Bioscience Solutions

Construction of a Full Thickness Skin Model Using RAFT™ 3D Cell Culture System

Therese Willstaedt[1], Jenny Schroeder[2], Lubna Hussain[1] and Theresa D'Souza[1]
[1]Lonza Walkersville, Inc., Walkersville, MD, USA; [2]Lonza Cologne GmbH, Cologne, Germany

Introduction

Historically, two-dimensional (2D) culture has been used to study human epidermal cell biology. Increasing experimental evidence shows that three-dimensional (3D) culture is a more appropriate *in vitro* format to utilize because it more closely mimics the *in vivo* cellular environment. Various studies have shown that *in vitro* skin micro-tissues comprising of a fibroblast layer containing dermis topped by a stratified layer of keratinocytes can be formed on polycarbonate transwells.[1] The total duration to obtain the full thickness skin models can vary from 28 – 30 days from seeding the fibroblasts, followed by keratinocytes and differentiation. The RAFT™ 3D Culture System could provide an advantage of shortening the culture period to 22 – 24 days. This culture system could potentially provide a scaffold for the addition of other skin cell types.

In this Technical Note, we describe the procedure in constructing full thickness (FT) skin models using the RAFT™ 3D Culture System with primary Human Neonatal Epidermal Keratinocytes (NHEK) and primary Human Neonatal Dermal Fibroblasts (NHDF). The RAFT™ Skin Model consists of a compressed collagen-type-I-based hydrogel that closely mimics the human dermis. Furthermore, we show analyses of the FT skin model to resemble the native skin by immunohistochemistry and immunofluorescence validated using key markers for epidermis and dermis.

Materials and Methods

Materials	Vendor	Cat. No.
RAFT™ 3D Cell Culture Kit	Lonza	016-0R95
RAFT™ Insert Absorbers for 24-well inserts	Lonza	016-1R33
24-well transwells	Corning	3470
Primary Human Neonatal Dermal Fibroblasts (NHDF)	Lonza	CC-2509
Primary Human Neonatal Epidermal Keratinocytes (NHEK)	Lonza	00192907
T150 cell culture flasks	Corning	430825
Accutase™	STEMCELL Technology	07920
FBM™-2 BulletKit™	Lonza	CC-3132
KBM-Gold™ BulletKit™	Lonza	192060
CnT-Prime Airlift Medium	CellnTec	CnT-PR-FTAL
Paraformaldehyde solution 16%, diluted to 4% (v/v) in PBS	Electron Microscopy Sciences	RT 15710
Ethyl alcohol, diluted to 70% (v/v) in deionized water	Sigma	459836
PBS (Phosphate Buffered Saline)	Lonza	17-516Q
Water for cell culture	Lonza	17-724Q
Xylene	Sigma	534056
Ethanol (ETOH)	Sigma	459844
Target retrieval buffer, universal reagent	R&D Systems	CTS015
Prolong Gold antifade reagent with DAPI	Thermo Scientific	P36931
Triton X-100	Sigma	X100-100 ml
Casein	Sigma	C4765
10% Normal Goat Serum (NGS)	KPL	71-00-27
Bovine Serum Albumin (BSA)	Sigma	A9543
Porcine Gelatin	Sigma	G-2500
Mouse anti human Cytokeratin 5/6 (1:50)	Agilent Technologies	M723729-2
Mouse anti human Cytokeratin 10 (1:50)	Agilent Technologies	M700201-2
Mouse anti human Cytokeratin 14 (1:200)	Abcam	ab45939-100
Mouse anti human Pan Cytokeratin (1:100)	Sigma	C2562
Mouse IgG1 (1:100)	Agilent Technologies	X093101-2
Rabbit anti human Vimentin	Abcam	ab45939-100
Rabbit anti mouse IgG Alexa Fluor® 568	Invitrogen	A-11061
Goat anti rabbit IgG Alexa Fluor® 568	Invitrogen	A-11011

Other Supplies and Equipment

- Standard cell culture incubator set to 37°C and 5% CO_2
- Olympus microscope
- Fluorescent microscope equipped with camera
- Centrifuge
- Steamer, such as Oster brand CKSTSTMD5-W
- Couplin jars or staining dishes
- Forceps
- Parafilm® M all-purpose laboratory film
- Coverslips

Fibroblasts Culture and Construction of Dermis Layer with the RAFT™ 3D Culture System

Fibroblast growth medium (FGM™-2 Medium) was prepared according to the Manufacturer's Recommendation, except Gentamicin/Amphotericin-B (GA-1000) was omitted. An ampule of NHDFs was thawed gently in water bath at 37°C until a crystal of ice was visible. The vial content was diluted in 9 mL medium and centrifuged at 200 × g for 5 minutes at room temperature (RT). The supernatant was aspirated, the cell pellet was suspended in 4 mL medium and cell counts were performed. The cells were seeded in FGM™-2 Growth Medium at 2,500 cells/cm^2 in one or two T150 flasks. The flasks were placed in a 37°C, 5% CO_2 incubator for 5 days, with alternate day feeds of 30 mL FGM™-2 Medium per T150 flask. Upon reaching 80–90% confluence, fibroblasts were harvested from the flask(s) with Accutase™, according to the manufacturer's instructions and diluted and centrifuged in a 50 mL conical tube at 200 × g for 5 minutes at RT. The supernatant was aspirated and NHDFs were suspended in 4 mL FGM™-2 Medium before performing cell counts.

A stock of NHDF was prepared at 1.64 × 10^6 cells/mL concentration and mixed with RAFT™ Reagents as per the RAFT™ Protocol according to manufacturer's instructions for the desired number of transwells. Instead of the full cell-collagen solution volume (240 µL) recommendation, half volume (120 µL) was pipetted gently in each transwell. The final concentration of NHDFs was at 8.25 × 10^3 per transwell (25,000 cells/cm^2). The hydrogels were formed and compressed according to RAFT™ Kit Instructions. A quarter volume (60 µL) of the cell-collagen solution was added on top of the compressed RAFT™ Cultures. The hydrogel was allowed to form without compression. FGM™-2 Medium was added to the chambers, 100 µL to the apical and 600 µL to the basal chamber. The dermis layer was allowed to develop for 5 days at 37°C, 5% CO_2, feeding the same volume on alternate days.

Note: All cultures are grown without antibiotics.

Keratinocytes Culture and Construction of Epidermis Layer with the RAFT™ 3D Culture System

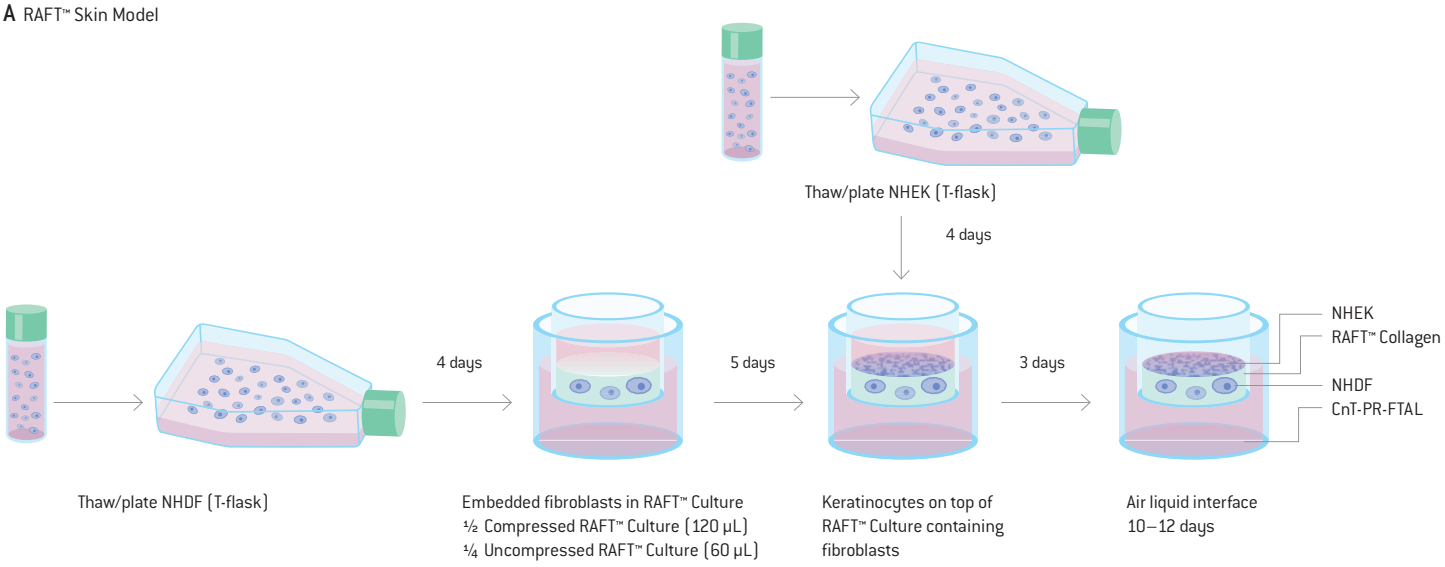
4–5 days prior to the addition of NHEK to the RAFT™ Dermis Layer, keratinocytes were cultured. Keratinocyte Growth Medium (KGM-Gold™ Medium) was prepared per the Manufacturer's Protocol by supplementing KGM-Gold™ Basal Medium with KGM-Gold™ SingleQuots™ of growth supplements, omitting GA-1000. An ampule of NHEK was thawed gently in water bath at 37°C until a crystal of ice was visible. The vial content was diluted in 9 mL medium and centrifuged at 200 × g for 5 minutes at RT. The supernatant was aspirated, the cell pellet was suspended in 4 mL medium and cell counts were performed. The cells were seeded in KGM-Gold™ Medium (without GA-1000) at 2,500 cells/cm^2 in one or two T150 flasks. The flasks were placed in a 37°C, 5% CO_2 incubator for 4–5 days, with alternate day feeds of 30 mL KGM™ Gold Medium per T150 flask. The cells were harvested from the flask(s) with Accutase™, according to the manufacturer's instructions, diluted and centrifuged in a 50 mL conical tube at 200 × g for 5 minutes at RT. The supernatant was aspirated and NHEKs were suspended in 10 mL KGM-Gold™ Medium before performing cell counts.

A stock of NHEK suspension at 3.5 × 10^5 cells/mL was prepared in KGM™-Gold™ Medium. FGM™-2 Medium was aspirated gently from the apical well of the prepared RAFT™ Cultures containing embedded NHDF. A volume of 100 µL NHEK cell suspension was added to the apical well. The final concentration of NHEKs was at 3.5 × 10^4 per transwell (1.06 × 10^5 cells/cm^2). FGM™-2 Medium from the basal chamber was removed and replaced with 600 µL of KGM™-Gold Medium.

The RAFT™ Skin Models were fed on alternate days with KGM™-Gold Medium for 2 days. Differentiation medium, CnT-PR-FTAL, was prepared as per manufacturer's instructions. The medium in the apical and basal chambers of the transwells were replaced with CnT-PR-FTAL for 24 hours (Figure 1). Thereafter, the medium from the apical chamber was removed to allow NHEK to differentiate at the Air-Liquid Interface (ALI). The RAFT™ Skin Models were fed with 600 µL of differentiation medium in the basal chamber on alternate days for 10–12 days. As an observation, we found at least for keratinocytes, usage in early passages was more optimal in developing the RAFT™ Skin Models.

Note: All cultures are grown without antibiotics.

A RAFT™ Skin Model



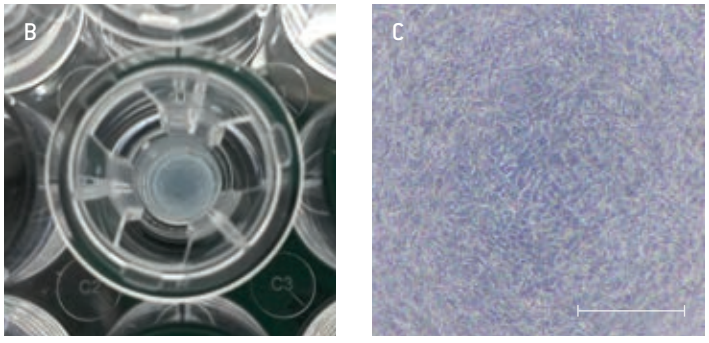


Figure 1
(A) RAFT™ Skin Model configuration using the RAFT™ Cell Culture System. NHDF were embedded in compressed/uncompressed RAFT™ Cultures. NHEK were seeded on top of the NHDF followed by air liquid interface (ALI) after 3 days for submerged culture. Top view of cultured NHEK on RAFT™ Culture in a transwell after 2 days in culture macroscopically **(B)** and microscopically **(C)**. Scale bar: 250 µm

Fixation of Skin Models for Histological Examination

The medium in the basal chambers of the transwells was aspirated followed by the addition of 4% PFA in PBS to the models: 100 µL in the apical chamber and 600 µL in the basal chamber. To allow for complete fixation, the models were stored at 4°C overnight (24 hours). Using a sharp scalpel, the transwell membrane was cut out and with the help of forceps the models were transferred to a 15 mL conical tube containing 70% ethanol. Histological processing, embedding, sectioning and Hematoxylin-Eosin staining was contracted to histology services.

Immunohistochemistry of Paraffin Embedded RAFT™ Skin Models

Deparaffinization and Rehydration

Paraffin sections of skin models mounted onto glass slides were deparaffinized and rehydrated for 5 minutes each in sequential order with the following treatments in Coplin jars: Xylene, 100% ETOH, 100% ETOH, 95% ETOH, 85% ETOH and 70% ETOH. The final rinse (a quick dip) was carried out in water for cell culture. Finally, the glass slides with deparaffinized sections were placed in PBS.

Antigen Retrieval (Steamer Method)

The Target Retrieval buffer was diluted to 1x and preheated in a container in a steamer before the last wash was completed. Following the PBS wash, excess liquid was removed from the slides by touching the edge of the slide to an absorbent paper. The slides were immersed in the container with pre-heated antigen retrieval solution and steamed for 30 minutes with the steamer lid on. The container with the slides was removed from the steamer and allowed to cool on the bench top at ambient temperature for 20–30 minutes. The slides were then transferred to PBS for two washes.

Blocking

Blocking buffer was prepared by mixing the following components at given concentrations: 0.2% Triton X-100, 0.2% BSA, 0.2% Casein, 5% Normal Goat Serum (NGS) and 0.2% Gelatin. Excess liquid was removed from the slides by touching the edge of the slide to an absorbent paper. Using a pipette, sections were covered with blocking buffer. A strip of Parafilm® was gently overlaid on the blocking buffer. Blocking was carried out at RT for 1 hour. The Parafilm® was removed and blocking buffer was drained.

Staining

Primary antibody diluted in blocking buffer was added to the sections and overlaid with a fresh strip of Parafilm®. Slides were incubated overnight at 4°C in a humidified chamber. The slides were then rinsed briefly in PBS, followed by three additional PBS washes for 10 minutes each.

Secondary antibody was diluted in Blocking Buffer, overlaid with a coverslip-sized piece of Parafilm® and incubated for 1 hour at RT. The slides were then rinsed briefly in PBS, followed by three additional PBS washes for 10 minutes each. It is important to note that paraffin exhibits autofluorescence in the green spectrum. Therefore, it is strongly recommended to use fluorescence dyes that emit light with a wavelength of 568 nm or higher e.g. Alexa Fluor® 568. Standard green fluorescent dyes with an emission wavelength below 568 nm are usually not compatible with paraffin-embedded tissue samples. Excess moisture was removed from the slide by touching the edge of the slide to an absorbent paper. The slide was counter stained and mounted by applying approximately 50 µL antifade reagent with DAPI to each section and covering with a coverslip. The sample was then cured by mounting on a flat dry surface and incubating for 24 hours at RT in the dark.

Results

The RAFT™ Skin Model developed over a span of 22 – 24 days showed a full thickness skin equivalent with epidermal and dermal layers observed in native skin. The morphologically differentiated epidermal layers showed basal, spinous, granular and cornified cell layers recognizable by the hemotoxylin and eosin staining of deparaffinized sections of RAFT™ Skin Models in Figure 2. The basal cells appear mostly columnar attached to the RAFT™ Fibroblast Collagen Scaffold. The spinous and granular layers are sandwiched between the basal and cornified layers with the latter observed as dense eosin staining and no nuclei. The data shown is representation of two lots of donors of keratinocytes and fibroblasts used in the study.

To demonstrate that the stratified skin equivalent resembled differentiation observed *in vivo,* immunohistochemistry was performed on de-paraffinized sections of RAFT™ Skin Models and native skin from an adult abdominal region. The sections were treated with target retrieval buffer and stained with antibodies for differentiation markers. Alexa Fluor® 568 was used as the secondary antibody. Appropriate isotype controls were incorporated and no significant fluorescence was observed in such samples (data not shown). Prolong Gold antifade reagent with DAPI was used to counterstain and mount slides. Cytokeratin 10 is an early marker of differentiation and is specific for suprabasal cells.[2] Basal cells in native skin and in RAFT™ Skin do not express this intermediate filament (Figures 3A and 3B). The pan-cytokeratin, cytokeratin 14 and cytokeratin 5/6 were localized in the epidermal layer of the RAFT™ Skin (Figure 4). Vimentin is a cytoskeletal protein expressed by fibroblasts and other mesenchyme-derived cells.[3] Some normal epithelial cells co-express cytokeratin and vimentin proteins.[4] Staining of vimentin was prominently observed in both native and RAFT™ Skin in the dermal component where the fibroblasts were shown to reside (Figure 5).

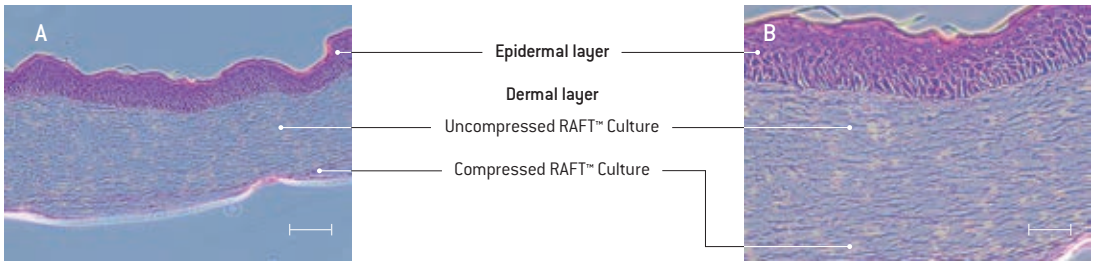


Figure 2
(A, B) Histology of RAFT™ Skin Model. The sample was cut perpendicular to the surface post fixation and embedding in paraffin. Hemotoxylin and eosin staining shows a distinct epidermal layer containing differentiated epidermal keratinocytes juxtaposed to the dermal layer containing dermal fibroblasts in RAFT™ Hydrogel. The scale bars represent 100 µm (A) and 50 µm (B)

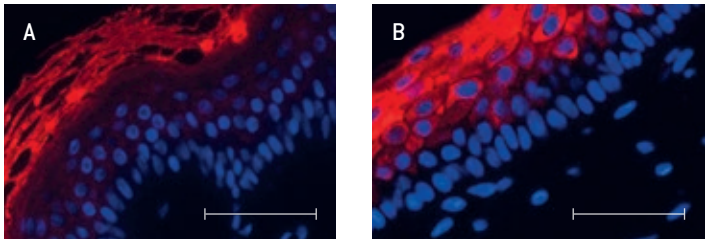


Figure 3
Immunofluorescent labeling of keratin 10 (CK10, red) in histological sections vertical to the surface of native skin (A) and RAFT™ Skin Model (B). The nuclei are stained with DAPI (blue). Scale bar: 50 µm

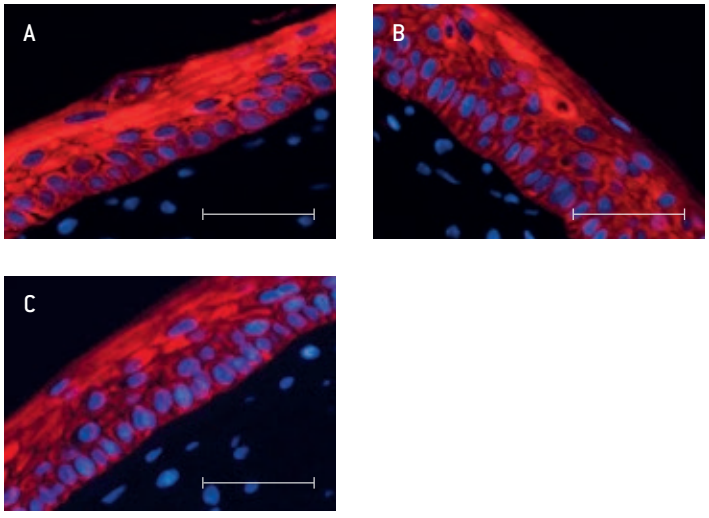


Figure 4
Immunofluorescent labeling of pan cytokeratin (A), cytokeratin 14 (B) and cytokeratin 5/6 (C) in histological section of RAFT™ Skin Model. The nuclei are stained with DAPI (blue). Scale bar: 50 µm

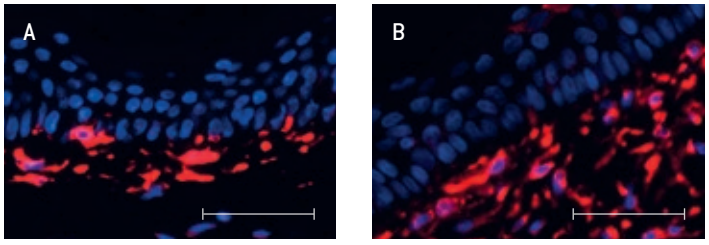


Figure 5
Immunofluorescent labeling of vimentin in histological sections vertical to the surface of native skin (A) and RAFT™ Skin Model (B). Scale bar: 50 µm

Conclusions

In this Technical Note, we show that the RAFT™ 3D Cell Culture System can be used to create skin models, which resemble native skin. The type-I-collagen-based RAFT™ Scaffold seeded with fibroblasts closely mimics the human dermis. Keratinocytes cultured on top of this scaffold at high cell density differentiate and form a stratified epidermis upon airlifting. The histological features and differentiation markers of the epidermal and dermal components of the RAFT™ Skin were similar to those of the native skin. Altogether, the data shown here demonstrates that a full thickness skin equivalent can be obtained within 22 – 24 days using the RAFT™ 3D Culture System, shortening the time-period in developing the model by 4 – 5 days. The RAFT™ 3D Culture System provides a platform to build models that are even more complex than a full thickness skin model. Its versatility also allows the investigator to potentially include other cell types in the skin model, e.g. adipocytes, melanocytes or endothelial cells. Hence, there's a clear advantage in using the RAFT™ 3D Culture System over simply using a transwell system alone.

References

1. Wojtowicz AM, et al. The importance of both fibroblasts and keratinocytes in bi-layered living cellular construct used in wound healing. Wound Repair Regen. 2014:22(2):246–255
2. Torma H. Regulation of keratin expression by retinoids. *Endocrinol.* 2011; 3(3):136–140.
3. Dave JM, Bayless KJ. Vimentin as an integral regulator of cell adhesion and endothelial sprouting. *Microcirculation.* 2014:21(4);333 – 344.
4. Velez-delValle C, et al. Epithelial cell migration requires the interaction between the vimentin and keratin intermediate filaments. *Nature Scientific Reports.* 2016;6(24389): 1 – 10.

Contact Information

North America

Customer Service: +1 800 638 8174 (toll free)
Fax: +1 301 845 8338
order.us@lonza.com
Scientific Support: +1 800 521 0390 (toll free)
scientific.support@lonza.com

Europe

Customer Service: +32 87 321 611
order.europe@lonza.com
Scientific Support: +32 87 321 611
scientific.support.eu@lonza.com

International

Contact your local Lonza distributor
Customer Service: +1 301 898 7025
scientific.support@lonza.com

Lonza Walkersville, Inc. – Walkersville, MD 21793

For research use only. Not for use in diagnostic procedures.

CD-SP074 03/18

www.lonza.com/research
www.lonza.com/raft