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Culture of human epithelial cells (skin, cornea, thymus) on 3T3J2 feeder layer cells V.1

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We use this protocol and it's perfectly working

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Abstract

This document is of vital importance for scientists attempting the culture of human keratinocytes derived from skin, cornea, thymus, esophagus etc. Please strictly adhere to the guidelines shared. Note that 3T3NIH ARE NOT the same as 3T3J2, which are way more efficient in sustaining keratinocyte culture.

Troubleshooting

Key steps for 3T3J2 feeders growth (Howard Green lab)

1 Obtain DMEM

2 Add 8% of bovine serum (DO NOT use fetal calf serum! key point)

3 Feed every 2 or 3 days

4 Pass the cells once a week

Culture of 3T3J2 feeder cells (Howard Green Lab)

5 When the cells have reached pre-confluency, trypsinize them according to the manufacturer. Split the cells as follows:

5.1 Set up one flask at low density to maintain the line (5*10^5 cells at early passages, 10^5 cells at late passages). This flask should reach pre-confluency in maximum of one week.

5.2 Set up several flasks for feeder layer cells (10^5 to 10^6 cells per flask). try either to use pre-confluent or just confluent flasks for feeder layer when growing keratinocytes)

6 Carry the line for no more than 14 passages.

7 If you continuously pass the feeders from post-confluent flasks some of the cells will begin to lose their contact inhibition. A slight rise in the saturation density of the flask is normal as you go on in passage number. The maximum number of cells from a just confluent 150 square centimeter flask is about 10-12 × 10^6 cells. If you're getting in the order of 15-20 × 10^6 cells your feeders are not being passed properly this can affect the quality of your keratinocyte culture.

Key steps for human keratinocyte cultures

8 Medium: DMEM +10% **fetal calf serum! DO NOT USE BOVINE SERUM**

9 Feeders: irradiated 3T3J2 feeder cells. Avoid Mitomycin.

10 37% CO^2 humid atmosphere incubator

11 Feedings: add EGF at the first and subsequent feedings. Plating efficiency should be fed every four days and stained on day 12 with rhodamine. Mass cultures should be fed every 2-3 days, depending on how fast the cultures are growing

Keratinocyte cultures

12 The mass culture should be passed and/or frozen between days 7 and 10 depending on how
well it is growing. Cultures should not be allowed to get confluent but also should not be kept in culture for more than 10 days before transferring them. It is best to feed the pre-confluent cultures the day before you plan to pass or freeze them, especially if the medium is very acidic looking.

13 **Procedure:**
Trypsinize the mass culture according to the standard procedure.
Count total number of cells.

- For plating efficiency: set up 2 ×100 mm Petri Dishes with 10^2 keratinocytes each.
- To amplify secondaries: add between 2-5 * 10^5 keratinocytes per 150 cm2 flask.

cFAD medium (Keratinocyte medium) = FAD, 10% fetal bovine serum + goodies

14 **Insulin**
Dissolve 50mg in 10 ml 0.005 N HCl (stock 5mg/ml)
Distribute in aliquots and store at -20°C.
Sterilize before use
Add 0.5 ml to 500 ml of complete medium (final concentration of 5 ug/ml)

T3
*Aliquots (concentrated stock 2*10^-4 M stocked at -20°C).*
Take 0.1 ml concentrated stock, make up to 10 ml with PBS
Distribute in aliquots and store at -20°C (stock 2*10^-6M)
Sterilize before use
Add 0.5 ml to 500 ml of complete medium (final concentration of 2*10^-9 M)

Hydrocortisone

Aliquots (concentrated stock 5mg/ml stocked at -20°C).
Take 0.4 ml concentrated stock, make up to 10 ml with serum-free medium (FAD)
Distribute in aliquots and store at -20°C (sotck: 200 ug/ml)
Sterilize before use
Add 1 ml to 500 ml of complete medium (final concentration of 0,4ug/ml)

Cholera Toxin
Aliquots (concentrated stock: 10^-5 M) stored at 4°C! DO NOT FREEZE!
Add 0.1 ml concentrated stock to 10 ml medium with 10% serum
Distribute in aliquots and store at -20°C (stock *10^-7 M*)
Sterilize before use
Add 0.5 ml to 500 ml of complete medium (final concentration of 10^-10 M)

EGF
100ul aliquots (concentrated stock: 100ug/100ul) stored at -80°C
Bring 100 ug aliquot concentrated stock to 10 ml sterile 01% BSA
Sterile filter (0.22um Millipore straining system)
Distribute in 100ul - 250ul aliquots and store at -20°C (stock 10ug/ml)

At feedings, add 0.1 ml to each 100 ml of complete medium (final concentration 10ng/ml)

Cell procedures

15 **Trypsinizing:**
Remove the culture medium and add trypsin /EDTA. Trypsinize at 37°C.
Collect the cells in a centrifuge tube, and add at least 0.5 volume of medium with 10% serum (cFAD) to stop tryptic action.
Centrifuge for 5 minutes 1300 rpm 5 min for 15 ml Flacon tube. Remove the supernatant and resuspend cells in the medium.
Determine the number of cells.

Freezing:
Change the medium of growing cells one day before freezing.
Trypsinize according to procedure.
Determine the total number of cells in suspension, centrifuge, and remove supernatant.
Resuspend the cells in cFAD + 10% DMSO (concentration of about 1-2*10^6 cells/ml)
Mix well and transfer about 1.2 ml to a freezing capsule and mark it indelibly.
Pre-freeze the cells at -80°C in isopentane freezing boxes for one night before moving to liquid nitrogen tank.

Thawing:
Remove the desired freezing capsule from the liquid nitrogen tank.

Quick thaw the capsule in 37°C water bath.
Transfer content with a Gilson tip into a 15ml tube with 10ml cFAD medium to wash the cells.
Centrifuge 5 mins at 1300 rpm, remove supernatant, resuspend in adequate cFAD medium, inoculate cells on 3t3J2 preirradiated dishes.

Protocol references

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