In Vitro Angiogenesis Assay Kit

Cat. No. ECM625

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Angiogenesis is the process of generating new capillary blood vessels. It is a fundamental component of a number of normal (reproduction and wound healing) and pathological processes (diabetic retinopathy, rheumatoid arthritis, tumor growth and metastasis).

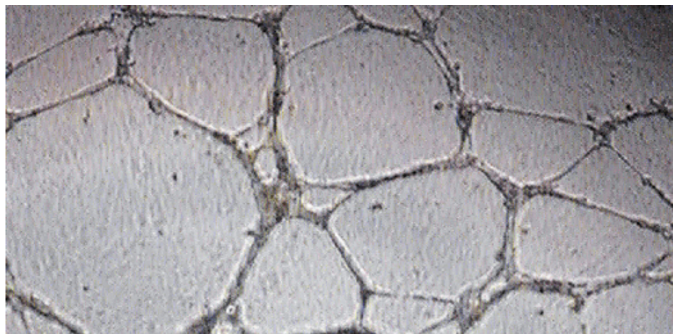
The MILLIPORE® *In Vitro* Angiogenesis Assay Kit provides a convenient system for evaluation of tube formation by endothelial cells in a convenient 96-well format. When cultured on ECMatrix™, a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor, these endothelial cells rapidly align and form hollow tube-like structures. Tube formation is a multi-step process involving cell adhesion, migration, differentiation and growth.

ECMatrix™ consists of laminin, collagen type IV, heparan sulfate proteoglycans, entactin and nidogen. It also contains various growth factors (TGF-β, FGF) and proteolytic enzymes (plasminogen, tPA, MMPs) that occur normally in EHS tumors. It is optimized for maximal tube-formation.

Application

The MILLIPORE® *In Vitro* Angiogenesis Assay Kit represents a simple model of angiogenesis in which the induction or inhibition of tube formation by exogenous signals can be easily monitored. For assaying inhibitors or stimulators of tube formation, simply premix the endothelial cell suspension with different concentrations of the inhibitor or stimulator to be tested, before adding the cells to the top of the ECMatrix™. The assay can be used to monitor the extent of tube assembly in various endothelial cells, e.g. human umbilical vein cells (HUVEC) or bovine capillary endothelial (BCE) cells.

The MILLIPORE *In Vitro* Angiogenesis Assay Kit is intended for research use only; not for diagnostic or therapeutic applications.



HUVEC Cells incubated 6-10 hours at 37°C on ECMatrix™

Kit Components

1. <u>ECMatrix™ Gel Solution</u>: (Part No. 90060) Five 1 mL bottles.
2. <u>ECMatrix™ Diluent Buffer, 10X</u>: (Part No. 90061) One 1 mL bottle.

Materials Not Supplied

1. 96-well Tissue Culture plate
2. Microcentrifuge Tubes, sterile
3. 37°C Tissue Culture Incubator
4. Inverted Light Microscope
5. HUVEC cells or other experimental cell line (any species)

Assay Instructions

CAUTION: ECMatrix™ will gel rapidly at 22-35°C. Thaw overnight on ice or in a +4°C refrigerator ("frost-free" refrigerators may generate temperatures above +4°C and cause ECMatrixTM to gel). Keep vials containing ECMatrix™ on ice all the time. Use pre-cooled pipettes, plates and tubes when preparing ECMatrix™. Undiluted ECMatrix™ is highly viscous and it might be necessary to cut off the tip of pipette tip with a sterile knife to ease pipetting. Gelled ECMatrix™ may be re-liquefied if placed on 0-4°C for 24-48 however the efficacy may be impaired.

1. Thaw ECMatrix™ Solution as suggested above. Thaw the Diluent Buffer on ice.

2. Add 100 µl of 10X Diluent Buffer to 900 µl of ECMatrix™ solution in a sterile microfuge tube. Mix well slowly; do not pipette air into the solution. Often it is best to work within a cold room. Keep solution on ice to avoid solidification.

3. Transfer 50 µl to each well of a pre-cooled 96-well tissue culture plate. Pipette tips and ECMatrix™ solution should be kept cold all the time to avoid solidification.

4. Incubate at 37°C for at least one hour to allow the matrix solution to solidify.

5. Harvest endothelial cells and resuspend in media. Use endothelial cell growth media, or a standard cell growth media supplemented with endothelial cell growth supplements. The presence of serum (0.5-10%) is optional. We prefer EGM (Endothelia Cell Growth Media) 150 microliters per well (96 well size) is usually fine.

6. Seed 5×10^3 - 1×10^4 cells per well onto the surface of the polymerized ECMatrix™.

7. Incubate at 37°C overnight (4-12h) in a tissue-culture incubator. Cellular network structures are fully developed by 12-18h, with the first signs apparent after 5-6h. After 24h the cells will begin to undergo apoptosis. To study the effect of pro-angiogenic factors, the incubation time should be decreased to 4-8h. Optimal times may vary depending on the cell type, cell age and media growth conditions.

8. Inspect tube formation under an inverted light microscope at 40X-200X magnification. Since the lack of contrast between unstained cells and ECMatrix™ could make visualization of the cellular networks difficult, it might be necessary to stain the cells using any of the common cell staining procedures (Diff-Quick or similar, see Quantitation note D).

Quantitation

Activated endothelial cells form cellular networks (mesh like structures) from capillary tubes sprouting into the stromal space (see Figure on p.2). The formation of these cellular networks is a dynamic process, starting with cell migration and alignment, followed by the development of capillary tubes, sprouting of new capillaries, and finally the formation of the cellular networks. Although *In vitro* Angiogenesis Assay Kit is designed as a qualitative assay, it is possible to quantitate to some degree the extent to which cellular networks have formed.

A. Pattern recognition

Define visual patterns by looking or photographing the cells at 40X – 200X magnification at set time at 37°C after seeding on ECMatrix™ (end-point assay). Assign a numerical value to each pattern. This way a numerical value is associated with a degree of angiogenesis progression. An example is presented in the table below.

Pattern	Value
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible. No sprouting.	2
Sprouting of new capillary tubes visible.	3
Closed polygons begin to form.	4
Complex mesh like structures develop	5

This pattern/value association criterion should be defined with the type of cells and experimental conditions that will be used in the angiogenesis assay. Several random view-fields (3-10) per well should be examined and the values averaged. This quantitation method will work best in assays involving potent inhibitors or activators of angiogenesis.

B. Branch Point Counting

A subtler, but more labor-intensive way to quantitate the progression of angiogenesis is to count the capillary tube branch points formed after a set amount of time (end-point assay). The length of the newly formed capillary tubes can also be taken into account when counting (do not count if shorter than an arbitrary predetermined length). Branch points in several random view-fields (3-10) per well should be counted and the values averaged.

C. Total Capillary Tube Length Measurement

An alternative method to branch point counting, suitable particularly for microscopes with imaging capabilities, is to measure the total length of all the capillary tubes in a view-field. The total capillary tube length in several random view-fields (3-10) per well should be examined and the values averaged.

D. Visualizing cell-tubes

Several commercially available cell stains can be used, such as Wright-Giemsa stain or crystal violet {0.5% crystal violet in a solution of 50% ETOH/PBS containing 5% formaldehyde}, or Masson's trichrome. Also MTT conversion can also be used to visualize the cells present on the matrix {Biotechniques 24:1038-1043, 1998}.

Storage

Store kit materials at -20°C for up to 6 months. Once ECMatrix™ has been thawed, store at 2-8°C for one week. Do not refreeze.

Related References

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