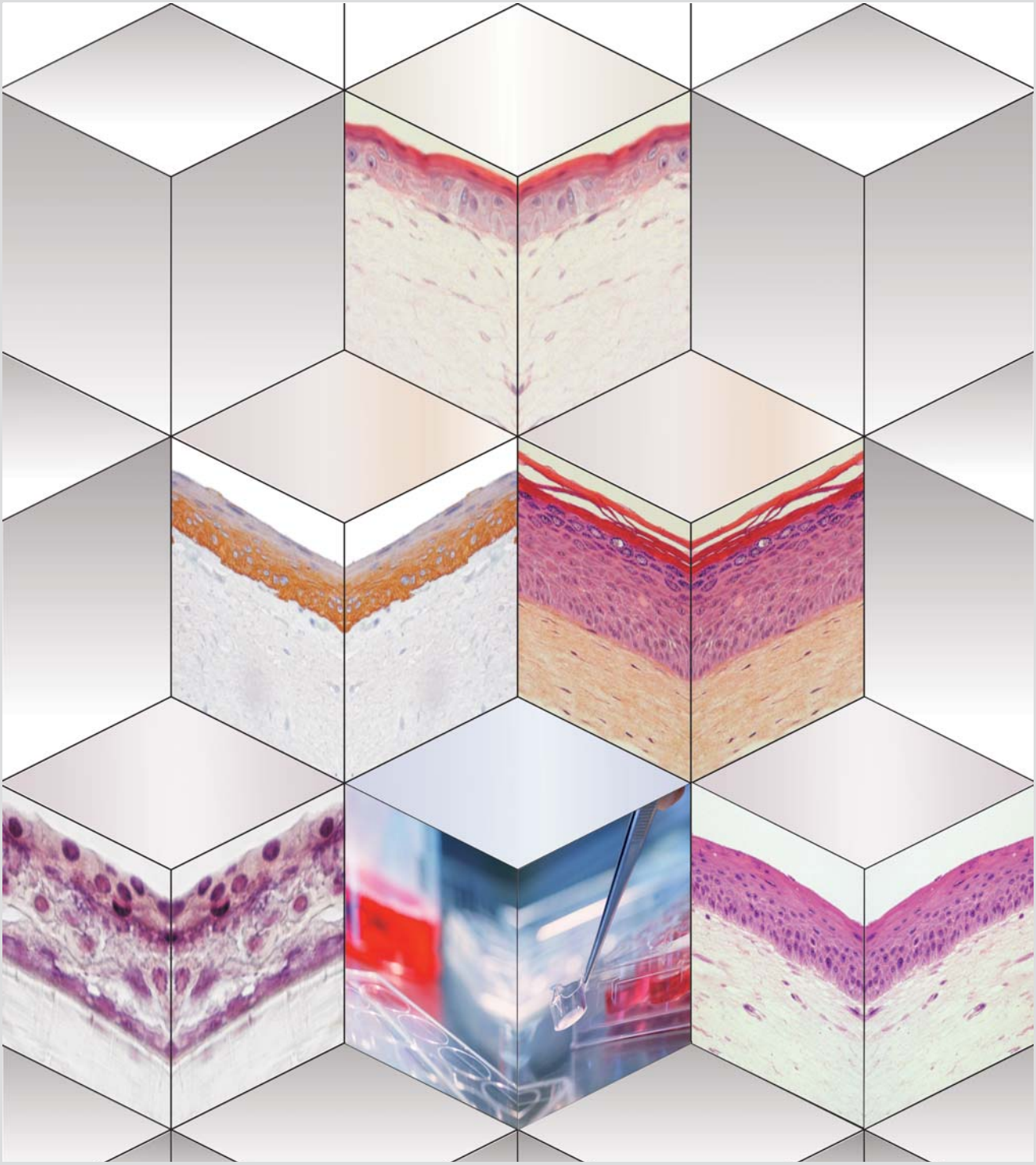
Application Note

Tissue reconstruction using ThinCert™ Cell Culture Inserts and Plates



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Introduction

For many years live-animal experimentation provided the singular means to access intact and living tissue for biological and pharmaceutical research. This situation changed with the introduction of new cell culture approaches that enabled reconstruction of tissue by multi-layering individual cell types *in vitro*.

Such approaches typically involve the use of porous membrane supports and/or three-dimensional collagen gels to re-create *in vivo*-like growth within a cell culture environment (Bell et al., 1979; Naughton et al., 1989). With the availability of primary cells from biopsies and organ donations, the spectrum of reconstructed tissues may be expanded to additionally include human material. Today, reconstructed renal and intestinal epithelia, epidermis, full thickness skin, airway epithelia, cornea and oral epithelia are frequently used to assess corrosive, irritating and phototoxic potential of substances as well as to study drug delivery and pathology.

One of the main challenges in tissue reconstruction is to restore specific tissue functions *in vitro*. Depending on the particular tissue type and scientific question, such critical features may encompass barrier function, the capability of substance transport, and the potential to express certain marker genes and signaling molecules. Optimal cell culture conditions must be well-established to re-create native function and maintain such features *in vitro*. For instance, it has been determined that cultivated epidermal cells differentiate and form a coherent *stratum corneum* only if they are exposed to air (Asselineau et al., 1985; Ponec et al., 1988). Specific cell culture labware, such as ThinCert™ Cell Culture Inserts with porous membrane supports, allows cultivation of epidermal cells at the air-liquid interface (air-lift culture) to ensure formation of a *stratum corneum*.

Figure 1 illustrates the two major steps of the formation of a skin equivalent *in vitro* including the submersed cultivation and the air-lift culture of the human skin model. With ThinCert™ cell culture inserts and the novel ThinCert™ Plate Greiner Bio-One offers an integrated solution for multiple tissue reconstruction applications. In the following, the opportunities and advantages of ThinCert™ Cell Culture Products are illustrated with two reconstructed tissue models – a multi-layered buccal mucosa and a full thickness skin.

Material

Item	Manufacturer	Cat.-No.
Accu-Chek® Aviva Blood Glucose Meter	Roche	3360578
Accu-Chek® Aviva strips for Glucose Meter	Roche	3360561
Bouin's fluid	Sigma-Aldrich Chemie GmbH	HT101128
CELLSTAR®, 12 well cell culture plate	Greiner Bio-One GmbH	665 180
Chondroitin 4-sulfate	Fluka	27042
Chondroitin 6-sulfate	Fluka	27043
DMEM, liquid medium	Invitrogen	41965-039
DMEM, powder	Invitrogen	52100-021
EnVision™+ System-HRP (AEC)	Dako	K4004
Fetal calf serum	Invitrogen	10270-106
Fibronectin	Invitrogen	33016-015
HEPES-Buffer	Sigma-Aldrich Chemie GmbH	H4034
KBM Basal Medium	Cambrex Bio Science	CC-3101
Keratinocyte-SFM Medium (Kit)	Invitrogen	17005-075
KGM SingleQuot Kit	Cambrex Bio Science	CC-4131
Mouse anti Cytokeratin 10 antibody	DakoCytomation	M7002
Mouse anti Cytokeratin 5/6 antibody	DakoCytomation	M7237
Mouse anti Filaggrin antibody	Biomeda	V10118
ThinCert™Plate, 12 well tissue culture plate	Greiner Bio-One GmbH	665 110
ThinCert™12 well Cell Culture Insert with 0,4 µm pores	Greiner Bio-One GmbH	665 640
Tissue-Tek® Cryo-OCT Compound	Fisher Scientific	14-373-65

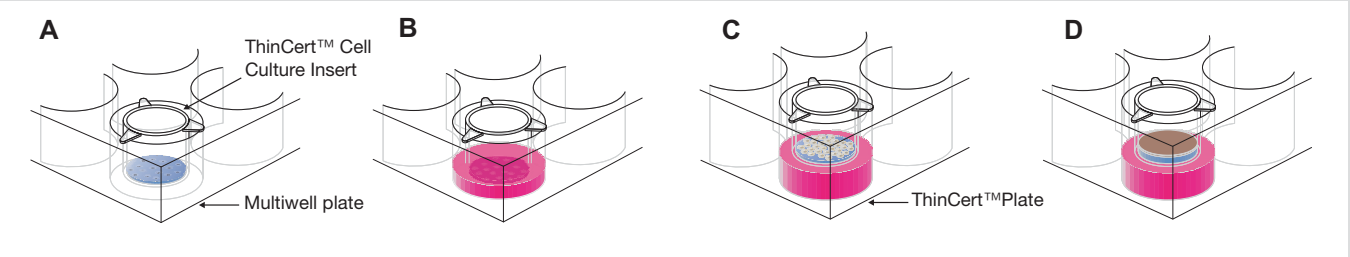

Figure 1: Steps involved in the reconstruction of a full thickness skin model in ThinCert™ Cell Culture Inserts.
A collagen gel containing primary dermal fibroblasts is poured onto the porous membrane of a ThinCert™ Cell Culture Insert (**A**). Subsequently, this dermis equivalent is cultivated under submersed conditions with cell culture medium reaching above the tissue (**B**). Primary epidermal keratinocytes are seeded on top of the dermis equivalent. After several days in submersed culture, the medium volume is lowered to the level of the membrane (**C**). The air-lift culture enables an epidermis with *stratum corneum* to form (**D**). For improved nutrient supply the air-lift culture is performed in a deep-well ThinCert™Plate to increase medium volume (**C, D**).

Methods

Cultivation of human oral squamous keratinocytes

ThinCert™ 12 well Cell Culture Inserts with 0.4 µm membranes were placed in the wells of 12 well standard plates or ThinCert™ Plates. 500,000 oral squamous keratinocytes were seeded in each insert. After overnight incubation at 37°C/ 5% CO_2 excessive medium was removed from the upper insert compartment and the cells were further cultivated at the air-liquid-interface using 0.5 and 4 ml Keratinocyte-SFM medium (with EGF, BPE) per well in the standard plate and ThinCert™Plate, respectively. Medium was exchanged every day in regular plates and every 4th day in ThinCert™Plates. Cells were cultivated for 29 days, thus forming a multi-layered buccal mucosa-like epithelium.

Preparation and cultivation of full thickness skin models

For gel solution 232.5 ml 2x DMEM cell culture medium, 7.5 ml HEPES buffer (4.76% in PBS, pH 7.3) and 1.25 ml chondroitin sulfate solution (5 mg/ml chondroitin 4-sulfate, 5 mg/ml chondroitin 6-sulfate in PBS+[1]) were mixed. The pH was adjusted to 7.8. Collagen gel (collagen isolated from rat tail tendon) containing 2 volume shares collagen solution (6 mg/ml in 0.1 % acetic acid) and 1 volume share gel solution was prepared according to patent WO 0192477 A2. 750 µl gel containing 75,000 human foreskin fibroblasts was pipetted on top of the membrane of a 0.4 µm 12 well ThinCert™ Cell Culture insert in a standard 12 well cell culture plate. Following gel formation at 37°C, the gel was covered with 50 µl fibronectin solution (5 µg/ml) and incubated for 10 min at 37°C. The fibroblast-containing gel was cultivated for 1 day at 37°C and 5% CO_2 under submersed conditions applying a total of 2.5 ml DMEM medium/5% FCS per well and insert[2].

150.000 human foreskin keratinocytes in 100 µl KBM basal medium/ 5% FCS were subsequently seeded on top of the collagen gel. After 1-2 h incubation at 37°C, a total of 2.5 ml KBM basal medium containing FCS (5%), hEGF (0.1 µg/ 500 ml) and BPE (15 mg/ 500 ml) was added to each insert and well. During submersed cultivation medium was exchanged every day, thereby gradually lowering the FCS concentration from 5% to 0%. After submersed culture the inserts carrying the skin equivalents were transferred into the wells of a conventional 12 well plate or a 12 well ThinCert™Plate. KBM medium with 1.88 mM $CaCl_2$ was filled into each well up to the level of the insert membrane (air-lift culture). The air-lift culture was performed for up to 13 days with one medium exchange at day 7.

Determination of the glucose content in cell culture medium

Medium samples from squamous keratinocyte cultures were drawn after 3, 7, 11, 15 and 19 days in culture (always prior to medium exchanges). The glucose content of the medium samples was determined using an Accu-Chek® Aviva test from Roche.

Histological analysis

Reconstructed buccal epithelia were snap frozen over liquid nitrogen embedded in Tissue-Tek® at cultivation day 29. Subsequently cryo-sections of 8 µm thickness were prepared and fixed with acetone (10 min, room temperature). Skin models were subjected to histological analysis after 3, 6, 10 or 13 days in air-lift culture. For this purpose, the tissue samples were fixed for 1 h with Bouin's solution, dehydrated and paraffin embedded according to standard protocols. Sections of 3 µm thickness were prepared. All tissue samples were stained with Hematoxylin and Eosin applying standard protocols.

[1] PBS+: phosphate buffered saline containing 1.8 mM $CaCl_2$ and 3.98 mM $MgSO_4$
[2] The total medium volume was splitted between insert and well, so that hydro-dynamic equilibrium was achieved between both compartments.

Immunohistochemical analysis

Immunhistochemical analysis was performed on 3 µm paraffine sections of skin models cultivated for 10 or 13 days in air-lift culture. Anti Cytokeratin 5/6, Cytokeratin 10 and Filaggrin antibodies were applied at concentrations of 1:1000, 1:2000 and 1:400, respectively. Immunohistochemistry was performed according to the instructions of the antibody manufacturers. For the primary antibody detection the HRP detection system EnVision™ + was used.

Results

In comparison to monolayer cell culture, tissue reconstruction requires cell culture in multiple layers at high densities. Hence, specific demands on the applied cultivation system arise, including an improved nutrient supply and enhanced gas exchange. In ThinCert™ Cell Culture Inserts tissue may be cultivated at the air-liquid interface with direct oxygen supply. With the upper insert compartment unavailable for medium deposition during the air-lift culture, the enlarged well size of the ThinCert™Plate provides a large medium and nutrient reservoir source directly below the insert membrane (**Figure 1C and D**).

Here, oral squamous keratinocytes were cultivated in air-lift culture using ThinCert™ Cell Culture Inserts in combination with conventional multi-well plates or ThinCert™Plates. Due to the limited medium reservoir of conventional multi-well plates, it was necessary to exchange the cell culture medium every day in these plates. In contrast, ThinCert™Plates allowed an expanded timeframe for medium exchanges to every 4th day. The improved nutrient supply achieved in ThinCert™Plates was well reflected in the glucose concentration of the culture medium, which was higher in samples drawn from ThinCert™ Plates than in samples from conventional plates at all examined stages (3, 7, 11, 15 and 19 days in culture, **Figure 2A**). As a result the cultivation in the ThinCert™Plate yielded a better tissue quality with thicker tissue and more cell layers (**Figure 2B**).

As mentioned above, air-lift culture not only guarantees proper oxygen supply of the cultivated tissue, it also provides an indispensable differentiation stimulus for the formation of terminal structures of air-exposed tissues such as the *stratum corneum* of skin.

Here, the cultivation of a full thickness skin model was used to further illustrate the capabilities of the ThinCert™ Cell Culture system. The dermis equivalent was first allowed to grow under submersed conditions for one week using ThinCert™ Cell Culture Inserts in conventional multiwell plates. The epidermis equivalent was then allowed to differentiate at the air-liquid interface for two weeks using ThinCert™ Cell Culture Inserts in ThinCert™Plates.

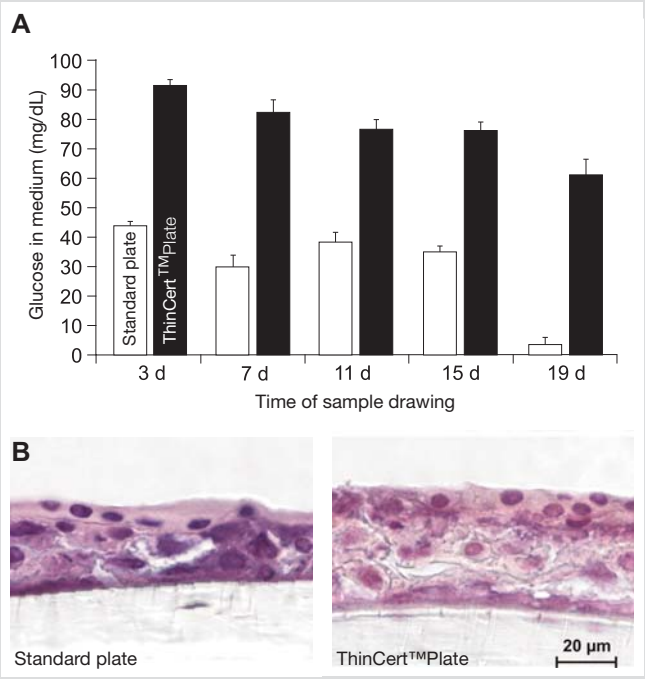

Figure 2: Improved nutrient supply with the ThinCert™Plate.
A *buccal mucosa* like epithelium was reconstructed from oral squamous cells using ThinCert™ Cell Culture Inserts in combination with either conventional multiwell plates or the ThinCert™Plate over a cultivation period of 29 days. The enlarged medium reservoir of the ThinCert™Plate allowed the reduction of medium changes from every day, as required by the conventional plate, to every 4th day. In addition to reducing the number of medium changes, a significantly higher glucose concentration was maintained in the ThinCert™Plate as compared to the standard condition (**A**, glucose measurements were performed prior to medium changes). Moreover, tissue generated in the ThinCert™Plate was thicker in appearance and contained more cell layers in comparison to tissue generated in the conventional plate (**B**, Hematoxylin-Eosin staining of the buccal mucosa epithelium after 29 days in culture).

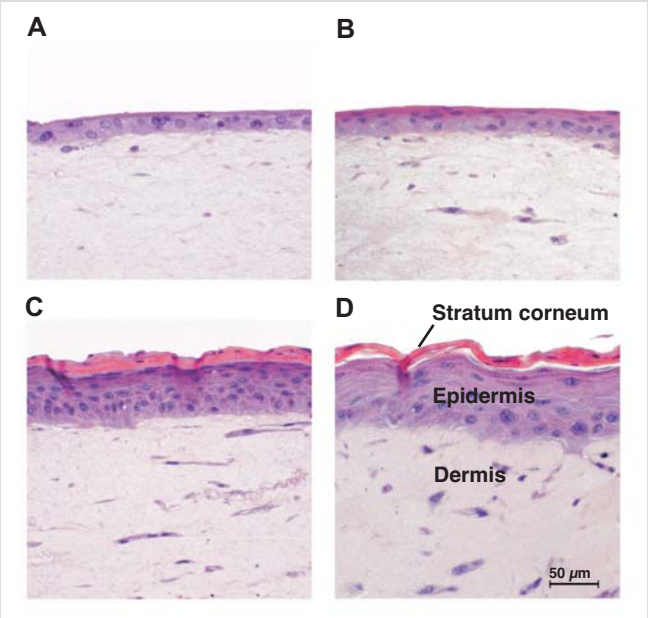

Figure 3: Maturation of a skin model cultivated in ThinCert™ Cell Cuture Inserts and the ThinCert™Plate.
The skin model was cultivated at the air-liquid interface and histologically analysed at cultivation days 3 (**A**), 6 (**B**), 10 (**C**) and 13 (**D**) using Hematoxylin-Eosin staining. First signs of cornification were detectable at cultivation day 6 (**B**). After 10 (**C**) and 13 (**D**) days a multi-layered epidermis with well defined *stratum corneum* had formed.

Histological analysis of the well stratified skin models revealed the first tendency of cornification at cultivation day 6 (**Figure 3B**). After 10 and 13 days in air-lift culture a multi-layered epidermis with defined *stratum corneum* had developed (**Figure 3C and D**), thus strongly resembling the stratified structure of native human skin. It is noteworthy that one medium exchange at day 7 was sufficient to provide the growing skin model with sufficient nutrients for the entire air-lift cultivation of two weeks.

For characterisation of the differentiation status of the cultivated skin models immunohistochemistry was performed at day 10 of the air-lift culture using antibodies against the early terminal differentiation markers Cytokeratin 5/6 and 10 and the late differentiation marker Filaggrin.

For comparison two sets of skin models were analysed – one using ThinCert™ Cell Culture Inserts in a conventional cell culture plate and one using ThinCert™ Cell Culture Inserts in the ThinCert™Plate for air-lift culture. Cytokeratin 5/6 and 10 were detectable in all skin models independent of the used plate (**Figure 4A, B, D, E**).

In contrast, Filaggrin was only detectable in the skin models cultivated in the ThinCert™Plate (**Figure 4C**), whereas those cultivated in the conventional plate were devoid of Filaggrin expression (**Figure 4F**).

The early onset of Filaggrin expression in the ThinCert™Plate suggests that culture conditions in this plate can promote and accelerate keratinocyte differentiation during the air-lift culture. At cultivation day 13 Filaggrin became also detectable in the skin models cultivated in the conventional plate (data not shown), thus indicating that terminal differentiation was slightly delayed, but not abolished in this control experiment.

Conclusion

The applications presented within this paper illustrate the multiple advantages and possibilities of ThinCert™ Cell Culture products for organotypic tissue culture and tissue reconstruction. The protocols provide detailed instructions that can easily be adapted to suit research interests other than buccal mucosa and skin reconstruction.

For additional information and continuative literature please refer to the website of Greiner Bio-One.

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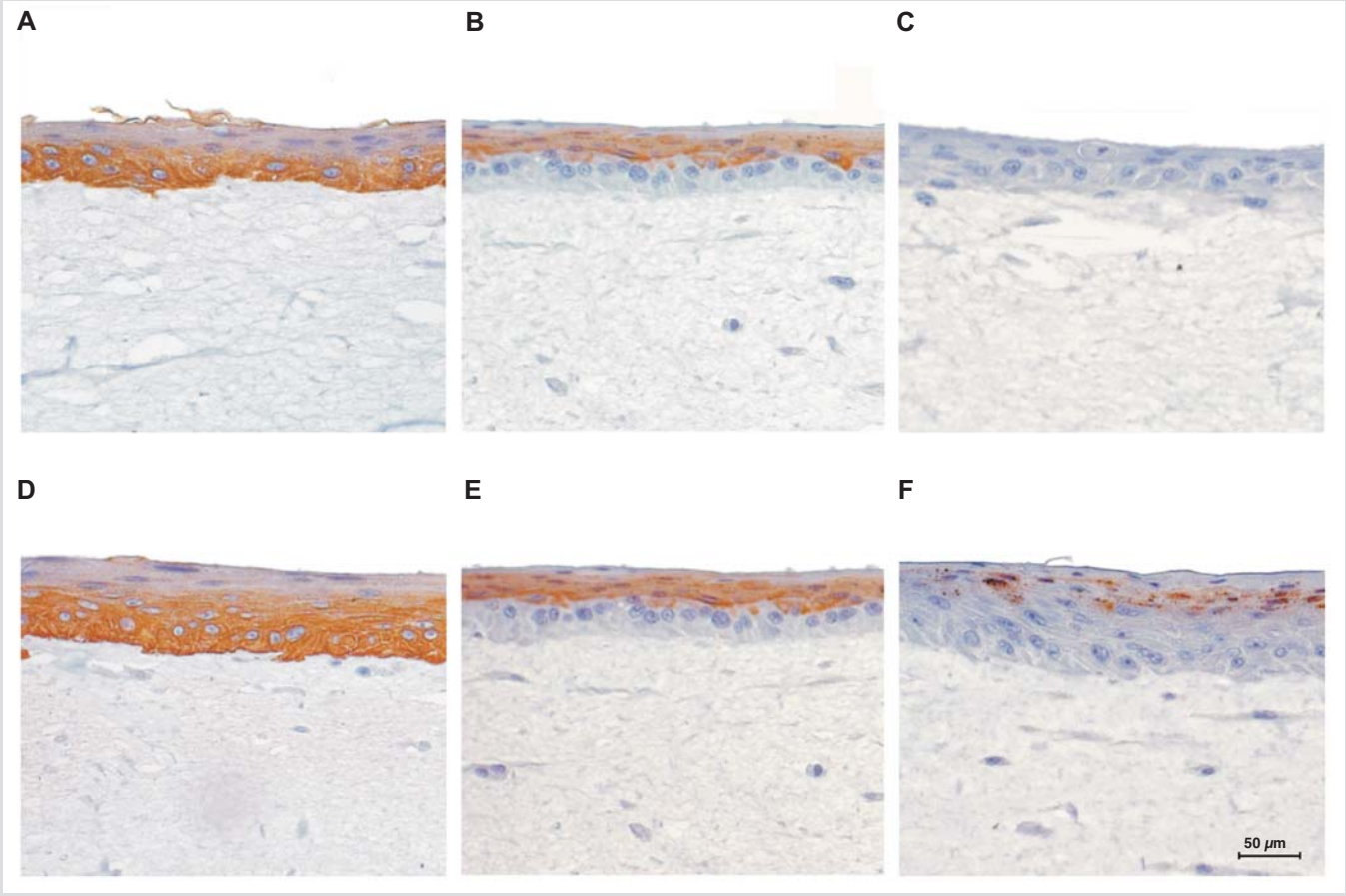

Figure 4: Immunohistochemical characterisation of skin models.
Skin models were cultivated in ThinCert™ Cell Culture Inserts and a conventional cell culture plate (**A–C)** or a ThinCert™Plate (**D–F**) and immunohistochemically analysed at cultivation day 10 using antibodies against Cytokeratin 5/6 (**A, D**), Cytokeratin 10 (**B, E**) and Filaggrin (**C, F**). The positive staining for the early differentiation markers Cytokeratin 5/6 (entire epidermis in **A, D**) and Cytokeratin 10 (suprabasel epidermal layers in **B**, **E**) indicates the onset of keratinocyte differentiation in both cell culture plates prior to cultivation day 10. Only the skin model that has been cultivated in the ThinCert™Plate showed immunoreactivity against the late differentiation marker Filaggrin (*Stratum granulosum* in **F**), thus indicating that in the ThinCert™Plate keratinocyte differentiation was more advanced than in the control plate (no immunoreactivity in **C**).

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Patents pending

In part, the skin reconstruction procedure described above is patent-protected (DE 10062623 B4, WO 0192477 A2).

Acknowledgement

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