3D Keratinocyte Starter Kit

This document describes the establishment of 3D epidermal keratinocyte cultures using the 3D Keratinocyte Starter Kit with Primary Keratinocytes (PR3D-HPEK-50). It includes the protocols required for both 2D submerged culture, as well as culture at the air-liquid interface.

The 3D Keratinocyte Starter Kit with Primary keratinocytes includes CELLnTEC's primary human epidermal keratinocytes (HPEK), CnT-Prime medium (CnT-PR) for routine 2D culture, CnT-Prime 3D Barrier medium (CnT-PR-3D) for use during the airlift culture, and 48 cell culture inserts.

General Notes

The key factors for successful establishment of 3D epidermal keratinocyte cultures are as follows:

- Early passage keratinocytes: Optimal proliferation and stratification is normally observed when using keratinocytes within the first 8-12 population doublings (approx 2-3 passages) after thawing
- 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 for 3D culture
- Keratinocytes must attach well to the inserts, and reach confluence in 2-3 days
- Medium volume and change frequency during air-lift culture must follow the guidelines below

The keratinocyte passaging protocol during routine 2D culture (prior to seeding for 3D culture) is also an important consideration. Acctuase is strongly recommended for the reasons outlined below. Trypsin is also possible, but requires stringent attention to timing, as valuable surface proteins can be over-digested with only a minute of excess digestion.

Accutase (Cat# CnT-Accutase-100) is a mixture of proteolytic and collagenolytic enzymes isolated from crustaceans. It is free of mammalian components. Accutase is the recommended detachment enzyme due to its gentle action that leaves most surface proteins intact, and the fact that it does not require a separate reagent to stop the reaction (simply dilute with medium immediately after detachment). Accutase is heat senstive, 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.

Trypsin / EDTA is a solution of proteolytic enzymes containing Trypsin, Chymotrypsin and Elastase. Trypsin is non-defined and of porcine origin. It may vary from lot-to-lot, and must be inactivated with a separate reagent immediately after cell detachment (preferably with an animal-component free inhibitor, alternatively with FCS). Trypsin is much more aggressive than Accutase, resulting in only a very small time window between succssful detachment and irreversible cell damage. If using Trypsin, extra special care must be taken to minimise the exposure time, and ensure rapid inactivation.

Immediately upon delivery

Check that the cell vial is still frozen and surrounded by dry ice. Thaw cells directly after arrival or transfer frozen vial to liquid nitrogen storage (transport on dry ice or liquid nitrogen to the storage site) until you are ready to thaw and begin culture.

Preparation

Before starting to work with the cells, ensure that the amount of medium, buffers and enzymes required

have been pre-warmed at least to room temperature.

Always use sterile instruments, aseptic technique, and work in a laminar flow to maintain sterility.

Special note about air-lift culture: the frequency of medium changes vs the volume of medium used are key factors for successful establishment of 3D epidermal cultures. Culturing multiple inserts per petri dish (e.g. 60 mm or 100 diameter) is an effective way to reduce the required handling time during air-lift culture, and obtain the recommended volume of medium per insert. Using this approach, only 3 medium changes are necessary per week (e.g. Monday, Wednesday, Friday), as long as at least 1 mL of medium is included per insert.

For example, a 60 mm petri dish typically requires 3.2 – 3.5 mL of medium during air-lift culture. If 3 inserts are cultured in each dish, this is equivalent to approx 1.1 mL of medium per insert. At this volume, cultures will establish quickly with only 3 medium changes per week. If the medium volume drops below 1 mL per insert, more frequent medium changes are required.

Thawing cells

1. Add the necessary amount of CnT-Prime medium to the desired culture vessels, and equilibrate them for at least 30 min in the 5% CO_2 incubator, to pre-warm the medium and to adjust to physiological pH.
2. Thaw vial 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 whilst still in the freezing medium (remove vial from water bath whilst some ice crystals remain). For more explanation about the features of an optimal thawing procedure, please review the knowledgebase 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 4,000 cells/cm^2.

IMPORTANT: Complete a medium change 6 to 24 h after seeding to remove the residual DMSO from the freezing medium.

Cultivation

During cultivation at 37°C and 5% CO_2 the CnT-Prime medium should be changed every 2nd to 3rd day.

Aspirate the old CnT-PR medium and replace it with the same volume of new CnT-Prime medium.

Cultures should be maintained without antibiotics / antimycotics. This is especially important during 3D culture, to avoid any inhibition of the differentiation process.

Passaging

1. Keratinocytes should be passaged when they reach 70 to 90% confluency. Do not allow the cells to become 100% confluent, as this will trigger differentiation, and poor growth in the subsequent passage.
2. Aspirate the CnT-Prime medium from the culture vessel.
3. Wash the cells by adding 100 µL / cm^2 of PBS (without calcium and magnesium), rotate the culture

vessel carefully to rinse the cells, then aspirate the PBS.

4. Add 50 µL / cm^2 of detachment enzyme (Accutase is recommended) to the cells.
5. Incubate at 37°C until cells are rounded and start to detach. Check for cell detachment under the microscope.
6. Tap the vessel to dislodge any remaining attached cells cells (slide vessel sideways into a solid object).
7. When using Accutase: add 2.5 volumes of CnT-Prime medium to the detached cells. When using Trypsin / EDTA: add the same volume of Trypsin inhibitor.
8. Rinse the bottom of the culture vessel 2 to 3 times with the suspension to remove all cells and to separate them into a single cell suspension.
9. Transfer the cell suspension to a centrifugation tube.
10. Centrifuge the cells 5 min, 200 x g at room temperature.
11. Aspirate supernatant and resuspend the pellet in 1 to 2 mL CnT-Prime medium.
12. Count the viable cells and seed according to the seeding density listed in the data sheet.

Establishment of 3-Dimensional Epidermal Cell Cultures

Epidermal keratinocyte growing well in CnT-Prime medium can be induced to differentiate into 3-dimensional cultures, which closely resemble the structure of the in vivo epithelium.

Cells within the first 8-12 doublings after thawing provide the most complete differentiation. It is important to use primary keratinocytes growing well in CnT-Prime medium, as this ensures there is no shock when transferring cells to the CnT-PR-3D medium.

For best results, we recommend that several inserts are placed in a single cell culture dish (as outlined below) during the initial period of 3D culture establishment. Inserts may be switched to multi-well plates after this period if required. We recommend polycarbonate inserts, 0.4 µm pore size, 12 mm diameter, (for example cat# PIHP01250 from Millipore, or cat# 140620 from Nunc).

CELLnTEC media do not contain antibiotics or antimycotics. It is not recommended to use antibiotic / antimycotic during 3D culture, as it can disrupt differentiation and stratification.

3D Culture Protocol

See also the training video demonstrating this protocol on www.cellntec.com, in the resources section of www.cellntec.com. Please note the additional recommendations found in this updated protocol with respect to medium volume and medium change frequency, that are not described in the video.

1. Place six inserts into a 60 mm cell culture dish.
2. Wet the inserts with 3 mL CnT-Prime medium.
3. Prepare a cell suspension (5 x 10^5 cells / mL in CnT-Prime medium). Seed cell suspension into inserts (2 x 10^5 cells per insert in 0.6 cm^2 Millicell inserts, 1.55 x 10^5 into 0.47 cm^2 Nunc inserts).
4. Add the appropriate amount of CnT-PR medium outside the inserts (into the cell culture dish, e.g. ~ 11 mL for cell culture dishes with 53 mm inner diameter, if dishes with another inner diameter are used adjust the quantity of medium accordingly), so that medium levels inside and outside the insert are equal and the cells are submerged.

Important note: make sure that no air bubbles are trapped underneath the membrane for the entire duration of the 3D culture.

5. Place the inserts in a humidified incubator at 37°C and 5% CO_2
6. Grow the cells until they reach confluencey. This is typically 2 days, when using early passage keratinocytes that have been proliferating well in the preceding week.
7. Optional: if you are unsure of the growth rate of the cells, it is advised to stain one insert to examine confluency, as cultures must be confluent before proceeding further with air-lift culture. It is recommended to fix the cells in the insert first (e.g. with 100% MeOH), and then stain with diff-quick, giemsa, or equivalent to allow visualisation of cell confluence. Stain is also available separately from CELLnTEC: see product CnT-ST-100.
8. If the monolayer is confluent, proceed with step 8, otherwise change medium in the remaining inserts, cultivate for another day to reach confluence. If confluence is not achieved in 3 days, the growth is unlikely to be sufficient to successfully develop a successful 3D culture.
9. Replace the CnT-Prime medium with 3D Barrier Medium (CnT-PR-3D) inside and outside the insert (same amounts as when seeding, Step 3 & 4).
10. Place the inserts in the incubator overnight (15 to 16 h) to allow cells to form intercellular adhesion structures.
11. Initiate 3D cultures by aspirating all the medium from inside the insert and replacing outside medium with fresh CnT-PR-3D differentiation medium up to the level of the membrane (e.g. ~ 3.2 mL for cell culture dishes with 53 mm inner diameter, if dishes with other inner diameter are used, adjust the quantity accordingly to obtain the correct medium level). Three inserts should be used per 60 mm dish (approx 1 mL of medium per insert) to allow 3 medium changes per week (i.e. Mon/Wed/Fri). Using this approach (1 mL medium per insert/medium change 3x per week) models are typically fully established on day 12. If inserts need to be incubated separately (for example in 24 well plates), the volume of medium per insert will be lower, and the medium should be changed daily.
12. Important note: The surface within the inserts should dry following air-lift and should remain dry for the rest of cultivation. Therefore it is very important to have a confluent monolayer before starting the air-lifted culture.
13. For histochemistry, immunohistochemistry or immunofluorescence analyses, 3D-cultures are fixed over night at 4°C by placing the insert in a separate 24-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 scientist@cellntec.com