Culture of human epithelial cells (skin, cornea, thymus) on 3T3J2 feeder layer cells V.2

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Protocol status: Working
We use this protocol and it's working

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.

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3T3J2 Growth Medium

1 Open DMEM bottle (ThermoFisher Scientific).

2 Add [M] 8 % (v/v) of bovine serum (ThermoFisher Scientific) (**DO NOT use fetal calf serum!**).

3 Add [M] 1 % (v/v) Pen/Strep and complete medium. Store the 3T3-J2 medium at 4 °C and heat it before use.

Culture of 3T3J2 feeder cells

4 Obtain 3T3-J2 from Kerafast (https://www.kerafast.com/item/1100/3t3-j2-cell-line). Plate the vial according the manifacturer's instructions in a humidified 37 °C 10% CO2 incubator. Feed the 3T3J2 flask every 3 or 4 days.

5 When the cells have reached pre-confluency, trypsinize them. Briefly, wash 1x with PBS and add Trypsin/EDTA and put the flask in a 10% CO^2 humidified incubator 37 °C for 00:15:00. If cells are not well detached, add a bit more Trypsin/EDTA and incubate for 00:05:00 minutes more. 40m
Collect the cells and add some 3T3-J2 medium to stop tryptic action. Count the cells.
Centrifuge the cells at 1300 rpm in a 15 ml tube for 00:05:00 or 00:15:00 for a 50 ml Falcon tube. Discard supernatant and add fresh 3T3-J2 medium.
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 and is precious for stock.

5.2 Set up several flasks for feeder layer cells (10^5 to 10^6 cells per flask) or expanding the stock. Try to use pre-confluent flasks for feeder layer when growing keratinocytes (human epidermis, cornea, thymus, esophagus). Carry the line for no more than 14 passages. If the cells grow too fast, discard the batch.

cFAD medium (Keratinocyte medium)

6 Prepare cFAD medium. The cFAD medium consists of a **3:1 mixture of DMEM and Ham's F12 medium** (Gibco-Invitrogen) supplemented with 10% **FETAL calf serum** (Gibco - Invitrogen), 0.4 µg/ml **hydrocortisone** (Calbiochem, VWR), 10-6M **cholera toxin** (Sigma), 5 µg/ml **insulin** (Novo Nordisk), 2 x 10-6 M 3,3',5-Triiodo-L-Thyronin (**T3**) (Sigma) as previously described (see references). Human recombinant epidermal growth factor (**rhEGF**; 10 ng/ml) (QED Biosciences) is added to the culture medium at the first feedings. **DO NOT USE BOVINE SERUM.**

Key steps for human keratinocyte cultures (Howard Green Lab)

7 Heat cFAD medium

8 Feeders: **irradiate** 3T3-J2 feeder cells when attached to the petri dishes (seeding density: 2.5 x 10^4 cells/cm2) with a total dose of **60 Gy**. **Avoid using Mitomycin** instead of irradiating, as we have observed transient fusion between keratinocytes and feeders under the microscope.

9 Once irradiated, put the dishes in a 37% CO^2 humid atmosphere incubator.

10 Remove the 3T-J2 medium from the irradiated dishes and plate keratinocytes on the irradiated 3T3-J2 feeder layers dishes with the cFAD medium. Wait until the next day to verify the attachment of the cells onto the layer. If the keratinocytes cells are plated "in mass", you will see them disperced in the dish. If they are few, you will see colonies growing in the following days.
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.

11 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. Count keratinocytes before freezing them and keep this record on the freezing vial/lab book.

trypsinization of keratinocyte cultures (Howard Green Lab)

12 Trypsinize the mass culture according to the standard procedure. 10m

Count total number of cells. Remove the culture medium and add trypsin/EDTA. Put the dish/flask in a humid incubator 37 °C

Pipet the cells up and down until they are almost all detached and puth them in a 15 ml Falcon tube, and add at least 0.5 volume of medium with 10% serum (cFAD) to stop tryptic action.

Centrifuge for 5 minutes 1300 rpm for 15 ml Flacon tube (15 min for a 50 ml falcon tube). Discard the supernatant and resuspend cells in fresh medium.

Determine the number of cells.

- For plating efficiency: set up 2 x100 mm Petri Dishes with 10^2 keratinocytes each. After colonies growth, fix the plate with formaldehyde at RT for 00:10:00 and stain the cells with rhodamine A.
- To amplify secondary cultures: add between 2-5 * 10^5 keratinocytes per 150 cm2 flask.

Cells freezing and thawing 5m

13 **Freezing:** 5m

Change the medium of growing cells one day before freezing.

Trypsinize according to procedure above and proceed to counting them.

Resuspend the keratinocytes cells in cFAD+10% DMSO (concentration of about 1-2*10^6 cells/ml).

If freezing 3T3-J2, resuspend the unirradiated feeder cells in 3T3-J2 medium +10% DMSO.

Mix well and transfer about 1.2 ml to a freezing capsule and mark it indelibly (use pencil if cells will go into an isopentane filled box).

Pre-freeze the cells at -80 °C in isopentane freezing boxes for one night before moving to liquid nitrogen tank -130 °C

Thawing:

Remove the desired freezing capsule from the liquid nitrogen tank. Quickly thaw the capsule in 37 °C water bath.

Transfer content with a Gilson tip into a 15ml tube with fresh 10ml heated cFAD medium to wash the cells for keratinocytes and 10 ml heated 3T3j2 medium for 3T3J2.

Centrifuge the Falcon tubes 00:05:00 at 1300 rpm, discard supernatant, resuspend cells in adequate volume of cFAD/3T3J2 medium.