Protocol for the isolation of primary murine keratinocytes from newborn mice

The isolation of keratinocytes needs an overnight incubation, therefore start it in the afternoon.

- Use newborn mice, not older than 2 days pp
- Kill by decapitation (I always make them sleep with ether before)
- Cool bodies (in a beaker) on ice for 1 hour
- In the meantime prepare: one 50ml falcon with half Beta. Iodide/ half 1x PBS
  one 50ml falcon with 1x PBS
  one 50ml falcon with 70% EtOH
  one 50ml falcon with 1x PBS
- Then perform the following procedure using one body after the other and keep the rest on ice:

Wash and disinfect bodies:

⇒ 1x PBS/ Betaisodona Iodide 1min
⇒ 1x PBS (rinse briefly)
⇒ 70% ethanol 1min
⇒ sterile water or PBS (rinse briefly)

- transfer to a sterile dish (everything must be sterile from here)
- cut off tail and limbs (throw away or use for genotyping)
- make a lengthwise incision on the back from the neck to the tail
- "unwrap" the mouse body using forceps and the dull parts of scissors, take off the skin in one piece
- transfer skin to a dish with antibiotic/antimycotic solution (GibCo) 1:100 in PBS without calcium and magnesium, incubate for a few minutes (you can do the next mouse in the meantime)
- take skin out of the antibiotic/antimycotic solution and transfer to a new 35 mm dish
- spread skin on the plastic surface, epidermal side up
- fill the dish with 2 ml trypsin solution for cell culture (0.025% trypsin in PBS/EDTA) ***from the side*** so that the skin floats on the trypsin solution. Do not cover the epidermis with trypsin, only the dermal side of the skin should come into contact with trypsin
- put the dish in a refrigerator over night.

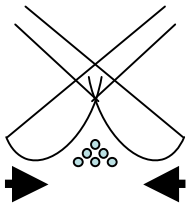
- At the same day coat cell culture dishes with collagen. We use bovine collagen I, either homemade or bought from Sigma. Dissolve the collagen in 0.1% acetic acid, e.g. at 2 mg/ml (stock solution), dilute with PBS without calcium and magnesium to a final concentration of 20 - 30µg/ml and spread 2 ml of this solution on a cell culture coated dish, 60 mm, Falcon (we've had problems with Greiner dishes). Incubate in the refrigerator over night. Next day aspirate the solution, wash twice with sterile PBS without calcium and magnesium. They are ready for use now (see below).

<u>Next morning:</u>

- Treat the feeder cells with mitomycin (???conc???; simply add concentrated mitomycin to the medium) and incubate cells for 2 hrs at 37°C, 5% CO_2.

In the meantime:

- Take the skins out and peel the epidermis from the dermis; use forceps, the epidermis comes off very easily, throw dermis away
- pool the pieces of epidermis in a 60 or 100 mm plastic dish
- Mince epidermis into very small pieces using two scalpels



- Suspend these epidermal fragments in 1,5 ml medium (FAD low Ca) with serum and shake in a 2ml sterile eppi for 30 minutes at RT, 1000rpm.
- (not necessary: pass the epidermis suspension through a sterile nylon filter 70 µm pore size (e.g. Falcon) and plate the flow through on the collagen coated dishes. Note: The density of the cells on the dish is very important, they have to be quite dense. When you isolate cells from a whole litter plate them on a maximum of two 60 mm dishes)
- wash the mitomycin treated feeder cells with 1x PBS, add trypsin/EDTA, rinse the plate with trypsin/EDTA, suck off the trypsin/EDTA and incubate the cells at 37°C, 5% CO_2 for a few minutes.
- Wash the collagen coated 6-well plate with 1x PBS, suck off the PBS
- Then resuspend the trypsinized feeder cells in FAD medium with 50µM Ca^{2+}.
- Apply the medium with the epidermal cells which had been shaking in FAD low Ca^{2+} to a 60 mm well per mouse and adjust the medium to 2ml with 0,5ml feeder cell suspension. Then incubate the cells at 32 Celsius, 5% CO_2 in a cell culture incubator. You won't see many cells growing out during the first week, don't worry about that. Colonies usually become visible in the second or third week.
- When the dish is confluent split 1:2 on collagen coated dishes. After passage 4/5 you can also split the cells at lower density, like 1:3 or 1:4. Murine keratinocytes are very sensitive, especially during the first passages after isolation. After 5-6 passages cells immortalize spontaneously and become less fuzzy.

The medium:

We use FAD medium with 50 µM Calcium concentration (FAD low Ca). The recipe for FAD medium with normal calcium concentration (1.8 mM) is published, I can fax it through. We have a calcium free mixture of DMEM and HAM's F12 manufactured by Biochrom and add calcium chloride ourselves to a final concentration of 50 µM.
The medium contains 10% FCS, in order to remove calcium ions this has to be pre-treated with Chelex (Bio-Rad; 2g/ 50 ml) overnight on a rotating wheel at 4 Celsius and then sterile filtered.

Good luck!

Für die Isolierung primärer Keratinozyten:

Material:

Ice – to cool dead mice

Beaker – put dead mice on its bottom to cool down

Scissors – one stumpf/stumpf, gerade - to decapitate mice

one stumpf/stumpf, gebogen – to peel off skin

Forceps - one stumpf – for handling mice and tissue samples

two spitz – for peeling off skin, its stretching on the petridish plate, peeling off epidermis…

50ml falcons – four of them

eppies - 1,5ml for tails, paws

2,0ml sterile for minced epidermis

Bacterial petridishes mit Nocken –

10cm – to keep mice inside while peeling off the skin

6cm – to mince epidermis into pieces

3,5cm – from Greiner

to float isolated skin in antibiotic/antimycotic solution

to spread skin with epidermis side up and float it onto trypsin/EDTA solution

6-well Zellkulturtestschalen- von Falcon oder TPP (über Renner GmbH #9206), KEINESFALLS Greiner

Lösungen:

1x PBS wo Mg^{2+}, Ca^{2+}

Betaisodona Iodlsg.

70% EtOH

Antibiotic/antimycotic sol. (GIBCO #15240-096 100x)

Collagen I (Sigma C9791 100mg; collagen type I from calf skin acid)

Trypsin/EDTA (GIBCO #25300-054 = ours = 1x = 0,5g/l trypsin + 0,2g/l EDTA.4Na in Hanks B.S.S.;
GIBCO #15090-046 = Ingos = 10x = 25g/l trypsin + 8,5g/l NaCl)

FAD medium + low Ca^{2+} (50µM)

Feeder Cells medium

Mitomycin (Sigma #M0503 oder Fluka #69824 je 2mg; stock: 0,4mg/ml in H_2O -> steril filt. kept at –20°C; f.c.: 4µg/ml; thawn aliqouts are kept at 4°C till they are used up)

Biologisches Material:

Newborn mice not older than 2 days pp

Feeder layer cells = J2 3T3

Preparation of FAD medium low Ca $^{2+}$ (50µM):

1. Removal of Ca $^{2+}$ from FCS GOLD (PAA):
   Thaw a 50ml aliquot of FCS GOLD (from Renate) at 37°C, then add 2g of Chelex 100 , 200-400 mesh (analytical grade chelating resin, sodium form; BioRad #142-2842) and mix o/n at 4°C on a wheel rotator. The next day spin at 2000 rpm for 10min, RT and filter FCS supernatant through a 0.20µm pore size filter (Einmal-Vakuum-Filtrationsgerät; 125ml Vol.; Renner GmbH #06 050). Now the FCS is considered Ca $^{2+}$ free and ready for use.
2. Addition of substitutes to the basic medium:
   500ml DMEM (2/3) + HAM's F12 (1/3) (Spezialanfertigung für die Hautklinik bei Biochrom : #FZ9995, Lot.No.: FF031)
   50ml Ca $^{2+}$ free FCS GOLD (PAA)
   5ml Pen/Strep (Biochrom #A2212)
   5ml L-Glutamin (Biochrom #K0282)
   0,025g Vitamin C (Sodium L-ascorbate; Sigma #A4034)
   500µl EGF (Sigma E9644 0.2mg; stock 10µg/ml in FAD + Suppl.; f.c.: 10ng/ml)
   500µl Insulin (Sigma I1882 100mg steril; stock 5mg/ml in steriler 5mM HCl; f.c.: 5µg/ml)
   50µl Hydrocortison (Sigma H4001 5g; stock 5mg/ml in EtOH; f.c.: 0,5µg/ml)
   5µl Cholera Toxin (Sigma C-8052, 1mg; stock 1mg/ml=10^{-5}M in dH_2O; f.c.: 10^{-10}M)
   750µl Adenin (zum Lösen auf 50°C erwärmen; Sigma #A9126 1g [1g gibt es nicht mehr im Katalog] bestellt: #A3159 5g; stock 1g/22,5ml = 1,2 x 10^{-1}M in 0,5N HCl; f.c.: 1,8 x 10^{-4} M)

Zusätze Z: Pen/Strep, L-Glu and Vitamin C in a mixture of

100ml P/S
100ml L-Glu
0,5g Vitamin C
steril filtriert und aliquotiert zu je 10ml

Feeder Cell Medium: 500ml DMEM (GIBCO #41965-039)
50ml FCS
10ml Zusätze Z

Feeder Cells: J2 3T3 do not grow on Greiner tissue culture dish, I used those from TPP