ab204726
In vitro Angiogenesis Assay Kit

Instructions for Use

For simple qualitative detection of angiogenesis.

This product is for research use only and is not intended for diagnostic use.
INTRODUCTION

1. BACKGROUND

In vitro Angiogenesis Assay kit (ab204726) provides a robust method to determine angiogenesis in vitro in less than 18 hours. This product provides a simple, easy to perform, qualitative tool for assessing angiogenesis.

Angiogenesis is the process of generating new blood vessels from the pre-existing vasculature. Angiogenesis is required for growth and development, wound healing, tissue granulation and formation of malignant tumours. The quick assessment of angiogenesis involves measurement of the ability of endothelial cells to form three-dimensional tube-like structures.
2. **ASSAY SUMMARY**

- Prepare endothelial cells
- Add cells to Extracellular Matrix gel
- Add Dye and incubate at 37°C for 30 minutes
- Analyze cells by light and fluorescence microscopy
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
6. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular Matrix Solution (2 vials)</td>
<td>1.25 mL</td>
<td>-20°C</td>
<td>-20°C / 4°C</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>10 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Staining Dye Concentrate</td>
<td>25 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Inhibitor Control- Vinblastine</td>
<td>10 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

7. **MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully perform this assay:

- Human umbilical vein endothelial cells (HUVEC) or other endothelial cells – primary cells or cell line can be used
- Appropriate endothelial cell culture media with serum
- Incubator at 37°C with 5% CO₂
- Inverted fluorescence microscope equipped with filter for Ex/Em = 490/540 nm (green fluorescence)
- General tissue culture supplies
- 96-well clear plate for cell culture
- Pipettes and pipette tips
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Keep enzymes, heat labile components and samples on ice during the assay.

- Make sure all buffers and solutions are at room temperature before starting the experiment.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Make sure all instrumentation needed for the assay is switched on.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 Extracellular Matrix Solution:
Ready to use as supplied. Always thaw slowly on ice or in a frost free 4°C refrigerator.

*NOTE:* temperature above 4°C will rapidly gel the Extracellular Matrix Solution. Thawing may take overnight at 4°C. The thawed matrix can be stored at 4°C for one week. For long term storage, aliquot Extracellular Matrix Solution under sterile conditions and store at -20°C.

9.2 Wash Buffer:
Ready to use as supplied. Equilibrate to 37°C before use. Store at -20°C.

9.3 Staining Dye Concentrate:
Ready to use as supplied. Aliquot Dye so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.4 Inhibitor Control-Vinblastine (2 µM):
Ready to use as supplied. Equilibrate to room temperature before use. Aliquot Vinblastine so that you have enough volume to perform the desired number of assays. Store at -20°C. Prior use, dilute Vinblastine in wash buffer as required.
10. ASSAY PROCEDURE and DETECTION

- It is recommended to assay all controls and samples in duplicate.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density to perform the experiment.
- Treat cells with test compounds for desired period of time to induce response.
- Appropriate incubation time depends on the individual cell type and cell concentration used. Optimize the incubation time for each experiment.

10.1 Cell Culture:

10.1.1 Grow endothelial cells in the appropriate culture media (37°C incubator containing 5% CO₂) so that they are approximately ~90% confluent on the day of the experiment.

10.1.2 Harvest cells under sterile conditions using basic cell culture techniques. Resuspend the cells in appropriate culture media containing 0.5 – 5% serum.

10.2 Tube Formation:

10.2.1 Add 50 µL of thawed Extracellular Matrix Solution to each well of a pre-chilled (on ice) 96-well plate sterile culture plate. Ensure the gel spreads evenly on the surface of the well (rock or tap gently to spread). Incubate for 1 hour at 37°C to allow the solution to form a gel.

**Background Control well** = no Extracellular Matrix Solution.

10.2.2 Plate 1 – 2 x 10⁴ cells/well onto the solidified extracellular matrix gel and the background control well.
10.2.3 Add appropriate treatment (angiogenesis factors/regulators and/or inhibitors) to the desired wells.

**Inhibitor Control well** = Vinblastine treated cells. **NOTE:** Treatment with Control-Vinblastine will vary depending on the cell type. For an endothelial cell line (EA.hy926 cells), we recommend using final concentration of 1 pmol/L.

10.2.4 Incubate cells for 4 – 18 hours in a 37°C incubator containing 5% CO₂.

10.3 **Tube Staining:**

10.3.1 Carefully remove incubation medium using a pipette without disturbing the cells or the extracellular matrix gel.

10.3.2 Gently wash the wells with 100 µL of Wash Buffer to remove serum. Remove Wash Buffer carefully.

10.3.3 Prepare a Staining Dye working solution by diluting Staining Dye Concentrate (from section 9.3) 1:200 in Wash Buffer (for example, 5 µL Dye Concentrate in 995 µL Wash Buffer. **NOTE:** Staining working solution is stable for 1 hour at 4°C). Add 100 µL Staining Dye working solution to each well.

10.3.4 Incubate dye with cells for 30 minutes at 37°C.

10.3.5 Examine the endothelial tube formation using light and fluorescence microscope (green filter).
11. **CALCULATIONS**

- We recommend acquiring several images per well. To ensure objective and quantitative analysis, we recommend using imaging software.

11.1 For manual analysis, if you do not have a specific software installed in your microscope, you can download ImageJ, an open source image processing designed for scientific multidimensional images by the National Institute of Health (NIH).

11.2 For automated analysis, we recommend using Wimasis WimTube Solution. This tool is based on tubule characteristics such as number of tubules, number of junctions, tubule length, and number of loops.
12. **TYPICAL DATA**

**Figure 1.** Endothelial Cell (EA.hy926 Cells) tube formation: Phase contrast (a, c, e) and fluorescent images (b, d, f) of endothelial cells in a tissue culture plate. (a, b) Untreated cells (c, d) Tube formation of endothelial cells on Extracellular Matrix Gel after induction with 0.1 µM PMA. (e, f) treatment with Vinblastine (1 pmol/L) inhibits tube formation.
Figure 2. HUVEC morphogenesis on Extracellular Matrix Gel. Cells (2 × 10⁴) were plated per 1 cm² well precoated with Extracellular Matrix Gel and grown for 18 hours (A) in the specific medium alone (positive control) or containing (B) PMA 10 µmol/L.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low or no fluorescent signal in positive control</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Dead or stressed (overcrowded) cells</td>
<td>Prepare fresh cell culture for the experiments. Make sure that cells are in the log growth phase.</td>
</tr>
<tr>
<td></td>
<td>Insufficient fluorescent dye concentration</td>
<td>Follow the procedure described in the protocol</td>
</tr>
<tr>
<td></td>
<td>Insufficient inducer concentration</td>
<td>Determine the appropriate concentration of inducer for the cell line(s) used in the study</td>
</tr>
<tr>
<td>Low or no fluorescent signal in samples</td>
<td>Inappropriate time point of detection</td>
<td>Ensure time of detection is optimized and samples are prepared immediately</td>
</tr>
<tr>
<td></td>
<td>Cell density is too low</td>
<td>Check cell count to confirm proper cell density. For suspension cells, remove supernatant carefully after washing step as cells may be dislodged and washed away</td>
</tr>
<tr>
<td>High fluorescence background</td>
<td>Stressed (overcrowded) cells</td>
<td>Prepare fresh cell culture for the experiments. Make sure that cells are in the log growth phase.</td>
</tr>
<tr>
<td></td>
<td>Omitting wash step</td>
<td>Make optional wash steps mandatory</td>
</tr>
<tr>
<td></td>
<td>Inappropriate cell conditions</td>
<td>Ensure you have viable cells at the beginning of the experiment, and that the inducer treatment does not kill the cells during the time frame of the experiment</td>
</tr>
<tr>
<td>No change in fluorescence signal after using a specific inhibitor</td>
<td>Inappropriate inhibitor concentration (too low or too high)</td>
<td>Optimize the concentration of the inhibitor and pretreatment time for each particular cell line.</td>
</tr>
</tbody>
</table>