Keratinocytes from keratin-deficient mice

General

Cells were derived from strain C57Bl6 on 3T3J2 feeders and spontaneously immortalized (Turksen (ed.) Epidermal Cells, Methods in Molecular Biology 585, chapter 5). Keratinocytes are highly adherent, and grow optimally on collagen I-coated dishes. To maintain their proliferative potential, Ca^{2+} in the serum must be adjusted to 50 µM. Avoid low density as they require cell-cell contact. Also, avoid prolonged time in suspension; loss of cell-matrix contact induces anoikis. Typical split ratio is 1:2, not more than 1:3! Cells are grown at 37°C. We use cell culture plastic from TPP (also possible), cell culture grade water and high quality serum (PAA Gold).

Thawing and plating cells

Coat a 6 cm dish with 90-120 µl of collagen I working solution per cm^2 growth area. Leave at 37°C for 30 min. Aspirate and rinse dish 2 x with PBS.

Thaw 1 freezing vial containing 1.5-2 million cells quickly by incubation in 37°C water bath and immediately transfer to sterile bench. Transfer cells into 50 ml plastic tube containing 10 ml of pre-warmed medium. Shake gently to mix and spin for 5 min at 1.000 rpm (RT). Re-suspend in 6 ml of medium and disperse cells by pipetting up and down a few times. Afterwards plate cells on collagen-coated 6 cm dish. Change medium next day. If they are confluent, split 1:2 and plate them onto a 6 cm dish until confluence. If large amounts of cell are required, switch to 10 cm dishes (to go from 6 to 10 cm means 1:3 splitting).

If you receive cells that stably re-express keratins (by lentiviral gene transfer), cells should always be cultured in selection (8 µg/ml of puromycin-or other, information is provided) medium. The frozen stocks we ship contain 95-99 % of stable transfectants. Regularly check presence of transfected keratins (e.g K5 or K6) by immunofluorescence and by Western blotting.

Subculture

Passage cells only when they are confluent (appr. 4-5 mill. cells on 10 cm dish). Typical split is 1:2, therefore coat 2 plates for each plate to be trypsinized (every 4-5 days).

Aspirate media and wash cells with PBS (Ca^{2+} and Mg^{2+}-free). Add 2 ml of trypsin solution per 6 cm dish (3 for 10 cm dish) (0.025 % trypsin/0.02% EDTA in PBS). Incubate cells for 7-8 min at 37°C until cells detach. Check detachment of cells by microscopy. Add same amount of fresh media and pipette the cells up and down several times to neutralize the trypsin/EDTA. Pellet the cells by low speed centrifugation (1000 rpm for 4 minutes, RT), aspirate media, resuspend cells in 10 ml of fresh media and replate them into 2 dishes. Add media up to 6 ml on 6 cm and 10 ml on 10 cm dish. Cells should not be seeded lower than 30.000 cells per cm^2.

Induction of terminal differentiation by addition of Ca^{2+}

Terminal differentiation of keratinocytes can be induced by culturing cells in FAD media containing high content of calcium. In general 2 million cells were seeded into a 10 cm collagen coated dish. When cells are used for calcium shift experiments the normal FAD media is changed to FAD+ Media containing 1.2 mM $CaCl_2$ on the next day. Experiments in high calcium are performed 3 days after plating and 48-50h after the calcium shift. (FAD+ - Differentiation media FAD media containing 1.2 mM $CaCl_2$)

Freezing

Cryopreserve keratinocytes at $2x10^6$ cells per vial. Trypsinize cells as described, spin down as above and resupend $2x10^6$ cells in freeze solution (90 % FCS Gold (Chelex treated) and 10% DMSO). To freeze, fill the cells into a cryotube and transfer it in a Nalgene freezing box immediately. Add 250 ml isopropanol in Nalgene freezing container. Place Nalgene container into -80° C ON (or at least 4 hrs). Thereafter, transfer cells to liquid nitrogen container.

Media, coatings, supplements:

Keratinocytes are routinely cultured in the presence of 10 % Chelex-treated serum.

FAD – Cell culture media

To prepare 500 ml of complete media, set up as follows:

DMEM/Ham's F12, low Calcium (50µM) Biochrom F.-9092 (US sales: Cedarlane Laboratories U.S.A.)

50 ml	FCS (Chelex-treated !)	- PAA A15-151 (500ml)
5 ml	Glutamax [100x]	- Invitrogen 35050-038 (500ml)
5 ml	Pyruvate [100x]	- PAA S11-003 (100ml)
2,5 ml	Pennicillin/Streptomycin	- Invitrogen 15140-122 (100mg)
2 ml	Adenin [250x]	- Sigma A8626-5G (5g)
500 µl	EGF [1000x]	- Invitrogen 53003-018 (100µg)
500 µl	Insulin [1000x]	- Sigma-Aldrich I-9278 (5ml)
250 µl	Hydroxycortison [2000x]	- Sigma-Aldrich H4001-1G (1g)
50 µl	Choleratoxin [10^{-5}M]	- Sigma C-8052 (1mg)

Preparation of stock solutions

Adenine (Sigma A8626-5G): stock (250x): 45mM in 0.05 N HCl; storage: - 20°C

2ml stock/ 460ml media > final concentration 0.18mM

Hydrocortison (Sigma-Aldrich H4001-1G): stock. (2000x): 1mg/ml in EtOH; storage: - 20°C

250µl stock/ 460ml media > end concentration 0.5µg/ml

Insulin (Sigma-Aldrich I-9278): stock. 5 mg/ml; storage: - 20°C

0.5ml stock/ 460ml media > final concentration 2.5 µg/ml

EGF (Invitrogen 53003-018): stock. (1000x): 10µg/ml in FAD Media; storage: - 20°C

0.5ml stock/ 460ml media > final concentration 10ng/ml

Cholera toxin (Sigma C-8052): stock. (10^{-5}M): 1mg/1,18ml sterile water; storage: 4°C

50 µl stock/ 500ml media > final concentration 10^{-10}M

Glutamax (Invitrogen 35050-038): stock. (100x): 200mM; storage: - 20°C

5ml stock/ 500ml media > final concentration 2mM

Pen/Strep (Invitrogen): stock. (100x); storage: - 20°C

2,5 ml stock/ 500ml media > final concentration 100U/ml P., 100µg/ml S.

Pyruvate [100x] (PAA S11-003), final conc. 1 x.

Penicillin/Streptomycin (Invitrogen 15140-122). Use at 1 x for final conc.

Collagen I (BD Biosciences 354236) Use at ~ 0,045 mg/ml 0,02 N acetic acid for final conc.

Use 90-120 µl of per cm^2 growth area.

Preparation of collagen I

Prepare always fresh in sterile bottle!

Stock solution 4,27 [3,91] mg/ml collagen I in 0,02 N acetic acid

Final solution: 0,5 ml stock solution
42,7 ml 0,02 N acetic acid

Prepare 0.02 N acetic acid from concentrated stock, using cell culture grade water. 0.02 N acetic acid is prepared from stock: to 500 ml of cell culture grade water, add 570 µl of concentrated acetic acid.

Use the following amounts of final solutions:

75 cm^2 flask	6-7 ml
10 cm dish	6-7 ml
25 cm^2 flask	3-4 ml
6 cm dish	2-3 ml
6 well	1 ml
3,5 cm dish	1 ml
24 well	0.25 ml

Serum

We routinely use FCS Gold (PAA A15-151). To lower Ca^{2+} concentration in the serum, it is treated with neutralized Chelex 100 (Bio-Rad) 5g/ 50ml FBS as described below.

Rinsing and neutralizing Chelex 100

1. Add 500ml Milli-Q dH_2O 500ml to flask or beaker
2. Add 100g Chelex (Bio-Rad) while stirring.
3. Turn off stirrer and let Chelex settle for 30 min.
4. Adjust pH to 7.35-7.4 with HCL (3-6 M)
5. Pour off dH_2O while being careful not pour off any Chelex
6. Again, wash one time with Milli-Q dH_2O: add dH_2O, stir 5 min, let Chelex settle for 30 min, then pour off dH_2O
7. Wash two times with 500 ml PBS as in step 6. On the last wash with PBS, stabilize the pH to 7.35-7.4 before letting Chelex settle.
8. Pour off PBS carefully.

Preparing chelexed Serum

9. Add 500ml of cold (4°C) FBS to 100g Chelex (in a flask or beaker)
10. Stir 1h at 4°C in the darkness (wrap with aluminum foil)
11. Filter FBS once through a 0.45 µm filter to remove remaining resin and again through a 0.22 µm filter to sterilize.
12. Set aside a sample to check that the pH is in a range of 7.35-7.4.
13. Label, aliquot into 15ml or 50ml sterile containers and store at -20°C in the dark.