alvetex
A REPROCELL BRAND

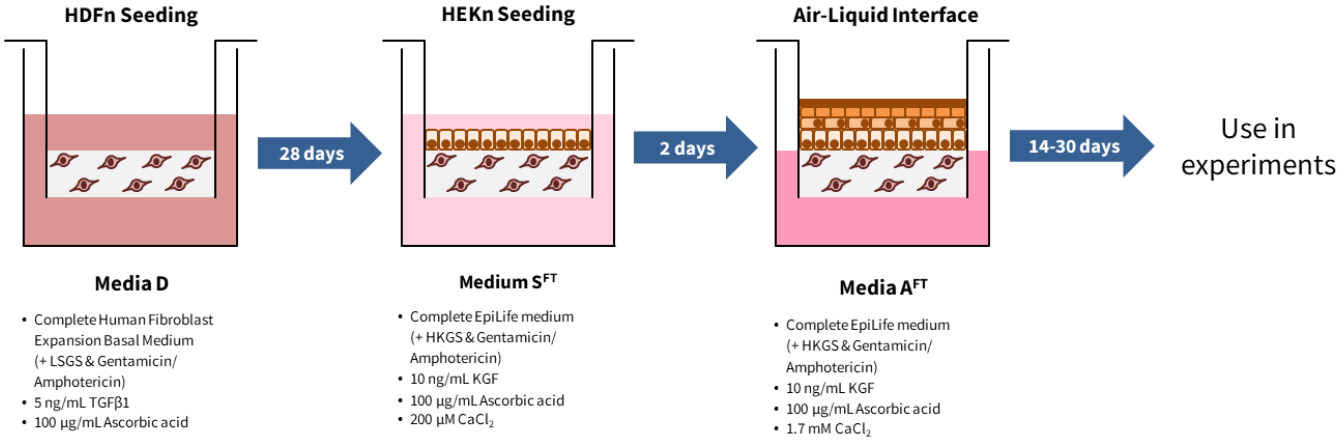
Protocol

Bioengineering of human full thickness skin for *in vitro* applications, screening and product development

1. Materials

Alvetex Scaffold 12 well insert (**note:** 12 well inserts can be suspended in wells of a 6 well plate)	REPROCELL AVP005
6 well plate	e.g. Greiner BioOne 657160
Alvetex well insert holder and deep Petri dish	REPROCELL AVP015-2
Human Fibroblast Expansion Basal Medium	Life Technologies M-106-500
Low Serum Growth Supplement	Life Technologies S-003-10
EpiLife Medium	Life Technologies M-EPI-500
Human Keratinocyte Growth Supplement	Life Technologies S-001-5
Gentamicin/Amphotericin Solution	Life Technologies R-015-10
KGF (diluted to 10 µg/mL in 1× DPBS)	Life Technologies PHG0094
$CaCl_2$ Solution	2 M Stock
TGFβ1 Recombinant Human Protein	Life Technologies PHG9214
Ascorbic Acid (Vitamin C)	Sigma A4544
Trypsin EDTA	Life Technologies R-001-100
Trypsin Neutraliser	Life Technologies R-002-100
Human Dermal Fibroblasts (HDFn, HDFa)	Life Technologies • neonatal: C-004-5C • adult: C-013-5C
Human Keratinocytes (HEKn)	Life Technologies • neonatal: C-001-5C

2. Schematic



3. Protocol

A. Preparation of Alvetex Scaffold

Alvetex Scaffold requires wetting pre-treatment as follows:

1. Soak in 70 % ethanol for 2-5 minutes in a deep Petri dish.
2. Soak in 1 × sterile PBS for 2-5 minutes in a deep Petri dish.
3. Place Alvetex Scaffold well inserts in a 6 well plate and pass 1 mL Human Fibroblast Expansion Basal Medium through each Alvetex Scaffold disc.
4. When ready to seed cells, aspirate excess media from the wells.

B. Seeding of fibroblasts onto Alvetex Scaffold

1. Prepare 11 mL **media D** per Alvetex Scaffold well insert.

Media D:

Complete Human Fibroblast Expansion Basal Medium (+ LSGS & Gentamicin/Amphotericin)

5 ng/mL TGFβ1

100 µg/mL Ascorbic acid

	Stock solution concentration	Final concentration	Dilution
TGFβ1	10 µg/mL	5 ng/mL	2000 ×
Ascorbic acid	10 g/mL	100 µg/mL	100 ×

2. Harvesting fresh HDFn cell cultures:
    a. Aspirate growth medium from the cultures.
    b. Wash once with 3 mL trypsin-EDTA (10 %, 0.25 % trypsin; 90 % versene) and aspirate off.
    c. Treat with 5 mL trypsin-EDTA (for a T175 flask) until detached (approx. 8-10 minutes).
    d. Add 5 mL complete media.
    e. Transfer cell suspension to a centrifuge tube.
    f. Rinse flasks again with complete media.
    g. Spin down cells at 200 g for 5 minutes.
3. Resuspend cell pellets in complete media and perform cell counts using a traditional hematocytometer and Trypan Blue exclusion assay (usually resuspend in 1 mL/T175 and 1:10 Trypan Blue dilution to ensure a reasonable density of cells for counting).
    a. 160 µL media
    b. 20 µL Trypan Blue
    c. 20 µL cell suspension
4. Adjust cell suspension volume to 500,000 cells per 100 µL.
5. Seed fibroblasts (100 µL) onto Alvetex Scaffold in a dropwise manner, ensuring even coverage.
6. Place in 37°C incubator (5 % CO_2, 95 % relative humidity) and allow cells to attach for minimum 2 hours.
7. Carefully add **media D** to the outer compartment (i.e. outside and below the Alvetex Scaffold insert while suspended in a well of a 6 well plate). When media reaches the bottom of the scaffold *gently* add ~0.5 mL to the inner compartment (within the insert, where the cells are). Continue to fill outer compartment to the maximum level (i.e. so that the outer and inner medium becomes continuous through the “side windows” of the well insert).

Note: 10 mL media used per 6 well plate well/scaffold. Ensure no air bubbles form under the scaffold when you add the media.

8. Place at 37°C (5 % CO_2, 95 % relative humidity), and change media every 3-4 days (usually Monday and Friday).
9. Culture for 28 days before seeding HEKn cells.

Note: Some fibroblasts may fall through the scaffold and begin to grow on the culture plate well floor. To avoid these cells affecting growth of the dermal equivalent, move scaffold to a fresh 6 well plate after 2 days.

Note: Over the 28-day culture period fibroblasts will start to grow in the culture well, it is recommended that models are transferred to a new well every 2 weeks.

C. Seeding of keratinocytes onto dermal component

1. Prepare 11 mL **media S^{FT}** per dermal equivalent.

Media S^{FT}:

Complete EpiLife medium (+ HKGS & Gentamicin/Amphotericin)

10 ng/mL KGF

100 µg/mL Ascorbic acid

200 µM $CaCl_2$

Note: EpiLife medium contains 60 µM $CaCl_2$; add 140 µM.

	Stock solution concentration	**Final concentration**	**Dilution**
KGF	10 µg/mL	10 ng/mL	1000 ×
$CaCl_2$	2 M	140 µM	14300 ×
Ascorbic acid	10 mg/mL	100 µg/mL	100 ×

2. Harvesting fresh HEKn cell cultures (p3 in flask → p4 in dermal equivalent model):
    a. Aspirate growth medium from the cultures.
    b. Wash once with 3 mL Trypsin-EDTA (10 %, 0.25 % trypsin, 90 % versine).
    c. Incubate at 37°C with 5 mL Trypsin-EDTA (for a T175 flask) until detached.
    d. Add 5 mL Trypsin neutraliser solution.
    e. Transfer cell suspension to a centrifuge tube.
    f. Further rinse flasks with complete EpiLife medium.
    g. Spin down cells at 200 g for 5 minutes.
3. Resuspend cell pellets in **media S^{FT}** and perform cell counts using a traditional hematocytometer.
4. Aspirate media from both the surrounding well and the top of the dermal equivalent. Keep the dermal equivalent in the 6 well plate and add HEKn cells.
5. HEKn cells are seeded at a density of 1.3×10^6 cells per 12 well insert (dermal equivalent model) in seeding volume 100-300 µL.
6. Place in 37°C incubator (5 % CO_2, 95 % relative humidity) and allow cells to attach for 2 hours.
7. Complete by adding 10 mL of **media S^{FT}** to each well containing an Alvetex Scaffold insert.
8. Carefully check the underside of each Alvetex disc insert, without lifting it out of the medium, to ensure that no air bubbles are trapped below the insert.

9. Incubate in submerged culture at 37°C, 5 % CO_2 and 95 % relative humidity for 48 hours.

D. Going to the Air-Liquid Interface

1. Prepare 36 mL **media A^{FT}** per deep Petri dish (**note:** the Alvetex well insert holder can hold 3 inserts).

Media A^{FT}:

Complete EpiLife medium (+ HKGS & Gentamicin/Amphotericin)

10 ng/mL KGF

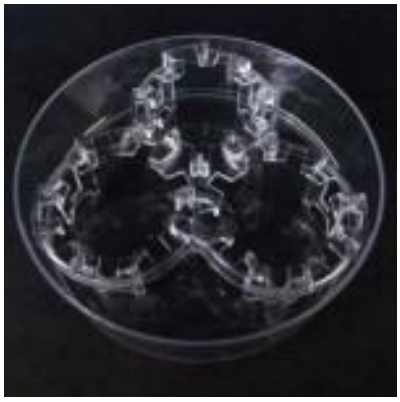
100 µg/mL Ascorbic acid

1.7 mM $CaCl_2$

Note: EpiLife medium contains 60µM $CaCl_2$; add 1.64 mM.

	Stock solution concentration	Final concentration	Dilution
KGF	10 µg/mL	10 ng/mL	1000 ×
$CaCl_2$	2 M	1.64 mM	1219 ×
Ascorbic acid	10 mg/mL	100 µg/mL	100 ×

2. Using a sterile pair of forceps transfer Alvetex Scaffold 12-well inserts to an Alvetex Well Insert Holder in a deep Petri dish on medium height. **Note:** three inserts can be cultured per deep dish.



An Alvetex well insert holder and deep Petri dish allows for three height settings. The full thickness model is cultured using the medium setting.

3. Tilting the plate to the side aspirate the medium from inside each insert, taking care not to puncture the membrane.
4. Add 35 mL **media A^{FT}** per dish. **Note:** the media should only just touch the bottom of the model.
5. Incubate at 37 °C, 5% CO_2 and change medium twice a week. **Note:** Monday and Friday media changes work well.

After 2 weeks at the air-liquid interface there will be a fully formed epidermis.