Mouse primary keratinocytes preparation

1. Fill a 150 X 25 mm petri dish with ice. Put newborn mice (2-3 days old) in the petri dish and insert it in an ice bucket. Leave the mice in the ice bucket for 1 hour.

2. Wash dead newborn once with water, then twice with 70% ethanol. Remove ethanol completely.

3. Using sterile techniques under the hood, amputate mice tails and limbs with surgical scissors.

4. Work with one mouse at a time. Cut on the dorsal side and along the length of the body of the mouse with a scalpel such that the skin is cut, but the internal parts of the body are intact. Carefully separate the skin from the rest of the viscera. Put the skin in a petri dish containing 20 ml of PBS.

5. Repeat the procedure for all the skins.

6. Flatten the skins in an empty 150 mm petri dish with the back of forceps. It's crucial that the skin is perfectly stretched, even at the edges, otherwise peeling is very difficult. The skins should have the dermis facing down.

7. Add approximately 10 ml of trypsin solution (Gibco #15050-065) to each petri dish, and store petri dishes overnight at 4°C. The skins should be floating on the trypsin solution.

8. Put one skin at a time on an empty petri dish, flatten it again and use forceps to separate the epidermis from the dermis, starting from one corner of the skin. Place the epidermis in a petri dish with Low Ca^{++} medium plus serum and EGF.

9. Mince epidermis with tweezers and scissors, until epidermis is reduced to very small fragments. Place the epidermis in a

flask with a sterile stirring bar; add enough medium (7 ml/skin). Stir slowly for 1-1.5 hours.

10. Filter cell suspension through 2-3 layers of sterile gauze.

11. Plate cells (1 skin/10 cm dish) on plates coated with collagen Type I. (Incubated at least 15 minutes in the incubator, after spreading coating solution evenly around the dish and removing as much as possible.)

12. Incubate at 34°C, 8% CO_2; change the medium everyday. Cells can be used 4-8 days after plating.

Amount of Medium / Dish

24 wells rack	500 µl
35 mm	1 ml
60 mm	3 ml
10 cm	7ml

DERMOFIBROBLAST PREPARTION

8 dermis	+	12 ml HBSS
		0.5 ml Collagenase solution (10 mg/ml H_2O)

Stir for 30 minutes at room temperature in a sterile flask
Filter through a sterile gauze
Plate 3-4 ml for each maxi dish
Incubate at 37°C, CO_2 5.0%

LOW Ca^{++}MEDIUM + SERUM + EGF (for keratinocytes)

For 1 liter:

Salts (5X)	200 ml
Glucose (10 g/L)	100 ml
Phenol red (2 g/L)	5 ml
Mem NE AA (100X)	10 ml
Mem vitamins (100X)	8 ml
Mem AA (50X)	18 ml
Chelex serum	40 ml
Antib./Antimycotics	10 ml
EFG (10 ng/ml final)	250 µl
CaC12 1M	45 µl
NaOH 5M	~0.9 ml for a final pH~7.0-7.2
+ H_2O MilliQ	up to 1 L

Filter 0.2 µm

SALTS 10X FOR 1 liter

KCl	4 g
$MgSO_4$-$7H_2O$	2 g
NaCl	68 g
$NaHCO_3$	22 g
NaH_2PO_4-H_2O	1.4 g

MEM NE AA	from Gibco
MEM VIT	
MEM AA	
ANTIB./ANTIM.	

EGF stock

100 µg	EGF (Collaborative Research)
10 ml	HBSS 1X
50 µg	BSA (100 mg/ml)

CHELEX FETAL BOVINE SERUM

Chelex treatment:

300 g Chelex 100 (Biorad; 100-200 mesh, sodium ferm)
4L ddH_2O

1.) Stir slow overnight at room temperature

2.) Adjust pH 7.0-7.5 with HCl (about 12 ml of HCl : H_2O 1:1 for 6 L)

3.) Filter using 3MM paper (after having eliminated most of the H_2O)

4.) Recover Chelex (Biorad) and add 1 liter FBS

5.) Stir at room temperature for 1 hour

6.) Sit undisturbed at room temperature for 30-60 minutes

7.) Recover supernatant and filter it 0.45 µm

8.) Aliquot and freeze at -20°C

COLLAGEN COATING SOLUTION

100 ml	HBSS 1X
100 µl	BSA (100 mg/ml)
2 ml	HEPES 1M pH 6.5
1 ml	VITROGEN 100 COLLAGEN (Vendor: Cell Trix 1-800-328-4901)

Filter 0.2 µm

TRYPSIN SOLUTION FOR SPLITTING KERATINOCYTES

1 L	PBS
60 ML	Trypsin 2.5% (Gibco, 610-5090AG)
6 ml	EDTA 0.4 M pH 8

TRYPSIN FOR SKINS:
GIBCO, TRYPSIN 0.25% CAT# 15050-065

TRYPSIN FOR SPLITTING FIBROBLASTS

1 liter	PBS
60 ml	2.5% Trypsin 10x (Gibco, #610-5090 AG)
6 ml	0.4 M EDTA pH 8.0

Filter through 0.20 µm

SPLITTING FIBROBLASTS

1 wash with PBS (6-8 ml)

1 ml Trypsin for fibroblasts 10 cm dish
Incubate at 37°C for 1-5 minutes
Wash dish thoroughly, and add to new dishes containing medium

SPLITTING OF KERATINOCYTES

1 wash with PBS (6-8 ml)
1 ml Trypsin for keratinocytes
Incubate
When cells are almost detached, aspirate Trypsin, add 1 ml PBS and split.

Note: Move dishes immediately after plating but not with a circular motion.

TRANSIENT TRANSFECTION PROTOCOL FOR MOUSE PRIMARY KERATINOCYTES

Protocol for 100 mm dishes (60 mm dishes).

Ethanol precipitate 20 µg of DNA/dish (10 µg) or less.
Wash keratinocytes with 5 ml (2 ML) 1XPBS and replace with 4ml (1.5 ml) low calcium medium without serum, with EGF(1 µl/ml), insulin and transferring (1 µl/ml)
Incubate cells at 34°C for at least 10 minutes before adding the DNA.
Resuspend DNA in 40 µl (20 µl)/dish of TBS and incubate at 65°C for 10 minutes.
Warm up at 37°C 80 µl (40 µl)/dish of DEAE-Dextran Solution for 5 minutes.
Mix resuspended DNA with DEAE-Dextran (add DNA solution on the side of the DEAE-D tube, close the cap and mix quickly).
Add immediately the DNA/DEAE-D. solution dropwise of cells.
Incubate for 2 hours at 34°C, then remove the medium.
Shock cells for 1 minute with 5 ml (2 ml) 10% DMSO-PBS.

Wash in 5 ml (2 ml) PBS and replace with 10 ml (4 ml) low calcium medium with serum
Change medium everyday, collect cells after 72 hours.

Virus Infection of Primary Keratinocytes

Materials

Adenovirus with known concentration
Keratinocytes in 60 mm dishes
Low calcium medium without EGF & serum
Low calcium medium

Procedure

1. Calculate the amount of virus needed for infecting the primary keratinocytes:

   Each 60 mm dish = 3×10^6 cells
   Multiplicity of infection = 100 virus per cell MOI = 100
   Virus per dish = 3×10^8
   1 ml of low calcium medium without EGF & serum with virus per dish

2. Add the appropriate amount of virus in low calcium medium without EFG & serum.
3. Remove medium from dish after incubating cells at 34°C for one hour. To avoid contamination, change Pasteur pipet for each type of virus.
4. Add low calcium medium.
5. Change the media everyday, and cells should be collected 48 hours after infection.

*For each freezing and thawing cycle, the concentration of virus is decreased by 20%.