In Vitro Angiogenesis Assay Kit

Item No. 10009964

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	100 Tests Quantity/Size	Storage
400145	Cell-Based PMA (1 mM)	1 vial/50 µl	-20°C
400146	Cell-Based Calcein AM (1,000X)	1 vial/20 µl	-20°C
400147	Cell-Based Extracellular Matrix Gel	1 vial/5 ml	4°C
400148	Cell-Based JNJ-10198409 (3 mM)	1 vial/100 µl	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed and used before the expiration date indicated on the outside of the box. The unopened kit may be stored at 4°C. Once the kit is opened, the extracellular matrix gel must be stored at 4°C and the other kit components may be stored at -20°C.

Materials Needed But Not Supplied

1. Calf pulmonary artery endothelial cells (CPAE) or human umbilical vein endothelial cells (HUVEC) (other endothelial cells may also be used)
2. Adjustable pipettes and a repeat pipettor
3. A 96-well plate for culturing cells
4. A microscope equipped with filters for excitation and emission at 485 and 535 nm, respectively, for measuring fluorescence

INTRODUCTION

Background

Angiogenesis is a physiological process that occurs during wound healing and normal development which involves the growth of new blood vessels from pre-existing vessels. These blood vessels form highly branched, tree-like tubular networks that ensure efficient and simultaneous transport of gases, liquids, nutrients, signaling molecules, and circulating cells between tissues and organs. Angiogenesis is complex and highly regulated, with tight coordination of cell proliferation, differentiation, migration, matrix adhesion, and cell-to-cell signaling.[1] Angiogenesis is regulated by several factors, most importantly growth factors such as vascular endothelial growth factors (VEGFs) and platelet-derived growth factors (PDGFs).

While normal angiogenesis is critical for homeostasis, abnormal angiogenesis is a significant component of several diseases, most notably cancer. Angiogenesis is essential for tumor development and metastasis. Inhibition of angiogenesis is one of the main therapeutic targets in cancer drug discovery. Angiogenesis inhibitors such as Tarceva have been widely used in the United States and other countries to treat lung cancer.[2] Most of these inhibitors target elements of growth factor-receptor signaling pathways. JNJ-10198409 is one of these molecules that inhibits the tyrosine kinase activity of the PDGF receptor.[3]

About This Assay

In vitro cell-based assays using calf pulmonary aortic endothelial (CPAE), human umbilical vein endothelial cells (HUVEC), or other endothelial cells grown on an extracellular matrix containing collagen are important tools for identifying factors that regulate angiogenesis. A number of such *in vitro* assays have been developed in the past, but these assays have been somewhat limited in their application due to the short duration which the cultured cells live, and the complexity of the assays. Cayman's *In Vitro* Angiogenesis Assay Kit uses a one-step model to study regulators of angiogenesis. Survival of the cells is improved compared to the other assays by the use of an extracellular matrix modified from Hermant, *et. al.*.[4] This matrix has been validated using both stimulators and inhibitors of angiogenesis, and in both short-term (a few days) and long-term (up to ten days) experiments.

This assay can be adapted to high throughput screening for angiogenesis inhibitors by using a 96-well plate format. The kit includes PMA as a stimulator of angiogenesis and JNJ-10198409 as an inhibitor of angiogenesis. The fluorescent dye Calcein AM is used to visualize cell organization. Calcein AM permeates the cell membrane and is hydrolyzed to calcein by intracellular esterases, resulting in a bright green fluorescence.

ASSAY PROTOCOL

Materials

1. Cell-Based PMA (1 mM) Item No. 400145
2. Cell-Based Calcein AM (1,000X) Item No. 400146
3. Cell-Based Extracellular Matrix Gel Item No. 400147
4. Cell-Based JNJ-10198409 (3 mM) Item No. 400148

NOTE

The Extracellular Matrix Gel may appear cloudy over time due to precipitation of collagen. However, this should not affect the performance of the product. To clarify, add 10-20 µl of concentrated HCl, then neutralize to pH 7.0 with 5 N NaOH just before you coat the plate.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical experimental plate will include wells without cells, wells with cells treated with experimental compounds, and wells of untreated cells. We suggest you record the contents of each well on the template sheet provided (see page 15).

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Assay Procedure

The following protocol is designed for a 96-well plate. For other sizes of plates the volume of medium/solution applied to each well should be adjusted accordingly.

1. Coat each well of a 96-well plate with 30-40 µl of Cell-Based Extracellular Matrix Gel (Item No. 400147). We recommend that you use a repeat pipettor to deliver the liquid gel to each well. Do not pipette the gel up and down as this will generate bubbles. Gently shake the plate for one minute to ensure that the gel is even.
2. Put the coated plate in a 37°C cell culture incubator for 30-60 minutes to allow the gel to solidify.
3. Prepare a single cell suspension at $1x10^5$ cells/ml in culture medium with or without angiogenesis activators and inhibitors. Cell-Based PMA (1 mM) (Item No. 400145) can be used at a concentration of 0.01-1 µM to induce angiogenesis; Cell-Based JNJ-10198409 (3 mM) (Item No. 400148) can be used at a concentration of 0.1-1 µM to inhibit angiogenesis.
4. Gently add 100 µl of the cell suspension prepared above to each gel-coated well.
5. Incubate the cells overnight in a 37°C cell culture incubator. Depending on the density of cell seeding, longer incubation times may be needed to obtain maximal cell network formation.
6. At the end of the experiment, prepare a calcein staining solution by diluting the Cell-Based Calcein AM 1:100 in culture medium. Add 10 µl of this solution to each well.
7. Examine the cell networking structure under an inverted fluorescence microscope. Images should be taken within two to three hours after cells are stained by calcein, as cells start to die after longer incubation in the staining solution.

PERFORMANCE CHARACTERISTICS

Cell Staining

An example of typical staining demonstrating angiogenesis by cell network formation is shown in the figure below. Your results may vary based on the type of cell used.

PMA induces and JNJ-10198409 inhibits angiogenesis in CPAE cells grown on Extracellular Matrix Gel. CPAE cells grown on Extracellular Matrix Gel tend to form web-like structures and PMA at concentrations as low as 0.064 µM increases this cellular network formation. JNJ-10198409 causes the withdrawl of cell extensions and thus attenuates cell-cell contact and network formation.

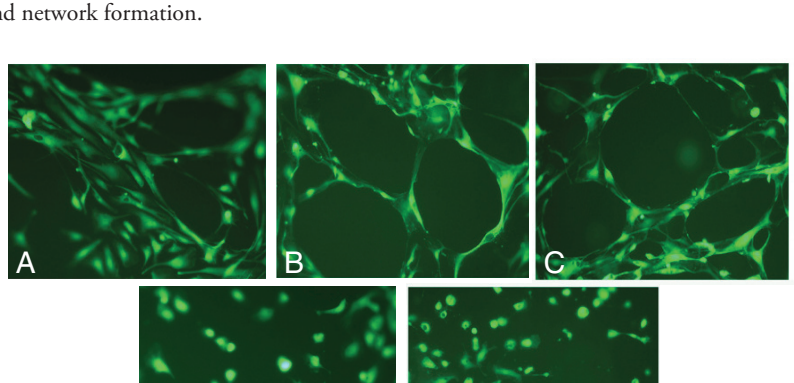

Figure 1: Cell network formation in endothelial cells. *Panel A:* CPAE cells suspended in culture medium containing vehicle, *Panel B:* 0.064 µM PMA, *Panel C:* 0.25 µM PMA, *Panel D:* 0.3 µM JNJ-10198409, or *Panel E:* 0.25 µM PMA + 0.3 µM JNJ-10198409 were seeded at a density of 10^4 cell/well in a 96-well plate and grown in a 37°C incubator for two days. On the third day, cells were stained with Calcein AM and the organization was examined under an inverted fluorescence microscope.

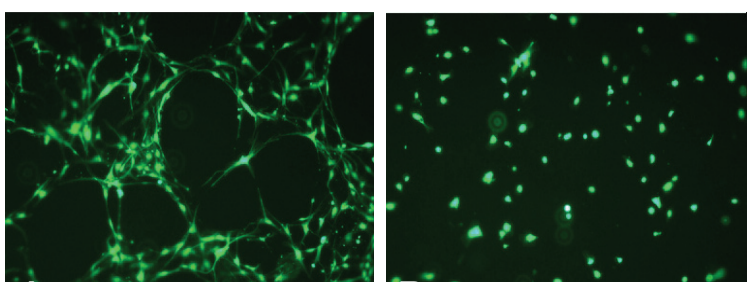

Figure 2: Inhibition of network formation by JNJ-10198409. *Panel A:* CPAE cells suspended in culture medium containing 0.064 µM PMA, or *Panel B:* 0.064 µM PMA + 0.3 µM JNJ-10198409 were seeded at a density of 6 x 10^3 cells/well in a 96-well plate and grown in 37°C incubator for four days. On the fifth day, cells were stained with Calcein AM and the organization was examined under an inverted fluorescence microscope.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No network formation in the positive control group	A. Cells may not be healthy or cells are too old B. Cell density is too high or too low	A. Use only healthy cells; use cells from a lower passage B. Use optimal cell density (in the case of CPAE, 10^4 cells/well in a 96-well plate
Cell layer curves up with the gel	A. Disturbance of the gel layer B. Bubbles in the gel layer C. Gel not adequately solidified	A. Do not disturb the gel layer. If you need to change the culture medium during the experiment, use a pipette to gently remove the medium rather than using aspiration B. Do not mix the liquid gel solution or pipette the solution up and down before coating the plate, as this will generate bubbles

References

1. Adams, R.H. and Alitalo, K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nature Reviews Molecular Cell Biology* **8**, 464-478 (2007).
2. Folkman, J. Angiogenesis. *Annu. Rev. Med.* **57**, 1-18 (2006).
3. Ho, C.Y., Ludovici, D.W., Maharoof, U.S.M., *et al.* (6,7-Dimethoxy-2,4-dihydroindeno[1,2-c]pyrazol-3-yl)phenylamines: Platelet-derived growth factor receptor tyrosine kinase inhibitors with broad antiproliferative activity against tumor cells. *J. Med. Chem.* **48**, 8163-8173 (2005).
4. Hermant, B., Desroches-Castan, A., Dubessay, M.-L., *et al.* Development of a one-step embryonic stem cell-based assay for the screening of sprouting angiogenesis. *BMC Biotechnology* **7(20)**, 1-9 (2007).

Related Products

Adipogenesis Assay Kit - Item No. 10006908
Adipolysis Assay Kit - Item No. 10009381
Apoptotic Blebs Assay Kit - Item No. 10010750
Genipin - Item No. 10010622
JNJ-10198409 - Item No. 10008131
LDL Uptake Cell-Based Assay Kit - Item No. 10011125

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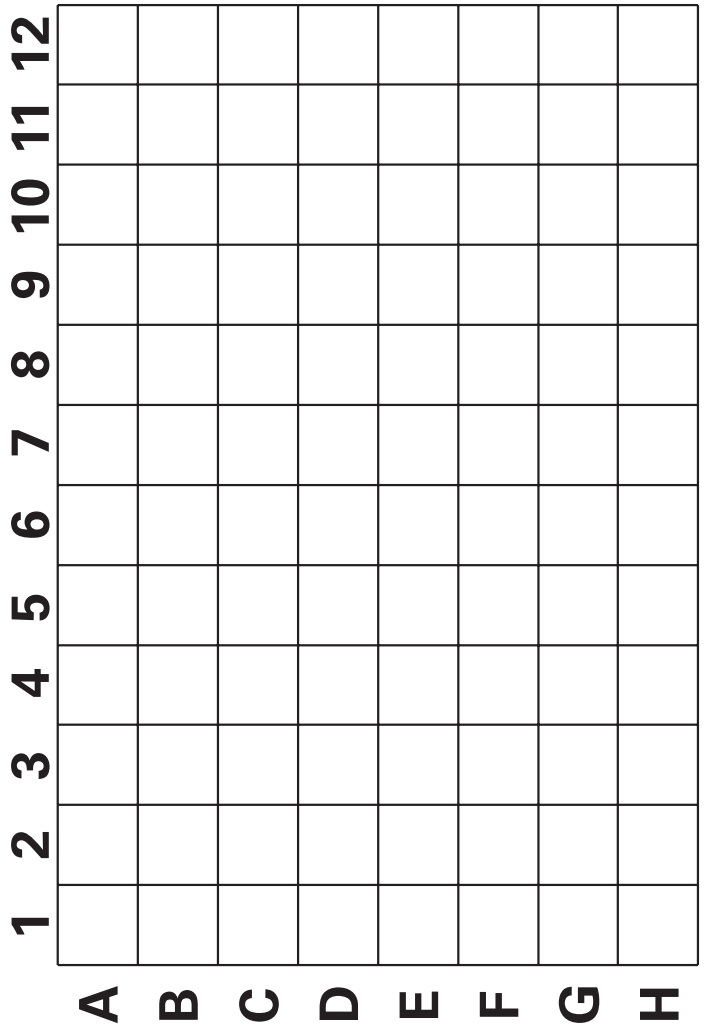
For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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