CHAPTER 2

DETAILED PROTOCOL TO GENERATE A HUMAN ORGANOTYPIC KNOCK-DOWN SKIN MODEL.

1. Materials

1.1. Reagents

Bovine collagen I (Advanced Biomatrix, San Diego, CA, USA)
BSA (essentially fatty acid free, Sigma, Vienna, Austria)
Calciumchloride (Sigma)
DMEM (1 g/l Glucose, PAA, Linz, Austria)
Ethanol 70% (Sigma)
FBS (PAA)
HBSS 10x (containing phenolred, Gibco BRL, Gaithersburg, USA)
Human neonatal primary KC (Cellsystems, Troisdorf, Germany; Lonza, Basel, Switzerland)
Human primary dermal FB (Cellsystems; Lonza)
KC basic medium (KBM, Lonza)
KC growth supplements (Lonza)
Dermalife medium (Cellsystems)
Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)
OptiMEM medium (Gibco)
PBS (PAA)
Pen Strep (Gibco)
Serum-free KC defined medium (SKDM)
Sodium hydroxide 1M (Sigma)
Stealth siRNAs (Invitrogen)
Transferrin (Sigma)
Trypsin neutralizing solution (TNS, Lonza)
Trypsin/EDTA (0.05% (Gibco)
Trypsin/EDTA (Lonza)
Vitamin C (Sigma)

1.2. Reagent setup

Fully supplemented DMEM medium: 50 ml heat inactivated FBS (1 hour at 57°C) and 5 ml PenStrep solution are added to 500 ml DMEM medium.

KC growth medium (KGM): One package of KC growth supplements, consisting of 0.1 ng/ml human recombinant epidermal growth factor, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.4 % bovine pituitary extract, 50 µg/ml gentamycin and 50 ng/ml amphothericin B, is added to 500 ml KBM.

Serum-free KC defined medium (SKDM): 10 µg/ml transferrin, 50 µg/ml ascorbic acid, 0.1% bovine serum albumin, 1,3 mM calciumchloride, 0.1 ng/ml human recombinant epidermal growth factor, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 50 µg/ml gentamycin and 50 ng/ml amphothericin B are added to 500 ml KBM.

1.3. Equipment

Inverted tissue culture microscope (x10 and x20 objectives)

Biosafety cabinet with aspirator for tissue culture

Tissue culture incubator maintained at 37°C, 5% CO_2 and 95% humidity

Incubator maintained at 37°C without CO_2

Water bath maintained at 37°C

Tissue culture centrifuge

Tissue culture flasks T75 cm and T225 cm (both PAA)

Tissue culture pipettes (5 ml, 10 ml and 25 ml)

Tubes (15 ml and 50 ml)

PIPETBOY Pipettor

µl-Pipettes (20 µl, 100 µl and 1000 µl)

Pipettes tips (20 µl, 100 µl and 1000 µl)

Inserts (3 µm pore size; BD Bioscience, Bedford, MA, USA)

Deep well plates (BD)

Forceps

2. Procedures

2.1. Culture of human primary dermal FB

Frozen ampules of cells are stored in liquid nitrogen. For use, the cells are thawed in a 37°C water bath. Thawing time should be one to two minutes. Wipe the outside of the vial with 70% alcohol and transfer it to the tissue culture hood.

nota bene: **After thawing, the cells should be further processed immediately.**

The cells ($1x10^5$) are transferred directly into a T75 cm culture flask containing 15 ml fully supplemented DMEM medium. The flask is then placed in a cell culture incubator at 37°C, 5% CO_2 and 95% humidity. Cells should attach after overnight cultivation. Medium changes are performed after overnight culture and subsequently every other day. When cells reach 90% confluency, they should be trypsinized and split for further expansion. For trypsinization first the culture medium is removed and the adherent cells in the culture flask are washed once with 5 ml PBS at room temperature. After removal of PBS, 3 ml of a 0.05% Trypsin/EDTA solution (room temperature) are added and the flask is put at 37° for 2-3 minutes.

nota bene: **Prolonged exposure of cells to Trypsin/EDTA can reduce their viability and life-span.**

The trypsinization process is checked under an inverted microscope. Once cells round up, gently tap the flask to detach them completely from the plastic surface. Subsequently 3 ml fully supplemented DMEM medium are added and the suspended cells are transferred to a 15 ml centrifuge tube. After centrifugation at 200 g for 2 minutes, the supernatant is removed and the cells are resuspended in fully supplemented DMEM medium and transferred to new culture vessels. The splitting ratio can range from 1:2 to 1:10.

2.2. Culture of human primary KC:

KC should be thawed as described in 2.1. Subsequently the cells ($5x10^5$) are transferred from the tube directly into four T75 cm culture flask containing 15 ml fully supplemented medium. The flasks are then placed in a cell culture incubator at 37°C, 5% CO_2 and 95% humidity. After overnight culture cells will be attached. The first medium change needs to be performed after overnight culture and then every other day. When cells reach 60-75% confluency, they can be split for further expansion. For splitting of cells the culture medium is removed and cells are washed by the addition of 5 ml PBS at room temperature. After removal of PBS from the flask 3 ml of Trypsin/EDTA solution (room temperature, Lonza) is added and the flask is put at 37°C for 5-7 minutes.

> ***nota bene:*** **Prolonged exposure of cells to Trypsin/EDTA can reduce the viability and life-span of keratinocytes.**

The trypsinization process is checked under an inverted microscope. Once cells have rounded up, they are detached them from the plastic surface by gently tapping on the culture vessels. Then the cells are resuspended in 3 ml of TNS-solution, transferred to a 15 ml centrifuge tube and centrifuged at 200 g for 2 minutes. After removal of the supernatant, cells are resuspend in KGM medium and transferred into a new culture vessels. The splitting ratio can range from 1:2 to 1:6.

2.3. siRNA transfection of KC

For transfection KC in passage 1-3 at 50–60% confluency in a T75 cm flask are used.

Prepare reaction-mix A: mix 2.5 ml OptiMEM medium with 50 µl Lipofectamine 2000 and incubate for 10-20 minutes.

> ***nota bene:*** **Be sure that Lipofectamine 2000 is a clear fluid and not cloudy.**

Prepare reaction-mix B: mix 2.5 ml OpitMEM medium with 100 µl of a 20 µM siRNA solution.

Prepare reaction-mix C: mix reaction-mix A and B together and incubate for 10-20 minutes. During this incubation period remove the old medium from KC and add 20 ml fresh KGM. Transfer reaction-mix C to the KC monolayer. Incubate KC for 24-48 h with reaction mix C.

Controls used are: untreated KC, KC transfected with scrambled siRNA, KC treated with reaction-mix C without adding any siRNA

2.4. Preparation of three-dimensional organotypic skin cultures

Pictures, representing the most important steps for the preparation of an organotypic skin culture, are shown in Figure 8. First, the inserts are transferred to deep-well plates using a sterile forceps (Figure 8, A-C).

Preparation of the collagen-matrix:

The matrix consists of 8 parts collagen, 1 part 10x HBSS and 1 part FBS containing FB ($1x10^5$/ml). For each insert 2.5 ml of the mix is needed. First the pure collagen solution is mixed with 10x HBSS by shaking well but avoiding foaming. Since the collagen solution is very acidic the color of this solution turns into bright yellow (Figure 8, D). The pH is adjusted by drop-wise adding 1M NaOH-solution until color turns into light red (Figure 8, E). The solution is put on ice, while FB are trypsinized as described above (2.1.). After washing FB in PBS once the needed number of FB is resuspended in the calculated amount of FBS and added slowly to the collagen solution. Shake well but avoid foaming. Pure 2.5 ml of the FB containing collagen solution into each insert. Avoid air bubbles (Figure 8, F).

> ***nota bene:*** **Avoid formation of air bubbles because in areas with air bubbles no epidermis will form.**

Subsequently, the well-plate is put into an incubator at 37°C without CO_2 for 2 hours.

> ***nota bene:*** **Incubation in a CO_2 incubator lowers the pH and will prevent gelation of the collagen matrix.**

After 2 hours collagen-gels should be solid (Figure 8, G) and are equilibrated with KGM medium by carefully adding 2 ml medium to the inserts and 14 ml to the outer wells (Figure 8, H). Then the well-plates are placed in a tissue culture incubator with CO_2 for 2-24 hour.

Preparation of the epidermal equivalent:

Trypsinize KC (either transfected or not) as described in (2.2.) (Figure 8, I). Remove the medium from the inserts and add 2 ml fresh KGM containing 1.5 x 10^6 KC to each insert (Figure 8, J). Place the wells in a tissue culture incubator with CO_2 for 24 to 48 hours.

Remove the medium from both the insert and the outer well and add 10 ml SKDM medium just to the outer well. The medium is changed every other day for up to 10 days.

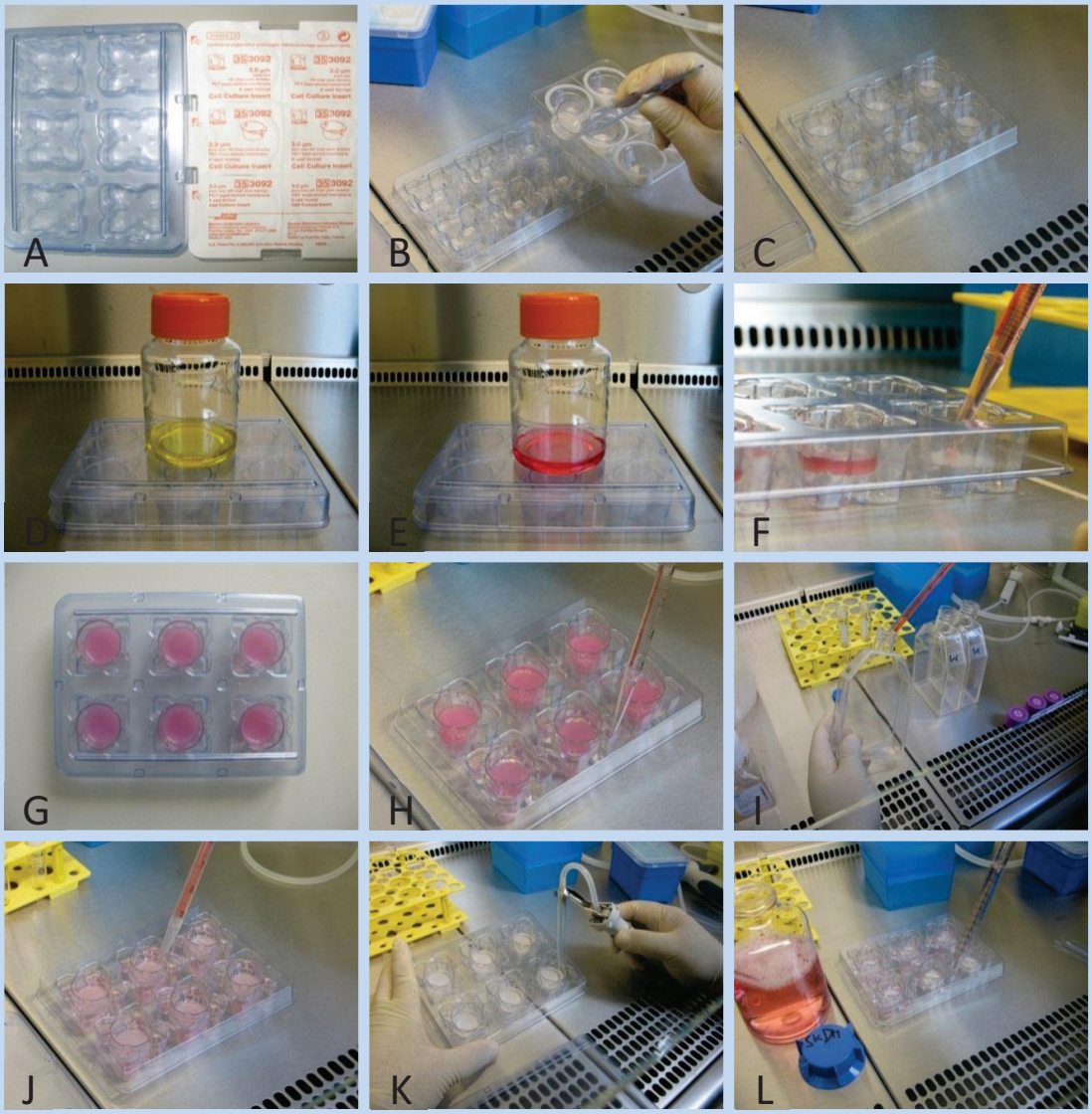

Figure 8: Important steps to setup an organotypic skin model

A-C: inserts are transferred to the deep-well plate
D: acidic mixture of collagen and HBSS
E: pH adjusted with NaOH (1M)
F: 2.5 ml of the collagen solution is poured into each insert
G: after 2 hours at 37°C gels become solid
H: gels are equilibrated with KGM medium
I: KC are trypsinized
J: medium is removed from the inserts and 2 ml fresh medium with KC is added
K: medium is removed from the insert and the outer well
L: 10 ml SKDM medium is added to the outer well only