Data Sheet

3dGRO® Skin Differentiation Medium

Cell Culture Media

SCM310

Pack Size: 500 mL

Store at -20 °C

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for human or animal consumption.

Background

Human skin contains both dermal and epidermal layers of cells connected by basement membrane ECM proteins. Dermal layers contain nerves, hair follicles, blood vessels, and fibroblasts, etc. Epidermal layers contain the squamous epithelium which consists of the basal, spinous, granular and cornified layers and serves as a barrier to the outside environment including pathogens and infections. In normal skin, keratinocytes replicate in the basal/parabasal layer and differentiate into the above layers. In the granular layer, keratinocytes undergo senescence and cornify generating the cornified layer.

Organotypic 3D skin models can be generated by differentiating primary human keratinocytes (PHKs) using transwell culture inserts (Millicell®). Briefly, PHKs are seeded onto a dermal equivalent consisting of rat tail collagen and fibroblasts (for example, collagen bed). The whole assembly is cultured until the PHK monolayer becomes confluent and then raised to the air-liquid interface for 10 or more days. PHKs then stratify and differentiate into terminally differentiated squamous epithelium. Organotypic 3D skin models have been used by the cosmetic industry as an alternative to animal testing.

3dGRO® Skin Differentiation Medium is an optimized formulation that supports robust human keratinocyte differentiation using organotypic 3D cultures during the air-liquid incubation step. The media produces complex stratified squamous epithelium containing both proliferating (BrdU+) and cornified (Filaggrin+) cell populations within 10 days. The medium is complete and does not require further supplementation.

Storage and Handling

3dGRO® Skin Differentiation Medium should be stored at -20 °C. After thawing, store at 4 °C and use within 2 weeks.

Quality Control Testing

- Appearance: Clear liquid/no particulates
- pH: 7.0–7.6
- Osmolarity: 326–360 mOsm
- Sterility: No growth
- Mycoplasma: Negative
- Endotoxin: < 2 EU/mL
- Functional Test: Pass

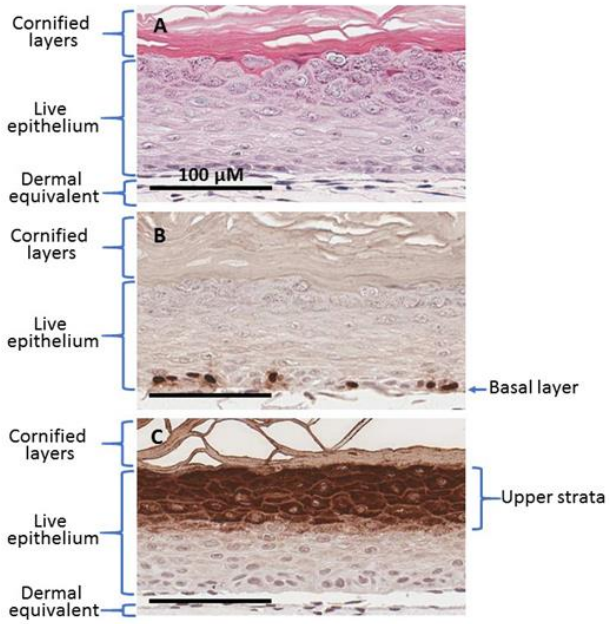


Figure 1. Morphology and biomarker staining of 10-day 3D skin culture. A. Hematoxylin and Eosin. Normal 10-day skin tissue has about 10 layers of live epithelium. **B.** Bromodeoxyuridine (BrdU) Staining. To mark cells in S phase, 100 µg/mL BrdU was added to the medium 12-16 hours prior to harvest. Basal keratinocytes are BrdU-positive (reddish brown). **C.** Filaggrin staining. Upper strata of live epithelium and cornified layer are filaggrin positive (reddish brown).

Required Materials for 3D Skin Culture

- Primary human epidermal keratinocytes (PHKs): 102-05N
- Keratinocyte Serum-Free Growth Medium (K-SFM): 131-500
- NIH3T3 cell line: 93061524-1VL
- DMEM: D6429-500 mL or SLM-120-B
- Fetal Bovine Serum (FBS): ES-009-B
- Penicillin-Streptomycin Solution (100X): TMS-AB2-C
- Rat Tail Collagen Type I: 08-115. Store at 4 °C
- 5X Reconstitution Buffer (5X RB)

  Final conc: 1.1% $NaHCO_3$, 0.025 N NaOH, 100 mM HEPES, 5X F12

  To make 200 mL, dissolve the following:
  - 2.2 g of $NaHCO_3$
  - 0.2 g of NaOH pellets
  - 4.76 g of HEPES (H-3375)
  - 1 bottle (10.6 g) of F-12 Nutrient Mixture (Ham) powder (N6760-10X1L) in water

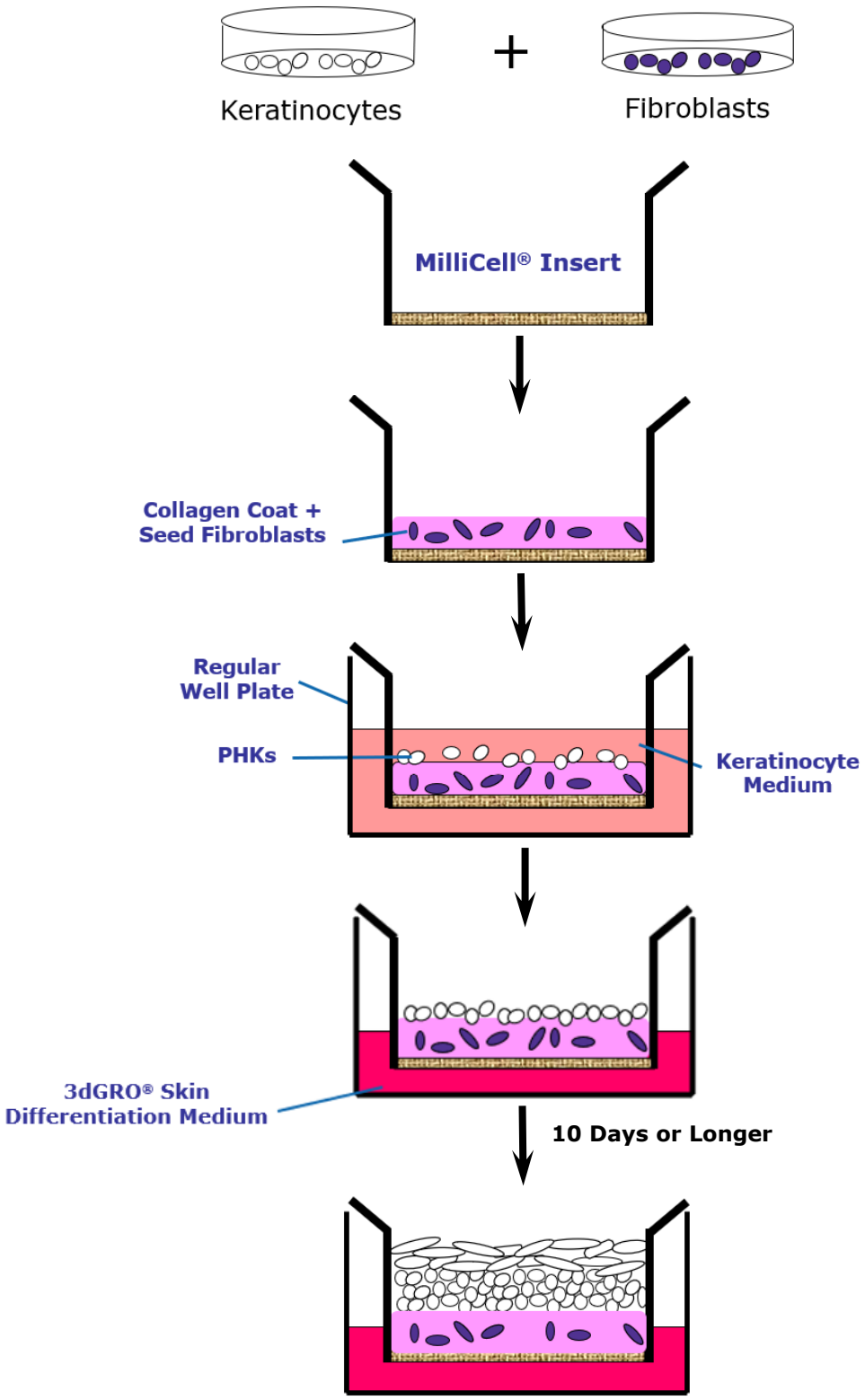
  Filter sterilize with 0.2 µm filter (S2GPU02RE).

  Store as 10 mL aliquots at -20 °C for up to 1 year. 5X RB can be freeze/thawed five times without affecting the quality of the collagen bed.
- Trypsin-EDTA Solution: T2601-100 mL
- 1X D-PBS w/o Ca^{2+} and Mg^{2+}: BSS-1006-B
- Millicell® Hanging Cell Culture Inserts, PET 0.4 µm, 12-well, 48/pk: PTHT12H48
- 12-well plates (CLS353043) and 10 cm culture plates
- Filter forceps: XX6200006P; For holding or transferring inserts
- 10% Neutral Buffered Formalin Solution: HT501320-9.5 L
- Scalpel (sharp tip). For tissue fixation
- Lens paper: WHA2105841. For tissue fixation. Cut into 3 x 3 cm squares before using.
- Tissue processing/embedding cassettes: Z672122-500 EA
- Xylene resistant markers: For tissue fixation. Z648191-12 EA
- 70% ethanol: For tissue fixation

Important Notes Before Starting

- Before starting, read the protocol through completely.
- While not necessary, it is highly recommended to add penicillin and streptomycin into the media to prevent contamination that may be introduced during the long culture process.
- The quality of the primary human keratinocytes (PHKs) is critical to generating high quality 3D skin cultures. Neonatal derived PHKs are preferred over adult PHKs. Only use primary keratinocytes that are at passage 1 or 2. Do not allow PHKs to be over-confluent.
- Except for the tissue fixation which is performed on the bench or chemical hood in the last step, all tissue cultures are handled using sterile techniques in a Class 2 biosafety tissue culture hood.

Workflow Schematic



1. Culture primary human keratinocytes (PHKs) and NIH3T3 fibroblasts in monolayers.
2. Prepare collagen bed containing NIH3T3 on culture insert.
3. Seed PHKs on top of collagen bed. Add K-SFM media outside of insert (inside multi-well). PHKs are in submerged culture for 3 days when PHKs are confluent.
4. Set up air: liquid interface culture. Aspirate K-SFM from insert. Transfer the insert assembly to a 12 well plate containing 3dGRO® Skin Differentiation Medium.
5. Change 3dGRO® Skin Differentiation Medium every day and harvest the culture on day 10.

Protocol for Human Skin Culture

Expansion of Primary Human Keratinocytes (PHKs) & NIH3T3 Fibroblasts

Based on the number of 3D skin cultures required, prepare the appropriate number of PHKs and NIH3T3 cells.

1. Thaw one vial of primary human keratinocytes (PHKs) into a maximum of three 10 cm tissue culture plates with Keratinocyte-Serum Free Medium (K-SFM). Refresh with K-SFM every other day until the culture reaches 80-90% confluence.

   Note: With a thaw ratio of 1:3, high quality PHKs should be > 90% confluency within 7 days of culture. If the PHKs are less than 80% confluence after 7 days of monolayer culture, the PHKs are of poor quality and suboptimal and you will need to re-start the experiment with a different vial or different lot of PHKs. Do not expand PHKs to higher passages as this will affect the quality of your 3D skin culture. Do not allow PHKs to be over-confluent. Discard the remaining PHKs or use for purposes other than 3D skin culture. In this protocol, 80-90% confluence of PHKs in one 10 cm plate can provide about ~20–25 skin cultures. Three 10 cm plates would provide ~60–75 skin cultures.
2. Thaw one vial of NIH3T3 fibroblasts into three 10 cm tissue culture plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin and streptomycin. Exchange with fresh medium every other day until the culture reaches 80-90% confluence.

   Note: NIH3T3 cells may be cultured up to five passages using a 1:3 split ratio. Do not allow the cells to be overconfluent as this will affect cell quality. In this protocol, 80–90% confluence of NIH3T3 in one 10 cm plate can provide approximately 80–90 collagen beds on inserts. Because NIH3T3 cells grow faster than PHKs, they can be thawed 2-3 days after thawing PHKs.
3. Adjust the confluence of PHKs and NIH3T3 to 80-90% on the same day when performing 3D skin culture. Timing may vary depending upon the user's experience and handling. Below is just a guidance.

   For example: On Monday, thaw 1 vial of PHKs into three 10 cm plates. With high quality PHKs, you should be ready to set up the 3D skin culture the following Monday (for example, 7 days later). NIH3T3 grow faster and thus 1 vial can be thawed on Thursday to be ready by the following Monday.

Setting up 3D Skin Culture System

Preparation of collagen beds on inserts

1. Estimate the numbers of inserts required for your experiment. Transfer the appropriate numbers of inserts into regular 12 well plates in advance. Maintain sterile techniques.
2. Thaw the 5X reconstitution buffer. Refer to "Required Materials for 3D skin Culture" for the formulation.
3. Maintain the following components on ice at all times:
   - 5X reconstitution buffer
   - Rat tail collagen and
   - Resuspended NIH3T3 cells

   Do not warm up to 37 °C until the end of Step 6.
4. Prepare NIH3T3 cell suspension: NIH3T3 cells should be 90-100% confluence at the time of trypsinization. Wash cells with 1X PBS to remove residual FBS which may interfere with trypsinization. Add 1 mL of trypsin per 10 cm plate and incubate for 2-3 minutes at 37 °C. Quench with DMEM containing 10% FBS. Collect the cells, centrifuge, and count the cells. Re-suspend the cell pellet to $4x10^6$ cells/mL with ice cold DMEM containing 10% FBS. Maintain cells on ice until ready to make up the collagen bed.

   Note: Trypsinize cells for minimum duration (approximately 2-3 min for NIH3T3 cells and 5-7 min for PHKs at 37 °C). Over-trypsinized cells may die or be hard to recover.

5. In a 15 mL Corning conical tube (on ice), strictly follow the order of addition for each ingredient. Total volume = 8 mL. Inappropriate order of addition or incomplete mixing of the mixture may kill feeder cells, as collagen is dissolved in 0.02 N acetic acid.
   - Add rat tail collagen to 6 mL into the 15 mL conical tube. Note: Rat tail collagen may be viscous and may vary in concentrations. Normal vendor range is 3-5 mg/mL. Use 6 mL of 3-5 mg/mL rat tail collagen. Read the scale on the conical tube to aid in assessing the collagen volume.
   - Add 1.6 mL of 5X reconstitution buffer. Mix well. Avoid introducing bubbles to the collagen.
   - Add 400 µL NIH3T3 ($4x10^6$ cells/mL) cell suspension. The mixing with collagen must be gentle but thorough. Avoid introducing bubbles to collagen.
   - Keep all components and the collagen mixture on ice to postpone the liquid collagen mixture from polymerizing. At any one time, prepare a maximum of 8 mL of liquid collagen mixture and aliquot the mixture to the inserts as soon as possible. Approximately 15-20 collagen-embedded inserts can be generated from 8 mL of the collagen mixture.
6. Using a 1- or 2-mL pipette, quickly aliquot 400 µL of the liquid collagen mixture directly to the center of each insert. Do not add the liquid collagen mixture along the side of the insert wall. Avoid introducing bubbles while aliquoting. Don't tilt the inserts while the collagen mixture is liquid. Incubate the inserts/plates at 37 °C for at least 30 min to allow the collagen beds to completely polymerize. After gelation, NIH3T3 in collagen beds can survive at 37 °C for up to 5 hours without adding any medium. PHKs should be seeded onto collagen beds within 5 hours.

   Quality Assessment (Optional): To assess NIH3T3 viability on 3D collagen beds, aliquot 400-800 µL of the collagen mixture in step 5 into a separate well of a 24 well plate. Allow the collagen mixture to polymerize before adding in 1 mL of K-SFM or DMEM containing 10% FBS. Observe the morphology of the NIH3T3 after overnight incubation. Live NIH3T3 should extend processes and be spread out in the collagen beds after overnight culture. If NIH3T3 cells are rounded without processes, this is indicative of poor quality or dead NIH3T3. The collagen beds should be prepared again.
7. Prepare PHK suspension: PHKs should be 80-90% confluent at the time of trypsinization. Wash cells with 1X PBS to remove any residual factors which may interfere with trypsinization. Add 1 mL of trypsin per 10 cm plate and incubate for 5-7 minutes at 37 °C. Quench with DMEM containing 10% FBS. Collect the cells, centrifuge, and count the cells. Re-suspend the cell pellet to $4x10^5$ cells/mL in ice cold K-SFM.
8. Add 0.5 mL of the PHKs ($4x10^5$ cells/mL) cell suspension to each insert. Total number of PHK = $2x10^5$ cells. Be careful to not damage the collagen bed as this may cause leakage of the medium. Add 2 mL of K-SFM media to the outside of the inserts (into the 12-well plate) so that the medium levels inside and outside the inserts are of equal volume and the cells are submerged. Incubate at 37 °C overnight.

   Quality Assessment (Optional): Because it is not possible to directly visualize the confluency of the PHKs cultured on collagen bed, an indirect assessment of PHK confluency is needed. To indirectly assess PHK's confluency on collagen beds, seed $2x10^5$ of PHKs into a well of a regular 24 well plate. A well of a 24-well plate has a similar surface area as the culture insert. PHKs cultured on a 24-well plate must be > 90% confluent by the third day. If the PHKs in the 24-well plate are slightly under 90% confluent by the 3rd day, refresh with K-SFM medium on both the inside and outside of the culture inserts and incubate for an additional day. If PHKs in 24-well plate are much less than 90% confluent by the 3rd day, the PHKs are in a poor condition, and you will need to restart with a new preparation.

   Note: Do not seed more than $2.5x10^5$ of PHKs onto the collagen bed; PHKs must be allowed to grow to confluency and form the natural structure on basal layer by themselves. PHK in submerged culture on collagen beds must grow to more than 90% confluence before shifting to air-liquid interface.

   The next day, without removing the original medium, add an additional 0.5 mL of K-SFM to the inside of each insert and incubate at 37 °C for 2 more days. PHKs should be in submerged culture on collagen beds for a total of three days. Normal to high quality PHKs will approach 100% confluence by the third day of submerged culture.

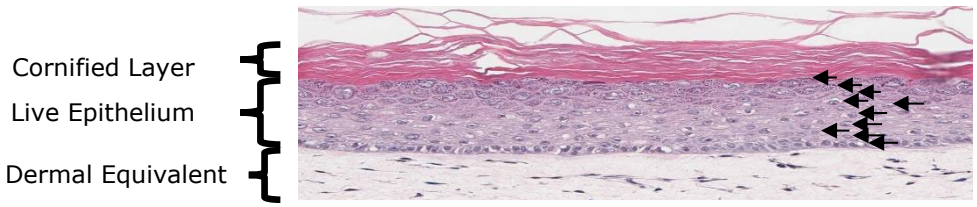
9. On the 3rd day, gently and thoroughly aspirate the media from each insert. Do not touch or damage the surface of the collagen bed. Use sterile forceps to transfer the inserts to a new 12 well plate containing 2 mL of 3dGRO® Skin Differentiation Medium in each well. Remove any bubbles from under the insert membrane.

   Note: You must add the exact amount of medium to the outside of the inserts to allow the skin cultures to be maintained at the air- liquid interface. The upper surface of the cells must be exposed to air while the lower surface of the cells is fed and wetted by media through the pores of the culture insert. If too much medium is added to the outside of the insert, this may force the extra medium to enter the insert and soak the skin surface. The result is disruption of the differentiation of the 3D skin culture.

10. The day when the insert is transferred onto the air-liquid interface is counted as day 0 for 3D skin culture. Incubate the skin culture at 37 °C for an additional 10 days. During the 10 days incubation, change the 3dGRO® Skin Differentiation Medium every day and remove any bubbles from under the insert membrane. Make sure that the inserts are at air-liquid interface and that the surface of the skin is not exposed to medium throughout the 10 days of differentiation. Use the 3dGRO® Skin Differentiation Medium within two weeks after thawing. Do not re-freeze or use the medium for longer than 2 weeks.

11. To harvest the mature skin cultures: Use sterile forceps to transfer the inserts to the corresponding wells in a regular 12-well plate. Soak the inside and outside of inserts with 10% buffered formalin phosphate at room temperature for 1-4 hours. Use a scalpel to detach the membrane with the skin tissue from the insert wall. To prevent the skin tissue from curling during the dehydrated tissue processing, transfer the skin tissues to a 3 x 3 cm lens paper (refer to "Required Materials for 3D Skin Culture"). Fold the paper without folding the skin tissue so that the skin tissue is protected inside the lens paper. Transfer the wrapped skin tissues into tissue cassettes labeled with xylene resistant marker and soak in 70% ethanol. If you are not planning on processing the samples immediately, keep the fixed tissues in 70% ethanol at 4 °C for up to 3 weeks. Tissue processing, embedding and section will follow normal histological procedures. Discard lens paper before embedding.

    Quality Assessment: 10 days of air-liquid culture should produce approximately 8 to 10-layers of the live epithelium (Arrows).



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2. Wilson JL et al. (1992) Epithelial-specific gene expression during differentiation of stratified primary human keratinocyte cultures. Cell Growth Differ 3(8): 471-483.

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