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Technical Datasheet

EZAssay™ Angiogenesis Cell Culture Kit (*In vitro*)

Product Code: CCK081

1. Introduction

Angiogenesis is a tightly regulated and complex process where new blood vessels are generated from pre-existing vasculature. Angiogenesis takes places in the following steps:1) disruption of the neighboring basement membrane, 2) migration of the cells, 3) proliferation and re organization of the cells in vessel structure; all in response to an angiogenic stimulus. This occurs during normal process such as development and wound healing as well as associated with pathological conditions such as cancer, rheumatoid arthritis, age-related macular degeneration, and atherosclerosis. It is regulated by several growth factors, .such as platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGFs), transforming growth factor-β (TGF-β) , fibroblast growth factor (FGF), and many more.

Cost effective *in-vitro* angiogenesis assay kits are needed for new therapeutic development. The assay kits is optimized for Human Umbilical Vein Endothelial Cells (HUVEC) to generate of endothelial cell tube networks on extracellular matrix.

The following protocol provides how to perform the tube formation assay, one of the most well-established in vitro assays for the formation of three-dimensional vessels.

2. Overview of the assay

1) Thaw/use fresh batch of the cells for experiment. Grow HUVEC cells upto ~80% confluence. Harvest and resuspend the cells in the appropriate medium.

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2) Add cells to the 96 well plate (1 x 10^4 cells per well) pre-coated with Extracellular Matrix Gel. The control well should have no matrix. Add Inhibitor Control CCK081(B) to the negetive control wells. Add test compounds (growth factors) to test wells.

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3) Incubate cells for 4-18 hours or as per the study in a 37°C incubator containing 5% CO_2.

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4) Add staining dye CCK081(C) and incubate at 37°C for 30 minutes. Image cells under fluorescence and phase contrast microscope.

3. Kit contents

The reagent supplied in the kit are sufficient for 100 assays.

Code	Description	Quantity	Storage	Detection method
CCK081(A)	Extracellular Matrix Solution	2x2.5ml	-20 °C	-
CCK081(B)	Inhibitor Control	10µl	-20 °C	-
CCK081(C)	Staining Dye Concentrate	2x25µl	-20 °C	Fluorescence
CCK081(D)	Wash Buffer	1x20ml	2-8°C upon receipt	-

4.Description of Kit Contents

Part A: Extracellular Matrix Solution CCK081(A) 2x2.5 ml
Source: Mouse
Storage: Long term ≤ -20 °C, short term at 4°C (for one week). Aliquot the matrix aseptically in pre-chilled tubes and store. Avoid freeze-thaw cycles as it leads to the loss of performance.
Note: Use as supplied. This is viscous solution. Thawing should be done on **ice or at 4°C refrigerator for overnight.** The matrix tends to form **gel at temperature higher than 10°C**, therefore care should be taken while handling the solution. The matrix once formed gel cannot be reverted back to solution. The tips used to handle the matrix should be **pre-chilled.**

Part B: Inhibitor Control CCK081(B) 10 µl
Storage: -20°C
Note: Ready to use, 5 mM stock solution. The 5 mM solution should be aliquoted and stored at -20°C, protected from light. The freeze-thaw cycle should be avoided. We recommend using CCK081(B) at a final concentration of 10-40 µM, however cell type specific concentration should be optimized.

Part C: Staining Dye Concentrate CCK081(C) 2 x 25µl
Storage: -20°C
Note: Aliquot dye to small quantity to avoid freeze-thaw cycle. Dilute in Dulbecco's Phosphate Buffered Saline (TL1006) just before use.

Part D: Wash Buffer CCK081(D) 1 x 10ml
Storage: 2-8°C upon receipt
Note: Ready to use as supplied. Equilibrate to 37°C before use.

5. Materials required but not provided in the kit

1. Human umbilical vein endothelial cells (HUVEC) or other endothelial cell line capable of tube formation
2. Tube formation inducers (e.g. VEGF, FGF-2, etc.)
3. HiEndoXL™ Endothelial Cell Expansion Medium, Reduced Serum (AL530)
4. Cell Harvesting Buffer, EDTA, trypsin, or other cell detachment buffer
5. Sterile DPBS(TL1006) to wash cells
6. Distilled H_2O
7. Trypan blue or equivalent viability stain
8. Deep-Multiple well Plate 96 wells, 1.6 ml/well, sterile.

6. General guidelines

It is important to optimize various experimental factors prior to use the EZAssay™ Angiogenesis Cell Culture Kit, as decribed below.

- We recommend all control to be assayed in duplicate.
- Cell lines should be checked for their optimal density for the angiogenesis assay.
- The time for incubation is dependent on the cell type and number of cells. This should be optimized according to the experiment.
- The treatment time and concentration with angiogenic substance and inhibitor should be carefully maintained.
- Cells either freshly thawed or from early passage (<4) are generally recommended for the assay.

- **Assay controls**

Include appropriate assay controls i.e.

1. Extracellular matrix control (cell seeded without matrix).
2. Inhibitor control (cells treated with angiogenesis inhibitor CCK081(B).

- **Measurement of fluorescence**

Fluorescence measurement is to be done using 488nm filter

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1. Preparation of cells

Always use freshly harvested or freshly thawed cells for assay. Seed the cells in a cell culture flask or dish which is appropriate for the assay and incubate at 37ºC in 5% CO_2 incubator. Allow the cells to grow till the desired confluence is achieved. Harvest the cells and use for assay. The kit has been optimized for HUVEC cells.

1. Revive HUVEC cells in a T25 flask using HiEndoXL™ Endothelial Cell Expansion Medium, Reduced Serum (AL530) or medium of choice without growth factor.
2. Change the culture medium 6 hrs after thawing of the cells and then at every 24 hrs.
3. After HUVEC cells reached ~80% confluency, seed them in 96 well plate for the angiogenesis experiment.

***(Note:** The quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in the assay.)*

7.2. Pre-assay optimization procedure

- Pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time.
- The concentration of angiogenic substrates for treatment is to be optimized as per the study.

7.3. Assay procedure

7.3.1.Endothelial Tube Formation Assay (*In Vitro*):

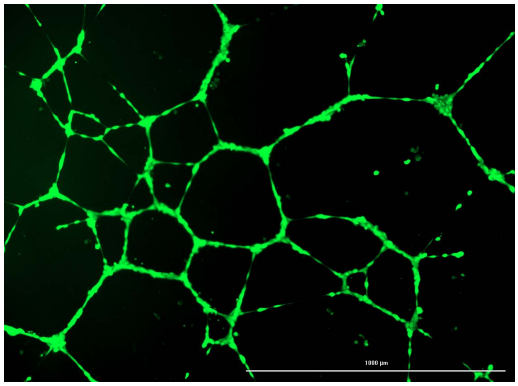
1. Add 50 µl of thawed cold Extracellular Matrix Solution CCK081(A) in each well of a pre-chilled 96 well plate. Tap the plate gently to ensure evel spreading of the gel on the surface of the well. Incubate 30 min at 37 ºC in 5% CO_2 incubator for formation of gel. In background control well, add no extracellular matrix solution.
2. Seed cells in the 96 well plates for mentioned conditions: a) test (treated with angiogenic substrates), b) negative control (cells treated with inhibitor control CCK081(B)), and c) background control (no extracellular matrix solution).We recommend 10^4 cells /well (100µl medium) for HUVEC.
3. Incubate the cells in 37°C incubator containing 5% CO_2 for 4-18 hours (according to the study protocol).

7.3.2.Tube staining and imaging:

1. Aspirate the media carefully out from the well without disturbing the cells or the Extracellular Matrix gel.
2. Wash the well gently using 100 µL of Wash Buffer CCK081(D).
3. Dilute 0.5 µL of staining dye concentrate CCK081(C) in 100 µL DPBS and add to the cells. Incubate for 30 minutes at 37°C incubator. Image the formation of endothelial tube using phase contrast and fluorescence microscopy (488nm green filter).

8. Typical Result

a)



b)

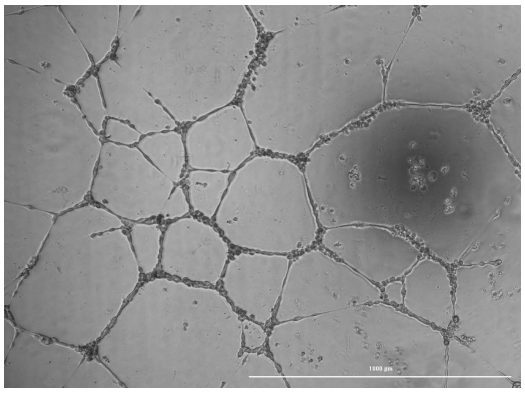


Figure Legend: HUVECs were seeded on 96 well polystyrene plate coated with extracellular matrix solution at 10,000 viable cells using HiEndoXL™ Endothelial Cell Expansion Medium, Reduced Serum, and incubated at 37°C and 5% CO2 in presense of 40ng VEGF. After 16 hours post-seeding, the cells were stained and incubated for 30 minutes at 37°C, 5% CO2, and then imaged at 10X magnification (Panel a). Panel b) is the phase contrast image of the field shown.

9. Storage and shelf life

- The components are recommended to store at -20°C except the CCK081(D) upon receipt. CCK081(A) can be stored at 2-8°C for one week. For longer storage -20°C is recommended.
- Repeated freezing and thawing may result in loss of reagent activity. Aliquot the components avoid to freeze-thaw.
- Use before the expiry date given on the label.

10. Advantages

- **Easy to use:** Ready-made kit for use.
- **Time saving**: Fluorescence can be measured without involving solubilization with organic solvent.
- **Reproducibility**: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results.
- **Safety**: No radioisotopes and organic solvent are required.
- **Fast:** Use of 96 well plate allows processing of large number of samples.

11. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

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).
		Use freshly thawed cells or cells having passage number less than 4 after thawing.
	Conditions are not suitable for the cell type(s) used in the study.	The concentration of cells per well, incubation times, cell culture medium, concentration of angiogenic factors (or growth factors) should be optimized for each cell type(s) used.
No or faint staining with dye concentrate	Dyes are quenched	Use fresh aliquot. Do not open the vial in the light. Keep the 96 well plate covered with aluminium foil after adding the dye.

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