invitrogen™

Endothelial Tube Formation Assay (*In Vitro* Angiogenesis)

Angiogenesis—the formation of new blood vessels from existing vasculature—is an integral part of both normal and pathological processes. Endothelial cells are the key cell type involved in this process. During angiogenesis, these cells:

1. Disrupt the surrounding basement membrane
2. Migrate toward an angiogenic stimulus
3. Proliferate to provide additional cells that make up a new vessel, and
4. Re-organize to form the necessary three-dimensional vessel structure.

One of the most well-established assays to model the formation of three-dimensional vessels is known as the tube-formation assay. The following protocol is designed to promote the formation of endothelial cell tube networks using Geltrex™ Reduced Growth Factor Basement Membrane Matrix.

The protocol uses the following Invitrogen reagents and cells:

Catalog #	Product	Size
C3099	Calcein, AM 1mg/mL solution in anhydrous DMSO	1 mL
C-003-5C	Cryopreserved Primary Human Umbilical Vein Endothelial Cell (HUVEC)	≥ 500,000 viable cells
12760-013	Geltrex™ Reduced Growth Factor Basement Membrane Matrix	1 mL
12760-021	Geltrex™ Reduced Growth Factor Basement Membrane Matrix	5 mL
S-003-10	Low Serum Growth Supplement (LSGS)	10 mL
M-200PRF-500	Medium 200PRF	500 mL
R-001-100	Trypsin/EDTA Solution (1x)	100 mL
R-002-100	Trypsin Neutralizer Solution (1x)	100 mL

Important

All procedures should be performed in a biological safety cabinet using aseptic technique to prevent contamination.

Day 0:

1. Prepare a bottle of supplemented Medium 200PRF by thawing a bottle of Low Serum Growth Supplement (LSGS) and transferring the entire contents of the LSGS bottle to the bottle of medium.

   Note: Once Medium 200PRF has been supplemented with LSGS, the supplemented medium should be stored in the dark at 4°C and should not be frozen. When stored in the dark at 4°C, the supplemented medium is stable for 1 month.

2. Seed cryopreserved endothelial cells at 2×10^5 viable cells per a 75-cm^2 tissue-culture flask using LSGS-supplemented Medium 200PRF (15 mL total volume).
3. Change culture medium 24–36 hours after seeding.
4. Change the medium every other day thereafter, until the culture is approximately 80% confluent (5–6 days).

Day 5:

5. Thaw Geltrex™ in a refrigerator (4°C) overnight.

   Note: Since refrigerator temperatures may vary, thaw Geltrex™ in an ice bath in a refrigerator.

Day 6:

6. **Optional step for fluorescent monitoring of tube formation using a cell-permeable dye (*e.g.* Calcein, AM):** Add the dye to the endothelial cells in a 75-cm^2 flask and incubate for 30 min at 37°C and 5% CO_2 (protect from light). Final dye concentration should be 2 µg/mL.
7. Add 50–100 µl of Geltrex™ per cm^2 to the growth surface and incubate coated surface for 30 minutes at 37°C to allow the gel to solidify.

   Note: 50 µL of Geltrex™ per cm^2 is sufficient for larger well sizes (*e.g.*, 12-well, 24-well). 100 µL per cm^2 is necessary for smaller well sizes (*e.g.*, 96-well).
8. Harvest the cells using the following procedure. Do not warm any of the following reagents prior to use. This procedure is designed for one 75-cm^2 flask. If different-sized culture vessels are used, adjust reagent volumes accordingly:
   a. Remove all culture medium from the flask.
   b. Add 12 mL of Trypsin/EDTA solution to the 75-cm^2 flask. Rock the flask to ensure the entire surface is covered.
   c. Immediately remove 9 mL of Trypsin/EDTA solution from the flask.
   d. Incubate the flask at room temperature for 1–3 minutes.
   e. View the culture under a microscope.
   f. When the cells have become completely round, rap the flask gently to dislodge the cells from the surface of the flask.
   g. Add 9 mL of Trypsin Neutralizer solution to the flask and transfer the detached cells to a sterile 50-mL conical tube.
   h. Add 9 mL of additional Trypsin Neutralizer solution to the flask and pipette the solution over the flask surface several times to remove any remaining cells.
   i. Add this solution to the 50-mL conical tube.
   j. Centrifuge the cells at 180 × g for 7 minutes, until the cells are pelleted.
   k. Remove the supernatant from the tube, being careful not to dislodge the cell pellet.
9. Add 4 mL of *non-supplemented* Medium 200PRF to the cell pellet and mix by pipetting up and down several times to ensure a homogeneous single-cell suspension.
10. Determine the concentration of cells in the non-supplemented Medium 200PRF.
11. Dilute cells in non-supplemented medium in the presence or absence of angiogenesis inducers and inhibitors. We recommend a concentration of $3.5–4.5 \times 10^4$ cells per 200 µL as a starting point and general guideline; the ideal plating density and media volume depends on cell type and should be determined experimentally. The final media volume should be ~200 µL/cm^2.

    Note: For a positive inducer control, we suggest diluting cells in LSGS-supplemented Medium 200PRF. LSGS-supplemented Medium 200PRF contains 2% (v/v) FBS and bFGF (3 ng/mL). For a positive inhibitor control, we suggest diluting the cells in LSGS-supplemented media and 30 µM Suramin.
12. Gently add cells at the selected density to the gel-coated well, at a final media volume of ~200 µL/cm^2.
13. Incubate the plate at 37°C, 5% CO_2 overnight.

    Note: Incubation times may vary. HUVEC, for example, develop well-formed tube networks after 4–6 hours. After 24 hours, endothelial cells typically undergo apoptosis.

14. **Optional Step**: If cells were not pretreated with a dye before harvesting, they can be stained at the end of the incubation period after the tube network has formed using a cell-permeable dye (*e.g.,* Calcein, AM):
    a. Add the dye to the cells and incubate for 30 minutes at 37°C and 5% CO_2 (protect from light). Final dye concentration should be 2 µg/mL.
    b. Gently remove the dye-containing media with a pipette, and replace with an equivalent volume of warm Medium 200PRF. The replacement media should be identical to the media the cells were incubated in during the tube formation.
15. Visualize the cells:
    - If a fluorescent dye was used, cells may be visualized using a fluorescence microscope.
    - If a fluorescent dye was not used, cells may be visualized directly using a light microscope.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
There is no tube network formation present in the positive inducer control well.	A. The cells may not be healthy or the cells may be too old. B. The cell density is too high or too low	A. Use only healthy cells or cells from an earlier passage. B. The optimal seeding density is cell-type specific. Therefore, it is necessary to optimize the seeding concentration.
The tubes at the edge of the well are out of focus.	There is a meniscus present at the edge of the well.	Use a larger well size in order to increase the observable area at the center of the well.
The tube network disappeared after staining.	A. The replacement media was different from the original media B. The replacement media was added too fast	A. Make sure that the replacement media being used is identical to the media the cells were originally cultured in during the experiment. B. Always add replacement media very gently to the cells.
There is high background fluorescence.	Following hydrolysis, Calcein, AM slowly leaks out of the cells.	Gently remove media and add replacement media.

Suggested References

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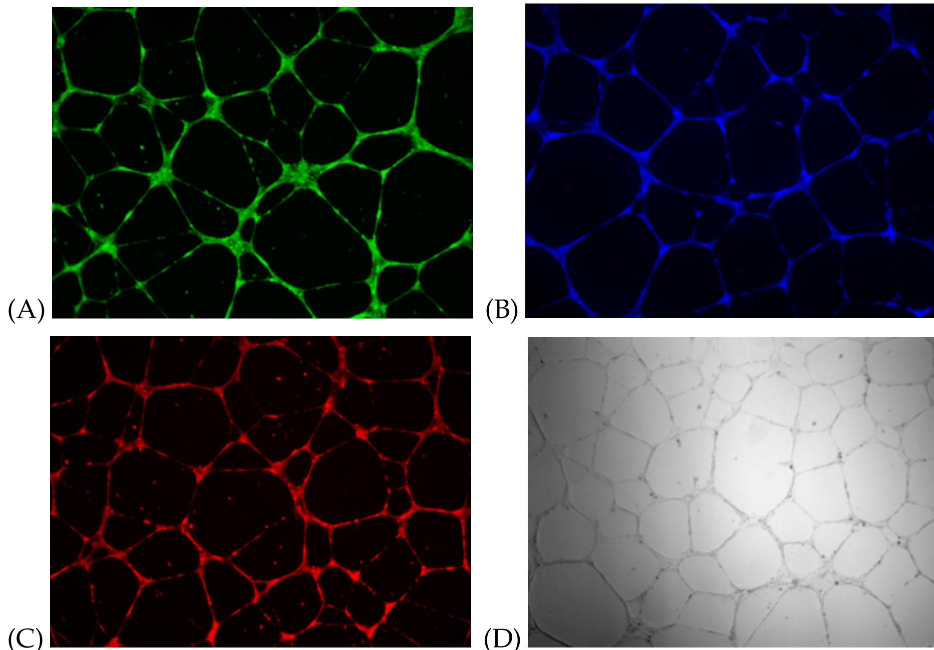

Figure 1. Positive Inducer Control Images

HUVEC (42,000 viable cells/cm^2) were seeded on a 24-well polystyrene plate coated with Geltrex™ (50 µL/cm^2) using LSGS-supplemented Medium 200PRF and incubated at 37°C and 5% CO_2. At 16 hours post-seeding, 2 µg/mL of **(A) Calcein, AM (Cat # C3099), (B) Calcein Blue, AM (Cat # C1429),** or **(C) CellTrace™ Calcein Red-Orange, AM (Cat # C34851)** was added directly to the culture well and allowed to incubate for 20 minutes (37°C, 5% CO_2) prior to taking these images at 4X magnification. **(D)** A representative **phase contrast** image.

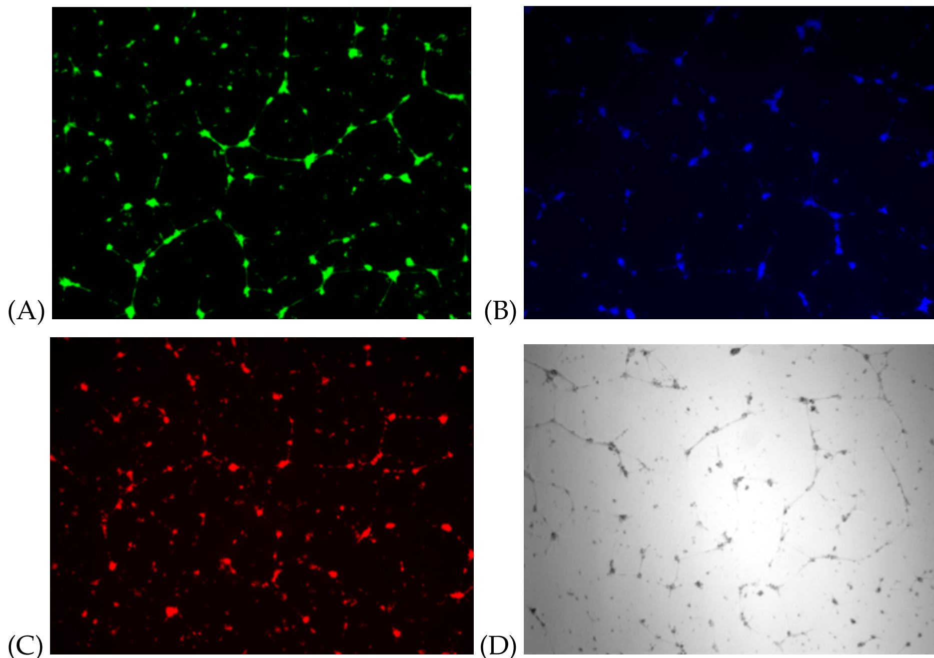

Figure 2. Negative Control Images (No Inducer Added)

HUVEC (42,000 viable cells/cm^2) were seeded on a 24-well polystyrene plate coated with Geltrex™ (50 µL/cm^2) using non-supplemented Medium 200PRF and incubated at 37°C and 5% CO_2. At 16 hours post-seeding, 2 µg/mL of **(A) Calcein, AM (Cat # C3099), (B) Calcein Blue, AM (Cat # C1429),** or **(C) CellTrace™ Calcein Red-Orange, AM (Cat # C34851)** was added directly to the culture well and allowed to incubate for 20 minutes (37°C, 5% CO_2) prior to taking these images at 4X magnification. **(D)** A representative **phase contrast** image.