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R&D SYSTEMS

Cultrex™

In Vitro Angiogenesis Assay Kit Tube Formation

Catalog Number: 3470-096-K

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

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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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.

R&D Systems® *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. The number of junctions or tubes between cells can be quantified and monitored in real time. This kit provides a high-throughput, and high-content screening platform to test multiple compounds for their effects on endothelial cell tube formation.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

Note: *The components for this kit require different storage/shipping temperatures and will arrive in separate packaging.*

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Cultrex Reduced Growth Factor Basement Membrane Extract (RGF BME)	3433-005-01	5 mL	Store at ≤ -70 °C.
Calcein AM	4892-010-01	50 µg	
Sulforaphane, 10mM	3470-096-02	15 µL	
Cell Staining Solution	3470-096-01	15 mL	Room temperature

OTHER SUPPLIES REQUIRED

Reagents:

- Human umbilical vein endothelial cells (HUVEC) or other endothelial cell line capable of tube formation
- Endothelial Cell Growth Media (R&D Systems®, Catalog # CCM027) or a similar medium
- Tube formation inducers
- Endothelial Cell Base Media (R&D Systems, Catalog # 390598)
- Cell Harvesting Buffer, EDTA, trypsin, or other cell detachment buffer
- Sterile PBS or HBSS to wash cells
- Distilled H_2O
- Trypan blue or equivalent viability stain
- DMSO
- Methanol

Equipment:

- Pipettes and pipette tips
- 37 °C CO_2 incubator
- Low speed centrifuge and tubes for cell harvesting
- Hemocytometer or other means to count cells
- ≤ -20 °C and 2-8 °C storage
- Standard light microscope
- Fluorescent 96-well plate reader, top reader\(485 nm excitation, 520 nm emission)

Disposables:

- Black walled or clear 96-well tissue culture plates
- Cell culture flask, 25 cm^2 or 75 cm^2
- 2, 5 and 10 mL serological pipettes
- Mircotubes
- Gloves

REAGENT PREPARATION

Thaw reagents completely before diluting

Cultrex™ RGF BME - Cultrex RGF BME is provided ready to use. Thaw in ice water bath at 2-8 °C overnight prior to use. Once opened, Cultrex RGF BME may be stored in aliquots at ≤ -20 °C for up to 3 months. Avoid repeated freeze-thaw cycles.

2 mM Calcein AM Working Solution - Briefly centrifuge Calcein AM 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.*

Cell Staining Solution - The cell staining solution comes ready to use. It provides for optimal contrast between the cells and Cultrex RGF BME.

100 µM Sulforaphane Working Solution - Add 2 µL of 10 mM Sulforaphane to 198 µL of Endothelial Basal Medium.

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.

1. Seed endothelial cells to be assayed at 0.1-1x10^6 cells per 25 cm^2 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 2-8 °C overnight.
   Note: *Refrigerator temperatures may vary; thaw extract in ice bath in a refrigerator.*

3. In the hood, working on ice, aliquot 50 µL of Cultrex RGF BME solution per well of 96-well plate. Place lid on plate, and visually assess each well. The Cultrex RGF BME should be evenly distributed across each well. Make sure air bubbles are not trapped in the Cultrex RGF BME by centrifuging the plate at 250 x g for 5 minutes at 2-8 °C.

4. Incubate the plate at 37 °C for 30-60 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 cm^2 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 CO_2 incubator.
6. Wash adherent cells two times with 5 mL sterile room temperature PBS.
7. Harvest cells.
   a. For 25 cm^2 flask, add 1 mL of Cell Harvesting Buffer and incubate at 37 °C for 3-5 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.
   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 $1x10^6$ 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-1α) at 100-200 ng/mL, or PGE2 at 10-7 to 10-8 M.
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 ($1-3x10^4$ 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 CO_2 incubator; incubation times may vary (4-24 hours). HUVEC develop well-formed tube networks after 4-6 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 CO_2 incubator.

d. Carefully aspirate Calcein AM solution and add 100 µL of PBS.

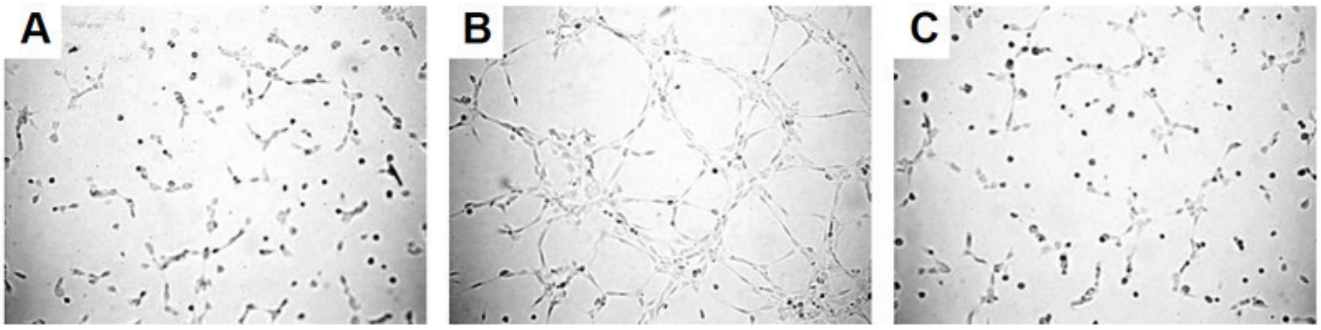
14. If Calcein AM was used at any time point during assay, visualize tube formation using a fluorescence microscope (485 nm excitation/520 nm emission).

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 seconds to 1 minute. Do not exceed 1 minute.
3. Carefully aspirate methanol and immediately add 150 µL of distilled water.
4. Wash twice with 150 µL per well of distilled water.
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 distilled water.

DATA EXAMPLE



HUVEC were harvested, counted and diluted in Endothelial Cell Base Media (which does not contain serum or angiogenic factors) or Endothelial Cell Growth Media, containing all supplements and growth factors necessary to support HUVEC growth. Trypsinized and harvested HUVEC were aliquoted at 10^4 HUVEC per well onto gelled Cultrex RGF BME and thereafter cultured for four hours at 37 °C and 5 % CO_2. Typical phase contrast images with HUVEC in Endothelial Cell Base Media without added angiogenic factors **(A)**, in Endothelial Cell Growth Media with added factors **(B)** and in Endothelial Cell Growth Media (with angiogenic factors) in the presence of 5 µM sulforaphane **(C)** are shown at 10X magnification.

TROUBLESHOOTING

Problem	Cause	Action
No tube formation in the positive control group.	Conditions are not suitable for the cell type(s) employed.	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.
		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.
Cultrex RGF BME is not transparent after fixation with methanol.	Methanol extracts water from Cultrex RGF 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 Cultrex RGF BME after Calcein AM staining	Different lots of Cultrex RGF BME may have variable levels of background staining with Calcein AM.	Do not add Calcein AM solution on Cultrex RGF BME. Pretreat cells with Calcein AM before harvesting. Use less Calcein AM.
	Contamination/esterases released by bacteria or mold may activate Calcein AM.	Start a new culture from seed stocks, and reassay. If seed stock is contaminated obtain new cells.
Tube network is lost after fixing and staining procedure.	The Cultrex RGF 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.

REFERENCES

1. Folkman J. (2007) Nat. Rev. Drug Discov. **6**:273.
2. Jaffe, E.A. *et al.* (1973) J. Clin. Invest. **52**:2745.
3. Folkman, J. and C. Haudenschild (1980) Nature **288**:551.
4. Kleinman, H.K. *et al.* (1986) Biochemistry **25**:312.
5. Lawley, T.J. and Y. Kubotan (1989) J. Invest. Dermatol. **93**:59S.
6. Ucuzian, A.A. and H.P. Greisler (2007) World J. Surg. **31**:654.
7. Salcedo, R. *et al.* (2003) Blood **102**:1966.
8. Bertl, E. *et al.* (2006) Mol. Cancer Ther. **5**:575.
9. Jackson, S.J. *et al.* (2007) Vascular Pharmacology **46**:77.
10. Chen, X. *et al.* (2003) Clin. Cancer Res. **9**:3115.
11. Fukumoto, S. *et al.* (2005) Clin. Exp. Metastasis **22**:31.
12. Caldas, H. *et al.* (2007) Blood **109**:1479.
13. Mochizuki, M. *et al.* (2007). Arch. Biochem. Biophys. **459**:249.