

In Vitro Angiogenesis Assay Kit Tube Formation

Catalog# 3470-096-K

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In vitro assay for investigating inhibitors and inducers of angiogenesis in a 96-well plate format

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I. Quick Reference Procedure for the *In Vitro* Angiogenesis Assay Kit, Tube Formation (Cat#3470-096-K): Read through the complete *Instructions for Use* prior to using this kit.

This page is designed to be copied and used as a checklist.

BME solution MUST be kept on ice at all times due to gel formation at temperatures higher than 8 °C.

Day 0:

- 1. Seed endothelial cells at 0.5 to 1.0 x 10⁶ cells per 25 cm² flask and culture overnight.
- 2. Thaw BME solution in ice-water bath at 2-4 °C in a refrigerator overnight.

Day 1:

- 1. Aliquot 50 µl of BME into each well of a 96-well plate.
- 2. Incubate for 30-60 minutes at 37 °C to allow BME solution to gel.
- 3. **Optional step:** for fluorescent monitoring of tube formation. Prior to harvesting, incubate endothelial cells for 30 minutes with Calcein AM at 37 °C (**Optional**).
- 4. Harvest, count cells, and prepare a single cell suspension at 1 x 10⁶ cells in 1 ml.
- 5. Dilute cells in Endothelial Medium in the presence or absence of angiogenic mediators and inhibitors (please see page 5).
- 6. Without disturbing gelled Cultrex[®] RGF BME, add 100 µl diluted (1 to 3 x 10⁴) endothelial cells per well.
- 7. Incubate cells 4 to 24 hours in a CO₂ incubator at 37 °C.
- 8. If Calcein AM was not used, carefully fix the cells in cold methanol and stain using CS solution without disturbing the cells (page 5).
- 9. Visualize tube formation under an inverted light microscope or fluorescent microscope (if Calcein AM was used).

II. Background

Angiogenesis is the process by which new capillaries arise from pre-existing vasculature. Pathogenic angiogenesis is a common feature of rheumatoid arthritis, age-related macular degeneration, atherosclerosis and cancer. Cost-effective assays are important tools in the development of new therapeutic strategies to modulate neovascularization. *In vitro* angiogenesis assays take advantage of the tendency of vascular endothelial cells to form tubular structures when cultured on a supportive matrix (basement membrane), and have been used successfully to identify pro-angiogenic factors and inhibitors. Basement membranes are continuous sheets of specialized extracellular matrix that form an interface between endothelial, epithelial, muscle, or neuronal cells and their adjacent stroma. Basement membranes not only support cells and cell layers, but also play an essential role in tissue organization that affects cell adhesion, migration, proliferation and differentiation.

Trevigen's ***In Vitro* Angiogenesis Assay Kit, Tube Formation** allows for the detection of inducers and inhibitors of endothelial cell tube formation. Cultrex[®] **Reduced Growth Factor (RGF) Basement Membrane Extract (BME)** is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm (EHS) tumor, which gels at room temperature to form a reconstituted protein matrix comprised mainly of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. Sulforaphane [1-isothiocyanato-(4R)-methylsulfinyl]-butane], found in broccoli and other cruciferous vegetables, is a naturally occurring cancer chemopreventive agent, and is provided as a control for inhibition of *in vitro* endothelial cell tube formation on Cultrex[®] RGF BME. Calcein AM is provided for rapid and accurate measurement of cell viability and/or cytotoxicity, and kinetic analysis of tube formation. Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of Calcein AM by intracellular esterases produces calcein, a hydrophilic and strongly fluorescent compound that is well-retained in the cell cytoplasm. Thereafter, the number of junctions or tubes between cells can be quantified and monitored in real time. Thus, Trevigen's **CAS[™] HT Tube Formation Kit** provides a high-throughput, and high-content screening platform to test multiple compounds for their effects on endothelial cell tube formation.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. The CAS[™] HT Tube Formation Kit contains reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

IV. Materials Supplied

<u>Component</u>	<u>Quantity</u>	<u>Storage</u>	<u>Catalog#</u>
BME Reduced Growth Factor	5 ml	≤ -20 °C	3433-005-01
Calcein AM	50 µg	≤ -20 °C	4892-010-01
Cell Staining Solution	15 ml	RT	3470-096-01
Sulforaphane, 10mM	15 µl	≤ -20 °C	3470-096-02

V. Materials/Equipment Required But Not Supplied

Equipment

1. 37 °C CO₂ incubator
2. Low speed centrifuge and tubes for cell harvesting
3. Hemocytometer or other means to count cells
4. -20 °C and 4 °C storage
5. Inverted light microscope (if Cell Staining Solution or phase contrast are used)
6. Fluorescence microscope (if Calcein AM used)

Reagents

1. Human umbilical vein endothelial cells (HUVEC) or other endothelial cell line capable of tube formation
2. Endothelial Growth Medium, as recommended by cell supplier
3. Tube formation inducers (e.g. VEGF, FGF-2, etc.)*
4. Endothelial Basal Medium without serum and supplements
5. Cell Harvesting Buffer, EDTA, trypsin, or other cell detachment buffer
6. Sterile PBS or HBSS to wash cells
7. Distilled H₂O
8. Trypan blue or equivalent viability stain
9. DMSO
10. Methanol

Disposables

1. Black walled or clear 96-well tissue culture plates
2. Cell culture flasks, 25 cm² or 75 cm²
3. 2 ml, 5 ml and 10 ml serological pipettes
4. sterile microtubes
5. Gloves
6. 1 - 200 µl pipette tips

*Available from Trevigen (Please see page 8).

VI. Reagent Preparation

Note: Thaw all reagents completely before preparation.

1. **BME Growth Factor Reduced without Phenol Red (Cat#: 3433-005-01)**
Cultrex BME is provided ready to use. Thaw in ice water bath at 2-4 °C overnight prior to use. Unused BME RGF may be stored in aliquots at -20 °C for up to 3 months. Avoid repeated freeze-thaw cycles.
2. **2 mM Calcein AM Working Solution** Briefly centrifuge Calcein AM (Cat#: 4892-010-01) microtube to pellet powder before opening tube, and add 25 µl of sterile DMSO. Pipette up and down to mix, and store solution at -20 °C. **Note:** A further dilution to 2 µM is made just prior to cell labeling by adding 5 µl of 2 mM Calcein AM Working Solution to 5 ml PBS.
3. **Cell Staining Solution (Cat#: 3470-096-01)** The cell staining solution comes ready to use. It provides for optimal contrast between the cells and BME.

4. **100 µM Sulforaphane Working Solution:** Add 2 µl of 10 mM Sulforaphane (Cat#: 3470-096-02) to 198 µl of Endothelial Basal Medium.

VII. Assay Protocol

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

Tube Formation Assay

The following procedure is suggested and requires optimization to suit cell type(s) of interest.

Day 0:

1. Seed endothelial cells to be assayed at 0.1 to 1.0 x 10⁶ cells per 25 cm² tissue culture flask 24 hours prior to assay. **Note:** Concentration may vary depending on cell type and has to be determined experimentally. On the day of assay, cells should not be more than 80% confluent. Avoid using HUVEC beyond passage 10.
2. Thaw Cultrex[®] RGF BME at 2-4 °C overnight. **Note:** Refrigerator temperatures may vary; therefore, thaw extract in ice bath in a refrigerator.

Day 1:

3. In the hood, working on ice, aliquot 50 µl of BME solution per well of 96-well plate. Place lid on plate, and visually assess each well. The BME should be evenly distributed across each well. Make sure air bubbles are not trapped in the BME by centrifuging the plate at 250 x g for 5 minutes at 4 °C.
4. Incubate the plate at 37 °C for 30 to 60 minutes.
5. **Optional step** for fluorescent monitoring of tube formation.
 - a. Prepare a 2 µM Calcein AM solution by adding 5 µl of 2 mM Calcein AM Working Solution to 5 ml Endothelial Basal Medium (serum-free and, preferably, phenol red free).
 - b. Wash cells with sterile PBS and add 5 ml of 2 µM Calcein AM solution per 25 cm² flask (**Note: Labeling before harvesting cells allows formed tube networks to remain intact and avoids numerous washing steps.**)
 - c. Incubate cells for 30 minutes at 37 °C in a CO₂ incubator.
6. Wash adherent cells two times with 5 ml sterile room temperature PBS.
7. Harvest cells.
 - a. For 25 cm² flask, add 1 ml of Cell Harvesting Buffer (see *Materials Required but Not Supplied*) and incubate at 37 °C for 3 to 5 minutes (until cells have dissociated from bottom of flask).
 - b. Transfer cells to a 15 ml conical tube, and add 1 ml of Endothelial Growth Medium (see *Materials Required but Not Supplied*).
 - c. Count cells and centrifuge at 200 x g for 3 minutes to pellet the cells and carefully remove supernatant.
8. Make a single cell suspension at 1 x 10⁶ cells per 1 ml.
9. Dilute cells in Endothelial Growth Medium or Endothelial Basal Medium in the presence or absence angiogenesis mediators of your choice. We suggest using VEGF at 5-15 ng/ml, FGF-2 at 20-50 ng/ml, AG73 peptide at 100-200 µg/ml, CXCL12 (SDF-1 α) at 100-200 ng/ml, or PGE2 at 10⁻⁷ to 10⁻⁸

M. Trevigen offers VEGF and FGF-2 sold separately (please see *Related Products*).

10. **Optional tested inhibitor as a Negative Control.** Sulforaphane at 1-10 μM can now be added as a control inhibitor of tube formation. Some optimization may be required to determine maximal inhibition levels, depending upon the cells being tested.
11. Slowly add 100 μl of diluted cells (1 to 3 $\times 10^4$ cells) onto each well containing gelled BME. Do not disturb the fragile surface of the gelled BME. The exact number of cells per well depends on the cell type and should be determined experimentally.
12. Incubate the plate at 37 °C in a CO₂ incubator; incubation times may vary (4 to 24 hours). HUVEC develop well-formed tube networks after 4 to 6 hours. After 24 hours, endothelial cells typically undergo apoptosis.
13. If cells were not pre-treated with Calcein AM before harvesting it can be stained with Calcein AM at the end of the assay, after the tube network has formed.

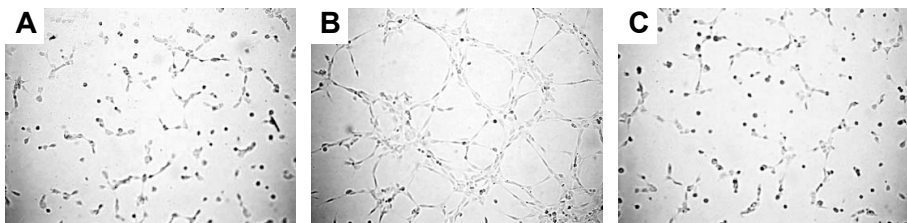
Method:

 - a. Remove medium from wells and wash once with 150 μl PBS per well.
 - b. Add 100 μl of 2 μM Calcein AM solution in PBS per well.
 - c. Incubate plate for 30 minutes at 37 °C in a CO₂ incubator.
 - d. Carefully aspirate Calcein AM solution and add 100 μl of PBS.
14. If Calcein AM was used at any time point during assay, visualize tube formation using a fluorescence microscope (485 nm excitation/520 nm emission).
15. If Calcein AM was not used, cells may be visualized directly on a light microscope, or for greater contrast, cells may be fixed, stained with Cell Staining Solution, washed and visualized using a light microscope.

Cell Staining Method:

- 1 Carefully aspirate the medium and wash once with PBS (100-150 μl per well). Do not disturb the tube network.
- 2 Fix cells with 150 μl per well ice-cold methanol for 30 sec – 1 minute. Do not exceed 1 minute.
- 3 Carefully aspirate methanol and immediately add 150 μl of dH₂O.
- 4 Wash twice with 150 μl per well of dH₂O.
- 5 Add 100 μl per well 1X Cell Staining Solution.
- 6 Incubate plate for 5-20 minutes at room temperature.
- 7 Remove Cell Staining Solution.
- 8 Wash 3 times with 150 μl of dH₂O.

VIII. Typical Results



HUVEC were harvested, counted and diluted in either Endothelial Basal Medium-2 (EBM-2) (which does not contain serum or angiogenic factors), or Endothelial Growth Medium-2 (EGM-2) (Cambrex), containing all supplements and growth factors necessary to support HUVEC growth. Trypsinized and harvested HUVEC were aliquoted at 10⁴ HUVEC per well onto gelled BME and thereafter cultured for four hours at 37 °C and 5 % CO₂. Typical phase contrast images with HUVEC in EBM-2 without added angiogenic factors (panel A), in EGM-2 with added factors (panel B) and in EGM-2 (with angiogenic factors) in the presence of 5 μM sulforaphane (panel C) are shown at 10X magnification.

IX. Troubleshooting

Problem	Cause	Solution
No tube formation in the positive control group.	Cells were not handled properly	Use freshly seeded cells (24 hours before tube assay).
		Do not use cells that were more than 80% confluent or passaged more than 10 times.
	Conditions are not suitable for the cell type(s) employed.	The initial concentration of cells per well, incubation times, cell culture medium, concentration of factors (endothelial growth factors or angiogenesis mediators) should be optimized for each cell type(s) analyzed.
BME is not transparent after fixation with methanol.	Methanol extracts water from BME and extracellular matrix proteins can precipitate and form aggregates.	Do not fix with methanol longer than 1 minute. Add sufficient amounts of water for washing. Try another reagent for fixation, for example 3.7% formaldehyde in PBS for 30 min at room temperature.
High background of BME after Calcein AM staining	Different lots of BME may have variable levels of background staining with Calcein AM.	Do not add Calcein AM solution on BME. Pre-treat cells with Calcein AM before harvesting. Use less Calcein AM.
High background of BME after Calcein AM staining (cont.)	Contamination / esterases released by bacteria or mold may activate Calcein AM.	Start a new culture from seed stocks, and re-assay. If seed stock is contaminated obtain new cells.

Problem	Cause	Solution
Tube network is lost after fixing and staining procedure.	The BME surface is very fragile and can be disturbed during fixing and staining.	Avoid fixing and staining too vigorously. Monitor the staining process using a light microscope or pretreat cells with Calcein AM before trypsinization and visualize tubes by using a fluorescence microscope.

X. References

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XI. Related products available from Trevigen.

Catalog#	Description	Size
3450-048-SK	Cultrex [®] Directed <i>in vivo</i> Angiogenesis Assay (DIVAA [™]) Starter	48 samples
3450-048-K	Cultrex [®] DIVAA [™] Activation Kit	48 samples
3450-048-IK	Cultrex [®] DIVAA [™] Inhibition Kit	48 samples
3471-096-K	Cultrex [®] In Vitro Angiogenesis Assay Endothelial Cell Invasion	96 samples
3455-024-K	Cultrex [®] 24 Well BME Cell Invasion Assay	24 inserts
3460-024-K	CultreCoat [®] 24 Well BME-Coated Cell Invasion Assay	24 inserts
3465-096-K	Cultrex [®] 96 Well Cell Migration Assay	96 samples
3465-024-K	Cultrex [®] 24 Well Cell Migration Assay	12 samples
3455-096-K	Cultrex [®] 96 Well BME Cell Invasion Assay	96 samples
3456-096-K	Cultrex [®] 96 Well Laminin I Cell Invasion Assay	96 samples
3457-096-K	Cultrex [®] Collagen I Cell Invasion Assay	96 samples
3458-096-K	Cultrex [®] Collagen IV Cell Invasion Assay	96 samples

Accessories:

Catalog#	Description	Size
3400-010-02	Cultrex [®] Mouse Laminin I, PathClear [®]	1 ml
3400-010-01	Cultrex [®] Mouse Laminin I	1 mg
3440-100-01	Cultrex [®] Rat Collagen I	100 mg
3410-010-01	Cultrex [®] Mouse Collagen IV	1 mg
3420-001-01	Cultrex [®] Human Fibronectin, PathClear [®]	1 mg
3416-001-01	Cultrex [®] Bovine Fibronectin, NZHD*	1 mg
3421-001-01	Cultrex [®] Human Vitronectin, PathClear [®]	50 μ g
3417-001-01	Cultrex [®] Bovine Vitronectin, NZHD	50 μ g
3438-100-01	Cultrex [®] Poly-L-Lysine	100 ml
3439-100-01	Cultrex [®] Poly-D-Lysine	100 ml
3445-048-01	Cultrex [®] 3-D Culture Matrix [™] BME	15 ml
3446-005-01	Cultrex [®] 3-D Culture Matrix [™] Laminin I	5 ml
3447-020-01	Cultrex [®] 3-D Culture Matrix [™] Collagen I	100 mg
3430-005-01	Cultrex [®] BME with Phenol Red	5 ml
3432-005-01	Cultrex [®] BME without Phenol Red	5 ml
3431-005-01	Cultrex [®] BME with Phenol Red; Reduced Growth Factor	5 ml
3433-005-01	Cultrex [®] BME; no Phenol Red; Reduced Growth Factor	5 ml
3415-001-02	Cultrex [®] Human BME, PathClear [®]	1 ml
3430-005-02	Cultrex [®] BME with Phenol Red, PathClear [®]	5 ml
3431-005-02	Cultrex [®] BME with Phenol Red, Reduced Growth Factor PathClear [®]	5 ml
3432-005-02	Cultrex [®] BME, PathClear [®]	5 ml
3433-005-02	Cultrex [®] BME; no Phenol Red; Reduced Growth Factor PathClear [®]	5 ml
3450-048-04	FGF-2	100 ng/ 10 μ l
3450-048-B9	FGF-2 (300 ng)/VEGF (100 ng)	10 μ l
3437-100-K	Cultrex [®] Cell Staining Kit	100 ml
3450-048-05	CellSpere [™]	15 ml

*New Zealand Herd Derived

XII. Appendices

Appendix A: Reagent and Buffer Composition

BME (Basement Matrix Extract) Growth Factor Reduced without Phenol Red (Cat# 3433-005-01)

A soluble form of basement membrane purified from Engelbreth-Holm-Swarm (EHS) tumor.

Calcein AM (Cat# 4892-010-01)

A non-fluorescent, hydrophobic compound that easily permeates intact, live cells. Once in the cell, Calcein AM is hydrolyzed by intracellular esterases to produce calcein, a hydrophilic, strongly fluorescent compound that is retained in the cell cytoplasm.

Cell Staining Solution (Cat# 3470-096-01)

A proprietary mixture of Azur A and Methylene Blue specially formulated to provide optimized staining of cells and structures grown on Cultrex[®] BME.

Sulforaphane, 10mM (Cat#3470-096-02)

Sulforaphane [1-isothiocyanato-(4R)-methylsulfanyl]-butane]