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Human Umbilical Vein Endothelial Cell and Normal Human Dermal Fibroblast Co-Culture and Angiogenesis Assay

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, 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 our 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. **FGM®-2 Fibroblast Growth Medium**
   Prepare as recommended in the Lonza Human Fibroblast Cell Systems Instructions for Use. Briefly, transfer the contents of each FGM®-2 SingleQuots® supplement kit vial to the bottle of FBM® basal medium with a pipette. Rinse each supplement vial with medium and pipette into the growth medium.
2. **EGM®-2 (Endothelial Cell Growth Medium) Without VEGF**
   Prepare as recommended in the Lonza Clonetics® Endothelial Cell System Technical Information & Instructions, except do not add the VEGF supplement. Briefly, before thawing all materials, **discard the VEGF supplement**. After thawing, transfer the contents of the remaining EGM®-2 SingleQuots® supplement kit vials to the bottle of EBM®-2 basal medium with a pipette. Rinse each supplement vial with medium and pipette into the growth medium.
3. **30 nM Working VEGF Solution**
   Combine 11.5 µL of stock VEGF (PeproTech® part no. 100–20) with 0.988 mL **EGM®-2 Without VEGF.**
4. **EGM®-2 With VEGF**
   Combine 522 µL of **30 nM Working VEGF Solution** with 49.478 mL **EGM®-2 without VEGF** (prepared in step 2 above) for a final concentration of 313 pM VEGF.
5. **CellTracker™ Dye**
   Prepare CellTracker™ Dye, from ThermoFisher Scientific, at a 10 mM working concentration with high-quality DMSO according to manufacturer instructions before use. Aliquot the dye and store at -20°C in a desiccator.

NOTE: Other fluorescent dyes compatible with live cell imaging systems may be used, but should be prepared according to manufacturer instructions and tested and optimized for use in this protocol.

6. **Suramin**
   Prepare 10 mg/mL suramin sodium salt in **EGM®-2 with VEGF** for a 7 mM stock concentration. Dose titrate suramin according to your experimental needs.

Part 1: Plating fibroblast cells

NOTE: Perform all work in a laminar flow hood.

NOTE: Cryopreserved cells are delicate and must be thawed and put into culture as quickly as possible with minimal handling.

1. Add 3–4 mL of complete **FGM®-2 medium** (for ≤4 amps of fibroblasts) to a 15 mL conical tube.
2. Wipe cryovials of Normal Human Dermal Fibroblasts (NHDF-Ad; Lonza part no. CC-2511) with 70% ethanol or isopropanol. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then retighten.
3. Quickly thaw cryovials in a 37°C water bath, being careful not to submerge the vial.
4. When the last sliver of ice melts, remove cryovials from the water bath.
5. For each donor, combine the contents of all cryovials into a single 15 mL conical tube using a pipette.

NOTE: Combine no more than 4 cryovials into a single 15 mL conical tube.

6. Centrifuge the cell suspension at 300xg for 5 minutes at room temperature.
7. Aspirate supernatant (removes DMSO).
8. Resuspend fibroblasts in 2 mL of room temperature **FGM®-2 Medium**.
9. Measure the total volume using a serological pipet.
10. Count fibroblasts with a hemocytometer and Trypan Blue stain. Recommended dilution with Trypan Blue is 1:2 (e.g., 20µL sample + 20µL Trypan Blue 0.4%).
11. Resuspend fibroblasts at a density of 150,000 cells/mL in room temperature **FGM®-2 Medium**.
12. Plate fibroblasts in a 96-well cell culture plate at a density of 30,000 cells/well by adding 200 µL of cell suspension to each well.
13. Let stand for at least an hour at room temperature. If cells sit longer than an hour (e.g. if preparation of HUVEC cells takes longer than an hour), place the plate in the incubator at 37°C, 5% CO_2, and 90% humidity until ready to seed HUVEC cells.

Part 2: Plating HUVEC cells with fibroblasts

NOTE: Perform all work in a laminar flow hood.

NOTE: Cryopreserved cells are delicate and must be thawed and put into culture as quickly as possible with minimal handling.

NOTE: The following directions are different from the instructions for the fibroblasts due to the smaller number of HUVECs to be plated and the more delicate nature of HUVEC cells.

14. For each donor, prepare a 15 mL conical centrifuge tube with 2–3 mL of 37°C **EBM®-2 Basal Medium.**

NOTE: Thaw no more than 3 cryovials at a time. If you will be thawing more than 3 cryovials per donor, prepare additional 15 mL conical tubes as above for every 3 cryovials you will thaw.

15. Thaw Human Umbilical Vein Endothelial Cell (HUVEC; Lonza part no. C2519AS) cryovials from one donor at a time, but thaw no more than 3 vials at a time.
16. Wipe cryovials HUVECs with 70% ethanol or isopropanol. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then retighten.
17. Quickly thaw cryovials in a 37°C water bath, being careful not to submerge the vial.
18. When the last sliver of ice melts, remove cryovials from the water bath.
19. Using a pipettor, gently resuspend the contents of each vial and then transfer to a single 15 mL conical tube containing EBM®-2.

NOTE: Combine no more than 3 cryovials into a single 15 mL conical tube.

20. Measure the total volume using a pipet.
21. Count HUVECs with a hemocytometer and Trypan Blue stain. Recommended dilution with Trypan Blue is 1:2 (e.g., 20µL sample + 20µL Trypan Blue 0.4%).
22. Add 1 µL of 10 mM **Cell Tracker® Dye** to the 15 mL conical tube.
23. Place the conical tube in a HulaMixer™ and set to low speed to keep the cells moving. Incubate for 45 minutes at 37°C, 5% CO_2, 90% humidity.
24. Fill each conical tube to approximately 10 mL using room temperature **EGM®-2 without VEGF.**
25. Centrifuge at 300xg for 5 minutes at room temperature.
26. Aspirate all supernatant from each 15 mL conical tube.
27. Using the cell counts for each tube from Step 19, resuspend cells in each conical tube with **EGM®-2 without VEGF** at $1x10^5$ cells/mL.
28. Retrieve the 96-well plate with fibroblasts from the benchtop or from the incubator (if applicable). Carefully aspirate 100 µL of media from each well.
29. Seed each well with 100 µL of HUVEC cell suspension from the appropriate donor.
30. The resulting co-culture should contain 30,000 fibroblasts and 10,000 HUVECs per well (ratio of 3:1 fibroblasts: HUVECs).

NOTE: A total seeding density of 40,000 cells/well is optimal for this specific 96-well co-culture format.

31. Place plate in an incubator at 37°C, 5% CO_2 and 90% humidity. Incubate overnight.

NOTE: If using live-cell imaging for quantification of angiogenesis, place the plate in the incubated live-cell imager (37°C, 5% CO_2, and 90% humidity) and equilibrate for 30 minutes to an hour before starting the first scan. If using a Sartorius Incucyte® SX5 Live Cell Analysis Instrument and the Sartorius Incucyte® Angiogenesis Software Module, we recommend using 4X magnification for quantification. Ensure that parameters are set to the correct type of 96-well plate used. Use Sartorius' guide with this module to initially establish parameters, and tune parameters as needed. Other live cell imaging instruments may require further optimization.

Part 3: Angiogenesis Assay

32. Prepare the **EGM®-2 with VEGF** medium as described in the Preparation of Reagents section Steps 3–4.
33. Prepare an 8-point suramin titration using the 7 mM stock solution described in the Preparation of Reagents section Step 6. Prepare according to the table below:

Suramin Titration	
Concentration (µM)	**Formula**
780	557.14 µL of 7 mM suramin + 4442.86 µL EGM®-2 with VEGF*
247	1.58 µL of 780 µM suramin + 3.42 mL EGM®-2 with VEGF
78	1.58 µL of 247 µM suramin + 3.42 mL EGM®-2 with VEGF
24.7	1.58 µL of 78 µM suramin + 3.42 mL EGM®-2 with VEGF
7.8	1.58 µL of 24.7 µM suramin + 3.42 mL EGM®-2 with VEGF
2.47	1.58 µL of 7.8 µM suramin + 3.42 mL EGM®-2 with VEGF
0.78	1.58 µL of 2.47 µM suramin + 3.42 mL EGM®-2 with VEGF
0	5 mL EGM®-2 with VEGF

*Requires both 1000 µL and 10µL pipettes to obtain correct volume

34. Carefully aspirate all medium from all wells in the plate without disturbing the cells.
35. Add 200 µL of **titrated suramin** to the appropriate wells for each concentration.
36. Replace in the incubator/live cell imager.
37. Change media with **titrated suramin** as in Steps 33–34 every 2 to 3 days. The experiment will run for 5 days.

General Note on Angiogenesis:

The degree of angiogenesis and response by HUVECs to angiogenesis inhibitors/promoters may be variable depending on the donor, and the passage number.

Ordering Information

Catalog No.	Description	Size
C2519AS	Human Umbilical Vein Endothelial Cells, Angiogenesis Qualified	≥500,000 cells/amp
CC-2511	Human Normal Dermal Fibroblast Cells - Adult	≥500,000 cells/amp
CC-3156	EBM®-2 Basal Medium	500 mL bottle
CC-4176	EGM®-2 SingleQuots® Supplement Kit	1 kit
CC-3162	EGM®-2 BulletKit®	500 mL basal medium + supplement kit
CC-3131	FBM® Basal Medium	500 mL bottle
CC-4126	FGM®-2 SingleQuots® Supplement Kit	1 kit
CC-3132	FGM®-2 BulletKit®	500 mL basal medium + supplement kit

VEGF (Recombinant Human VEGF; PeproTech® 100-20) mentioned is a product of PeproTech®.

Invitrogen™ CellTracker™ Dye (ThermoFisher C2925) mentioned is a product of ThermoFisher Scientific.

Corning® 96-Well plate (Corning® 3596) mentioned is a product of Corning®.

Incucyte® SX5 Live Cell Analysis Instrument mentioned is a product from Sartorius.

Incucyte® Angiogenesis Software Module (Sartorius 9600-0011) mentioned is a product from Sartorius.

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

Suramin sodium salt (Sigma-Aldrich S2671-100MG) mentioned is a product from Sigma-Aldrich.

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