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Matched Donor Keratinocytes and Fibroblasts 2D Co-Culture Protocol

Instructions for use

Safety Statements

These products are not for use in GMP manufacturing, nor human or animal *in vivo* use, including use as a diluent or as an excipient, or for diagnostic use.

These products are for research use *only.*

WARNING: LONZA PRIMARY CELLS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA-approved method for the presence of HIV-I, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV-1, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human-sourced products should be handled at the biological safety level 2 to minimize exposure to potentially infectious products, as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5th edition. If you require further information, please contact your site safety officer or Scientific Support.

Preparation of Reagents

All work should be performed in a laminar flow hood. Decontaminate the external surfaces of all supplement vials and the medium bottles with 70% ethanol or isopropanol.

1. **KGM® Gold Keratinocyte Growth Medium**
   Add the contents of each KGM® Gold SingleQuots® Supplement to the KBM® Gold Keratinocyte Basal Medium. Rinse each supplement vial with medium and pipette into completed KGM® Gold Keratinocyte Growth Medium.
2. **FGM® 2 Fibroblast Growth Medium**
   Add the contents of each FGM® 2 SingleQuots® supplement to the FBM® Basal Medium. Rinse each supplement vial with medium and pipette into the completed FGM® 2 Fibroblast Growth Medium.
3. **CnT-Prime Epithelial/Stromal Co-Culture Medium**
   Prepare according to manufacturer instructions.

Thawing and Initial Monocultures

NOTE: All work is to be performed in a laminar flow hood.

NOTE: All steps apply to both keratinocytes and fibroblasts unless otherwise noted.

Prepare an appropriate number of culture flasks (T-25, T-75 or T-150) by adding the appropriate volume of Growth Medium to each flask (**Table 1**).

a. KGM® Gold Growth Medium for keratinocytes or FGM® 2 Fibroblast Growth Medium for fibroblasts.

Vessel Size	Standard Feed Volume	Weekend Feed Volume (or flasks with ≥ 45% confluence
T-25	5 – 7 mL	8 – 10 mL
T-75	15 – 18 mL	20 – 25 mL
T-150	30 – 35 mL	40 – 50 mL

Table 1: Media volume for each flask type.

2. Prepare a 15 mL conical tube with 1 mL of the appropriate pre-warmed 37°C Growth Medium.
3. Thaw one cryovial of cells (keratinocytes or fibroblasts) at a time in a 37°C water bath for no more than 2 minutes. DO NOT SUBMERGE VIAL. Remove when only a sliver of ice remains.

NOTE: Thawing for more than 2 minutes may lead to less-than-optimal results.

4. Disinfect ampule of cells and transfer to BSC.
5. Carefully resuspend cells and avoid making bubbles.
6. Transfer cells to the 15 mL conical tube with appropriate Growth Medium.
7. Rinse cryovial with 1 mL of Growth Medium from the conical tube and return contents.
8. Measure volume of cell suspension with a pipette.
9. Count cells using 0.4% trypan blue solution and a hemocytometer. Recommended dilution with trypan blue is 1:2 (e.g., 20 µL sample + 20 µL trypan blue).
10. Calculate the volume of cell suspension needed to seed cells in the flasks (e.g., T-25, T-75 or T-150) prepared in Step 1 at 3,500 cells/cm^2.
11. Place in a humidified incubator at 37°C, 5% CO_2.
12. Change medium 1 day after initial plating, following the feeding guidelines in **Table 1**.
13. Feed every other day after that until confluence of 70 – 90% is reached.

Trypsinization and Passaging into Co-Culture

NOTE: The following instructions are for a 150 cm^2 flask size. Adjust all volumes accordingly for other flask sizes. Trypsinize and passage all flasks of matched donor pairs of keratinocytes and fibroblasts at the same time.

1. For each T-150 flask of keratinocytes or fibroblasts:
   a. Thaw 8 mL of Trypsin/EDTA and allow it to warm to 37°C.
   b. Allow 20 mL of HEPES Buffered Saline (HEPES-BSS) to come to room temperature.
   c. Thaw 23 mL of TNS and keep at 2 to 8°C until ready to use.
2. Prepare 96-well plates with 150 µL CnT-Prime Epithelial/Stromal Co-Culture Medium per well that has been thawed and warmed to come to room temperature. Set plates aside until ready to seed plates.
   a. **Note:** The media does not require any additional supplements/additives. Thaw/warm media using a room temperature water bath. Alternatively, thaw in the fridge overnight, and allow it to come to room temperature the following day by sitting it on the benchtop/ room temperature water bath. Follow all manufacturer protocols regarding this medium.
3. Aspirate medium from the T-150 flask.
4. Rinse the T-150 flask with 20 mL of HEPES-BSS. Let the HEPES-BSS sit for 2 minutes before aspirating off.
5. Add 7 – 8 mL Trypsin/EDTA to the T-150 flask.
   a. **For Keratinocytes:** place flasks in an incubator at 37°C, 5% CO_2 for about 4 minutes for cells to release.
   b. **For Fibroblasts:** keep flasks at room temperature for about 2 – 3 minutes, checking at the 2 minute mark for release.
6. Once 90% of the cells are released or rounded up, tap the sides of the flask to fully remove all cells from the flask surface.
7. Immediately quench with 16 mL of TNS.
8. Pool all cells from the same donor and cell type (e.g., all keratinocytes from the same donor) into an appropriately sized conical tube or tubes.
9. Wash all flasks of the same donor and cell type with a serial rinse of fresh 5 – 7 mL TNS (i.e., pipet 5 – 7 mL into first flask, then transfer to subsequent flasks). Transfer rinse to the conical tube with the rest of the cell suspension.
10. Spin cells at 300 Xg for 5 minutes at 4°C.
11. Aspirate supernatant.
12. Resuspend the cells from each individual donor and cell type in 1 – 2 mL of Co-Culture Medium and count using 0.4% trypan blue and a hemocytometer. Recommended dilutions with trypan blue are 1:10 (10 µL of sample + 90 µL of trypan blue) or 1:20 (10 µL sample + 190 µL trypan blue).
13. Calculate the appropriate volume of cell suspension to add to each of the wells of the 96-well plate such that:
    a. 1,280 keratinocytes are added to each well.
    b. 3,840 fibroblasts are added to each well.
    c. This is a 1:3 keratinocyte:fibroblast ratio. The ratio is based off a baseline of 4000 cells/cm^2.

NOTE: Dilute the cell suspension with Co-Culture Medium as needed in order to increase pipetting volume if cell count is large.

14. Place co-cultures into an incubator at 37°C, 5% CO_2 for up to 7 days. Change medium every 2-3 days.

15. Healthy cultures should show fibroblasts growing across the entire well, while keratinocytes form islands of cells amongst the fibroblasts.
16. Further imaging can be performed using ICC, staining keratinocytes with a fluorescent anti-cytokeratin 14 antibody and fibroblasts with a fluorescent anti-CD90 / Thy1 antibody.
    a. **Note:** Cultures can be fixed and stained at 7 days.

Ordering Information

Catalog No.	Description	Size
CC-2511	NHDF – Cryopreserved Normal Human Dermal Fibroblasts	≥ 500,000 cells
00192627	NHEK-Ad – Cryopreserved Normal Human Epidermal Keratinocytes; Single Donor	≥ 500,000 cells
00192060	KGM® Gold Keratinocyte Growth Medium BulletKit®	500 mL KBM® Gold Basal Medium plus KGM® Gold SingleQuots® supplements
00192151	KBM® Gold Basal Medium	500 mL bottle
00192152	KGM® Gold SingleQuots® Kit	1 kit
CC-3132	FGM® 2 Fibroblast Growth Medium BulletKit®	500 mL FBM® Basal Medium plus FGM® 2 SingleQuots® supplements
CC-3131	FBM® Basal Medium	500 mL bottle
CC-4126	FGM® 2 SingleQuots® Kit	1 kit
CC-5012	Trypsin/EDTA	100 mL bottle
CC-5002	Trypsin Neutralizing Solution (TNS)	100 mL bottle
CC-5024	HEPES Buffered Saline Solution (HEPES-BSS)	500 mL bottle

PBS without Calcium or Magnesium (ThermoFisher Scientific 10010023) mentioned is a product of GIBCO®.

GIBCO® Trypan Blue 0.4% (ThermoFisher Scientific 15250061) mentioned is a product of Thermo Fisher Scientific.

CnT-Prime Epithelial/Stromal Co-Culture Medium (CellIntec CnT-PR-CC) mentioned is a product of CellnTec.