Full Thickness Skin Models

This document describes the establishment of full thickness (FT) in vitro skin models using primary human keratinocytes and fibroblasts grown at the air-liquid interface in Prime media.

The following products are required to establish the FT models:

- #CnT-PR: Fully defined keratinocyte proliferation medium
- #CnT-PR-F: 1% serum fibroblast proliferation medium
- #CnT-PR-FTAL: Fully defined co-culture medium for culture at the air-liquid interface
- Inserts: 0.4 um pore size, PET membrane. It is strongly recommended to use lots provided by CELLnTEC, as they are known to succeed in this model (see below)
- Accutase: a gentle detachment enzyme that does not require a stop reagent (#CnT-Accutase-100)
- #CnT-FT-CELLS: Value pack of primary human HPEK keratinocytes and HDF dermal fibroblasts (juvenile donors) that have been tested in FT culture
- 12-well plates: two alternative options are possible. (i) Standard plates (e.g. TPP #92012) used with re-usable plate spacers (#CnT-SP) to raise inserts 2.5 mm and increase medium volume per well during air-lift culture. Or (ii) deep-well plates (e.g. Greiner #665110) that enable a larger volume of medium during air-lift culture with hanging inserts.

For first-time users wishing to test the system, free samples of the culture media and inserts are available. Please see www.cellntec.com/products/ordering/free-samples/ for details.

Primary cells (keratinocytes and fibroblasts), plus the plate spacers (to increase medium volume in standard plates) are available for purchase from CELLnTEC.

General Notes

The key factors for successful establishment of full thickness skin models are as follows:

- Early passage, juvenile donor keratinocytes and fibroblasts: Optimal proliferation, ECM production and differentiation in air-lift culture is observed with primary cells within the first 8-10 population doublings after thawing. It is thus recommended to seeding into the inserts for 3D culture after 1 or 2 passages of expansion post thawing (depending on number of cells required). Comparing fibroblasts from different donors for their ECM production can also be valuable.
- Keratinocytes must be proliferating well in CnT-PR medium (approx. 4 doublings per week) and show regular cobblestone morphology in the passage prior to seeding into the inserts. They must always be passaged at 70-90% confluency to ensure good growth in the subsequent passage.
- For convenient medium change frequency (Mon/Wed/Fri) during culture in 12-well inserts, it is

critical that each insert be exposed to at least 1.8 mL of culture medium, also during air-lift culture. If the medium volume drops below this volume per insert, daily medium changes are required.

- The passaging protocol during routine 2D culture (prior to seeding for 3D culture) is also an important consideration. Accutase is strongly recommended because as it is much gentler than trypsin, does not require a separate reagent to stop the reaction (simply dilute with medium immediately after detachment), and has a larger safe timing window between effective cell detachment and the point where cells are over-digested and damaged. Accutase is heat sensitive and must be stored at 4°C. Pre-warm only the amount required to 20°C just prior to use. Exposure to higher temperatures or repeated warming / cooling cycles may lead to reduced activity.
- Inserts can vary from lot-to-lot, at times significantly, due to changes in pore density, pore-size distribution, and also the evenness of the pore distribution across the track-etched membrane. To avoid costly and time-consuming failure due to membrane-related issues, it is strongly recommended to purchase inserts through CELLnTEC, as these lots have been tested for suitability in this model.

Preparing Fibroblasts and the Dermal Layer

1. Prepare medium: add the appropriate amount of CnT-PR-F medium to the culture flasks in which the HDF fibroblasts will be expanded. Allow medium to equilibrate pH and temperature for at least 30 min at 37°C in the 5% CO_2 incubator.
2. Thaw vial of HDF fibroblasts with gentle swirling in water bath at 37°C until just melted. Vials must be thawed rapidly, whilst ensuring that the temperature of the cells inside does not rise above 4°C (remove vial from water bath whilst some ice crystals remain). For more explanation about the features of an optimal thawing procedure, please review the knowledge base article in the resources section of www.cellntec.com.
3. Resuspend the cells in the cryo vial by pipetting up and down two to three times and transfer the whole content (1 mL) to the equilibrated cell culture vessels. The DMSO content in the freezing medium is 10%, after seeding in medium this must be diluted to less than 1% (10x dilution).
4. The volume seeded per vessel should deliver the recommended seeding density of 1'000 cells/cm^2. Complete a medium change 6 to 24 h after seeding to remove the residual DMSO from the freezing medium.
5. Expand the cells for one or two passages in CnT-PR-F medium at 37°C and 5% CO_2 with 3 medium changes per week (Mon/Wed/Fri). Cultures should be maintained without antibiotics / antimycotics. This is especially important during subsequent 3D culture, to avoid any inhibition of the ECM production and differentiation processes. Passage the cells prior to reaching full confluency using Accutase according to the general cultivation protocol available on www.cellntec.com. Seed fibroblasts at 1'000 cells per cm^2 after passaging.
6. Latest when fibroblasts reach 90% confluency, detach using Accutase, dilute and centrifuge

according to the general cultivation protocol, and then seed cells into inserts (PET 0.4 um pore size) at 200'000 to 500'000 cells per insert. Add 1 mL of medium inside the insert and approximately 2 mL of medium outside the insert. Ensure there are no air bubbles trapped below the membrane. Grow the fibroblasts for 14-21 days, with 3 medium changes per week, prior to seeding keratinocytes on top (see below). Pay special care during medium changes not to disturb the fibroblast layer - tilt the plate to a 45° angle and ensure the Pasteur pipette tip does not get too close to the cells on the membrane surface.

7. Culture the inserts in regular CnT-PR-F for the first three days. During this time prepare a sufficient amount of CnT-PR-F that contains an extra 1 mM ascorbic acid. Use this medium for the remainder of the dermal formation period.

Preparing Keratinocytes and Seeding into Inserts

1. Thaw a vial of HPEK keratinocytes with gentle swirling in water bath at 37°C until just melted. Vials must be thawed rapidly, whilst ensuring that the temperature of the cells inside does not rise above 4°C (remove vial from water bath whilst some ice crystals remain).
2. Resuspend the cells in the cryo vial by pipetting up and down two to three times and transfer the whole content (1 mL) to cell culture flasks in which the cells will be expanded – ensure that the CnT-PR medium in the culture flasks has been equilibrated for at least 30 min at 37°C in the 5% CO_2 incubator. The DMSO content in the freezing medium is 10%, after seeding in medium this must be diluted to less than 1% (10x dilution)
3. The volume seeded per vessel should deliver the recommended seeding density of 4'000 cells/cm^2. Complete a medium change 6 to 24 h after seeding to remove the residual DMSO from the freezing medium.
4. Expand the cells without antibiotics / antimycotics, with medium changes every 2-3 days for one or two passages. Passage the cells with Accutase following the general cultivation protocol on www.cellntec.com.
5. For seeding the inserts detach keratinocytes using Accutase, dilute and centrifuge according to the general cultivation protocol, re-suspend in CnT-PR-FTAL medium and seed 120'000 keratinocytes per insert on top of the established dermal fibroblast layers. Add a total of 1 mL of CnT-PR-FTAL medium inside the insert and add the appropriate amount of medium outside the insert such that the surface of the medium is at an equal level inside and outside the insert (this normally requires approx. 2 mL of medium outside the insert). Grow the keratinocytes in this submerged culture for 3 days (medium change on day 2).
6. Three days after seeding the keratinocytes, sterilize the plate spacers by soaking for 30 minutes in 70% alcohol, allow to air-dry (keep sterile), and then place them on top of new 12-well plates. Carefully transfer the inserts into the new plates, where the spacer lifts them 2.5 mm higher than in the plate alone. Raise the models to the air-liquid interface by adding medium outside the insert into each well, such that the surface of the medium is at an equal level to the insert

membrane, and the surface of the model is now exposed to the air (approx. 2 mL per well). Models must have at least 1.8 mL per well, to enable the convenience of Mon/Wed/Fri medium changes.

7. Grow the models at the air-liquid interface for a further 12 days, with medium changes 3x per week. At this point, the models are fully established and stratified and can be used for experimentation.

9. For histochemistry, immunohistochemistry, or immunofluorescence analyses, 3D-cultures are fixed over night at 4°C by placing the insert in a separate 12-well plate and filling the well and the inside of the insert with 4% Paraformaldehyde / PBS. Cut the skin / membrane out and proceed for paraffin- embedding and H & E staining (for detailed information see separate protocol on our resources section).

Further questions? CELLnTEC scientists work regularly with these cultures and are available in case further questions arise. To contact them directly, please email **support@cellntec.com**