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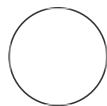
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**Protocol status:** Working  
We use this protocol and it's working

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

Pierluigi Giuseppe Manti<sup>1</sup>

<sup>1</sup>Università degli Studi di Milano, Dipartimento di Oncologia ed Emato-Oncologia



Pierluigi Manti

### DISCLAIMER

The author declares no conflict of interest.

### ABSTRACT


Despite several protocols having been published in the past in a fragmented way, this protocol represents a harmonious recollection and reorganization of those, which proves to be a useful tool for any scientists attempting the culture of human keratinocytes derived from skin, cornea, thymus, esophagus, etc. in any laboratory. This protocol is also of utter importance as the generation of engineered skin, cornea, and thymic mini-organs becomes more and more feasible.

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





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## 3T3J2 Growth Medium

- 1 Open DMEM bottle (ThermoFisher Scientific).
- 2 Add [IM] 8 % (v/v) of bovine serum (ThermoFisher Scientific) (**DO NOT use fetal calf serum!**).
- 3 Add [IM] 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 manufacturer's instructions in a humidified  37 °C 10% CO<sub>2</sub> 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<sub>2</sub> 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. 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<sup>5</sup> cells at early passages, 10<sup>5</sup> 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<sup>-6</sup>M **cholera toxin** (Sigma), 5 µg/ml **insulin** (Novo Nordisk), 2 x 10<sup>-6</sup> 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:  $2.5 \times 10^4$  cells/cm<sup>2</sup>) 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<sub>2</sub> 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 dispersed 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)

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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 put 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 Falcon 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  $2-5 \times 10^5$  keratinocytes per 150 cm<sup>2</sup> 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  $1-2 \times 10^6$  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.

