Frequently Asked Questions about Keratinocyte Cell Cultures

1. **What growth medium is used to culture human keratinocytes?**
   The preferred keratinocyte medium is CNT-07. This medium includes growth factors and is available from Zen Bio Inc. (Cell-N-Tech catalog #CNT-07).

2. **Is it necessary to grow keratinocytes on collagen-coated cultureware? If so, what supplier is recommended?**
   Yes culture keratinocytes on collagen IV coated cultureware. Extensive testing has shown that collagen IV (Sigma C7521) at a concentration of 0.67 µg/cm2 is optimal for enhancing the plating efficiency of keratinocytes.

3. **How do I coat cultureware with collagen IV?**
   Follow the manufacturer's method for diluting collagen IV. Create a stock solution of 500 µg/mL which can be frozen at -70°C for up to six months. Using the stock solution, make a working solution of 100 µg/mL in sterile water. This working solution can also be frozen at -70°C for up to six months. Dilute the working solution 1:15 in PBS (phosphate buffered saline) and use 2.5 ml to coat a T25 flask or 7.5 ml to coat a T75 flask. Collagen IV coated flasks can be stored at 4°C for up to 1 month. Just before use, incubate the flasks at 37°C for 30 minutes and rinse with PBS.

4. **What is the procedure for thawing and recovering frozen keratinocytes?**

   1. Prepare the culture medium.
   2. Place the frozen cell culture ampule in a 37°C water bath and agitate vigorously.
   3. Once completely thawed, wipe ampule with 70% alcohol. Score the neck of the glass ampule and open by snapping the neck where the score had been made or by using an ampule opener.
   4. Remove the contents of the ampule using a sterile transfer pipette and place in a collagen IV coated T25 tissue culture flask containing 5 ml of the appropriate fresh growth medium for keratinocyte cultures.
   5. If a cell count is required, mix the contents of the flask gently with a 1 ml pipette and remove 0.2 ml for a 1:5 diluted cell count. Place the flask in the 37°C incubator lying cell surface down. Gently swirl the flask to distribute the cells evenly over the flask surface. Adjust the cap to allow appropriate gas exchange (depending on buffering system of the medium).
   6. Re-feed the culture with fresh medium on the following day.
   7. Some cell lines recover better if all traces of cryoprotectant are removed by washing and centrifugation. Transfer the contents of ampule or cryovial to a 15-ml centrifuge tube with 3 ml of growth medium. Centrifuge for 5 min at 200 x g and 20°C. Remove supernatant, resuspend pellet, and transfer to a collagen IV coated T25 flask with a final volume of 5 ml.

5. **How is a keratinocyte subcultured?**

   Feed keratinocyte cultures 3 times a week (typically Monday, Wednesday, and Friday) with keratinocyte medium. When keratinocytes are 80-90% confluent, subculture them.
   <u>For sub-culturing</u>:

   1. Wash cells with 3 mL D-PBS.
   2. Rinse with 3 mL Versene solution (maximum 5 minutes).
   3. Trypsinize cells with 1 mL 0.05% trypsin/0.53 mM EDTA.
   4. Neutralize with 5 mL 2 mg/mL soybean trypsin inhibitor (STI) or culture medium

5. Centrifuge the cells at 200 x g for 5 minutes at 20°C
6. Resuspend in 5-6 ml keratinocyte medium for counting
7. Subculture at 1 x 10^4 cells/cm^2

6. What is the freezing medium used to cryopreserve human keratinocytes?

Keratinocyte growth medium + 10% FBS + 10% DMSO

7. Is it possible to use the keratinocyte growth medium for freezing the cells?

It is possible to freeze the cells in growth medium supplemented with 10% DMSO.

8. What protocol should be used for freezing keratinocytes?

To freeze keratinocytes, Versene (0.2 g/L EDTA•4Na in phosphate-buffered saline, Invitrogen 15040), 0.05% trypsin/0.53 mM EDTA in HBSS, soybean trypsin inhibitor (STI, 2 mg/ml), keratinocyte growth medium, and 2x keratinocyte freeze medium (growth medium, 20% FBS, and 20% DMSO) are needed for the following procedure for T75 flasks of confluent cells:

1. Prepare the 2x freeze medium and keep it refrigerated or on ice until use.
2. Each flask that is to be pooled for the freeze (freeze pool) should be examined microscopically for contamination and any unusual growth pattern.
3. Aspirate the growth medium from each flask. Wash the cells with 7 ml D-PBS then rinse with Versene solution (maximum 5 minutes at room temperature). Trypsinize the cells with 2.5 ml 0.05% trypsin/0.53 mM EDTA.
4. Examine the flasks microscopically to make sure the cells have rounded up - typically within 1-3 minutes.
5. Once the cells have lifted, add 10 ml STI to each flask to inactivate the trypsin. Gently triturate and then transfer the cell suspension from all flasks. Pool the cells.
6. Remove an aliquot of the freeze pool, count the cells, and calculate the total viable cells.
7. Centrifuge the cells at 60-100 x g for 10 minutes at 8-10°C.
8. Using culture medium without serum or DMSO, resuspend the cell pellets in half the volume needed for a cell density of 500,000 cells/ml.
9. For the second half of the volume, add the 2X freezing medium slowly (drop by drop) while gently mixing the cells.
10. Dispense 1 mL of the cell suspension to each glass ampule or plastic cryovial.
11. Freeze the ampules or cryovials at a rate of 1°C per minute.
12. Frozen cell stocks are stored in liquid nitrogen tanks. Glass ampules are submerged in liquid. Plastic cryovials are stored in the vapor phase.