Angiogenesis Co-Culture Assay Kit Protocol (HUVEC/HDF)

Product Code: ZHA-4000-24

The cells in this kit require immediate attention

For research use only – not for diagnostic or therapeutic use

Please read the entire protocol prior to use and take care to follow the instructions carefully

Kit Contents

Room temperature box contents:

- Angiogenesis Co-Culture Assay Kit Protocol (HUVEC/HDF)
- Certificate of Conformity
- KC1001 24 well tissue culture plate
- KC1003 Angiogenesis Basal Medium (125ml)

Dry ice box contents:

- KC1011 Angiogenesis co-culture cells (HUVEC/HDF)
- KC1036 Growth supplement (2.5ml)
- KC1016 Antibiotic supplement (125µl)
- KC1006 VEGF 2ng/ml (20µl)
- KC1007 Suramin 1mM (220µl)
- KC1004 Mouse anti-human CD31 primary antibody (40µl)
- KC1005 Goat anti-mouse IgG1-alkaline phosphatase conjugate secondary antibody (30µl)
- KC1010 BCIP/NBT tablets (x2)

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Contents

1. Introduction

Angiogenesis is the multistep process whereby new blood vessels develop from pre-existing vasculature. Angiogenesis plays a key role in numerous physiological and pathological processes including wound healing and the development of collateral circulation following an ischaemic episode[1], reproduction-associated neovascularisation[2], growth of solid tumours[3] and diabetic retinopathy[4]. Understanding the mechanism of angiogenesis will therefore provide new approaches to the treatment of a wide range of pathologies.

Angiogenesis is a complex process in which the following events are believed to play a critical role:

- Proteolytic degradation of the extracellular matrix[5]
- Directed migration of endothelial cells[6,7]
- Proliferation of endothelial cells[8]
- Deposition of new extracellular matrix[5]
- Formation of tubules and anastomosis of the newly formed vessels[5,7]

Experimental approaches to the study of these events have been limited by the lack of suitable models of angiogenesis. Several *in vivo* systems have been developed including the chick chorioallantoic membrane (CAM) assay[9] and the rabbit cornea model[10] but these systems are impractical for the study or screening of large numbers of samples and are far removed from angiogenesis in a human system. The *in vitro* methods currently in use have generally isolated the different component parts of the angiogenic process and have studied endothelial cell proliferation[11], endothelial cell migration[12] or the ability of endothelial cells to associate into tubules when in contact with various matrix proteins[13]. None of these assay systems accurately reflect the angiogenic process in its entirety.

In the Cellworks Angiogenesis Co-Culture Assay Kit, human endothelial cells are co-cultured with human fibroblasts in a specially formulated medium. The endothelial cells initially form small islands within the culture matrix. They subsequently begin to proliferate and then enter a migratory phase during which they move through the matrix to form threadlike tubule structures. These gradually join up to form a network of anastomosing tubules which closely resembles the capillary bed found in the CAM assay[14]. The tubules stain positively for PECAM 1 (CD31).

The angiogenesis co-culture is responsive to known micro- and macro-molecular inhibitors and stimulators of angiogenesis and so, unlike some other models, can be used to measure both positive and negative effects on angiogenesis. It yields reproducible dose response curves permitting comparison of different treatment regimens and reagent concentrations.

In addition to cryogenically-preserved co-culture cells, a 24 well tissue culture plate, growth medium and associated supplements, the kit also includes:

- Validated antibodies and staining reagents for tubule visualisation
- Validated positive control (VEGF)
- Validated negative control (Suramin)

The kit is designed so that test compounds, conditioned media or tissue explants can be added to the culture at any time during the angiogenic process. To determine the resulting effects on tubule formation, the kit contains reagents to detect and stain endothelial cell marker CD31 with permanent chromogenic BCIP/NBT substrate. Parameters of tubule networks can be measured from images of the plate using Cellworks Image Analysis Software, AngioSys 2.0. A demonstration version of this software is available free of charge (see section ‘Analysing Results’ for details).

Handling and Storage of Kit Components

Code	Component	Storage Temperature
KC1001	24 well tissue culture plate	Ambient
KC1003	Angiogenesis Basal Medium	2-8°C
KC1011	Angiogenesis co-culture cells (HUVEC/HDF)	-135-196°C (vapour/liquid nitrogen)
KC1036	Growth supplement	-20°C
KC1016	Antibiotic supplement	-20°C
KC1006	VEGF control	-20°C
KC1007	Suramin control	-20°C
KC1004	Mouse anti-human CD31 antibody	-20°C
KC1005	Goat anti-mouse IgG1-Alk. Phos. conjugate	-20°C
KC1010	BCIP/NBT tablets	-20°C

Ensure kit components are stored at the indicated temperatures immediately and are used prior to expiry dates elapsing

Contact techsupport@caltagmedsystems.co.uk for kit component MSDS

BIOHAZARD NOTE

This kit contains cells of human origin that test negative for HIV-1, hepatitis B, hepatitis C, mycoplasma, bacteria and fungi. No test procedure can guarantee the absence of known and unknown pathogenic agents. Human cells should be considered potentially biohazardous and appropriate precautions taken for their handling and disposal.

Implement good laboratory practice and use aseptic technique at all times.

2. Additional Materials

The following equipment and reagents are required in addition to the kit contents

For the assay:

- Class 2 laminar flow hood
- Incubator at 37°C with 5% CO_2 humidified atmosphere
- Aspirator and sterile aspirator pipettes
- Micropipettes and sterile pipette tips
- Electronic power pipette pump and sterile serological pipettes
- Sterile tubes
- Test compounds etc. for test conditions

For the staining:

- Ice-cold 70% ethanol
- Phosphate buffered saline (PBS)
- Bovine serum albumin (BSA)
- Deionised water
- Disposable syringe and 0.22µm filter disc
- Vortex

3. Guidelines for Test Conditions

Test Compounds

Test compounds should be added directly to angiogenesis growth medium whenever possible. If necessary, compounds may be dissolved or reconstituted in a solvent such as DMSO or ethanol. A concentrated stock solution can then be diluted in angiogenesis growth medium to the required working concentration. Final solvent concentrations should be kept constant and the experimental design should include control wells treated with solvent alone. Final solvent concentrations should not exceed 0.1% (v/v).

Tissue Explants

Ensure sterility of tissue explants has been maintained prior to addition to the assay. Small explants of approximately 2-3mm^2 can be placed in wells upon a medium change. The plate should not be disturbed until the next medium change. Extreme care must be taken to avoid dislodging explants or disturbing cell sheets.

Conditioned Media

Conditioned cell culture media can be diluted in angiogenesis growth medium and added directly to the plate. Optimal dilution ratios should be determined by the end-user but as a starting point it is recommended that conditioned media are diluted 1:1 in angiogenesis growth medium.

4. Protocol Summary

The following provides an outline of the angiogenesis co-culture assay kit protocol

We recommend three replicate wells per control and test condition

Day 1
- Angiogenesis growth medium preparation
- Angiogenesis co-culture cell seeding

Day 2
- Control and test compound dilutions (as required)
- Medium change

Days 3 to 10
- Medium change every 2 to 3 days

Day 11
- Tubule fixing and staining

Day 12 or later
- Tubule analysis

5. Angiogenesis Co-Culture Assay Protocol

The following provides details of the angiogenesis co-culture assay kit protocol including use of control and test compounds. This protocol can be adapted for an end-user's specific requirements, for example testing tissue explants and/or conditioned media.

Use good laboratory practice and aseptic technique at all times

Day 1 – assay set-up

- Thaw growth supplement and antibiotic supplement at 2-8°C (overnight) or at room temperature
- Equilibrate Angiogenesis Basal Medium to room temperature
- Add growth supplement and antibiotic supplement to Angiogenesis Basal Medium and mix well to formulate angiogenesis growth medium (AGM)
  Omission of antibiotic supplement may lead to excessive tubule formation
- Add 0.5ml AGM to each well of the 24 well plate and incubate at 37°C/5% CO_2 for 30 min to equilibrate
- Aliquot 12ml AGM into a sterile tube
- Rapidly thaw the co-culture cells by agitating the vial in a 37°C water bath
- Immediately add the cells to the AGM aliquot
- Mix gently using a serological pipette or by inverting the tube
- Add 0.5ml cell suspension to each well of the 24 well plate, ensuring the cell suspension remains evenly mixed during this process
- Ensure even dispersion of the cells within each well by holding the plate horizontally and rotating clockwise, anticlockwise and in a figure-of-eight several times
  This is important to reduce cell clumping and uneven distribution of tubules
- Place the plate on the bench for 20-30 min to allow the cells to adhere
  Avoid leaving the cells at room temperature for more than 30 min
- Place the plate in an incubator at 37°C/5% CO_2 humidified atmosphere

Day 2 – addition of compounds

- Equilibrate AGM to room temperature
- Thaw control compounds at room temperature
- Dilute control and test compounds as follows:
  - VEGF 1:1000 (e.g. add 11µl VEGF solution to 10.989ml AGM)
  - Suramin 1:50 (e.g. add 220µl suramin solution to 10.78ml AGM)
  - Aliquot 11ml AGM (or equivalent volume to control solutions) for untreated control conditions
  - Dilute any test compounds as required

  Prepare adequate volumes for all medium changes for the required number of wells for the duration of the assay (11ml is ample for four medium changes for four wells per condition)
- Change the medium for up to four wells at a time:
  - Aspirate the medium from each well, taking care to avoid contact with the cells
  - Gently add 0.5ml of the required equilibrated medium down the side of each well

  Avoid possible cell desiccation by changing the medium for no more than four wells at a time
  Do not mix test compounds directly in the wells as this will likely damage the cell culture
- Return the plate to the incubator at 37°C/5% CO_2 humidified atmosphere
- Store medium aliquots at 2-8°C when not in use

Days 3 to 10 – medium ***changes every 2 to 3 days***

- Equilibrate medium aliquots to room temperature
- Change the medium for up to four wells at a time as Day 2
- Return the plate to the incubator at 37°C/5% CO_2 humidified atmosphere

Day 10 or 11 – preparation

- At least one day prior to the assay end-point, prepare 70% ethanol and store at -20°C
- Prepare PBS (0.20g KCl, 0.20g KH_2PO_4, 8.00g NaCl, 1.15g Na_2HPO_4 in 1 litre deionised water) either fresh on the day or in advance (store at 2-8°C and equilibrate to room temperature prior to use)
- Prepare 1% BSA/PBS (1g BSA in 100ml PBS) fresh on the day of the assay end-point

Day 11 – fixing and staining

Do not allow the cell sheet to dry out – prepare each reagent during the previous incubation step or retain wash solution in the wells until the next reagent is ready to dispense down the side of each well

- Retrieve 70% ethanol from storage at -20°C
- Aspirate the medium from each well, taking care to avoid contact with the cell sheet
- Gently add 0.5ml PBS per well
- Aspirate the PBS from each well, taking care to avoid contact with the cell sheet
- Gently add 0.5ml ice-cold 70% ethanol per well
- Incubate at room temperature for 30 min
- Aspirate or decant the ethanol and wash each well by adding 0.5ml 1% BSA/PBS per well
- Aspirate or decant the 1% BSA/PBS and repeat the wash step twice for a total of three washes
- Dilute mouse anti-human CD31 primary antibody 1:400 in 1% BSA/PBS (e.g. 32.5µl antibody in 13ml 1% BSA/PBS) and mix thoroughly
- Aspirate or decant the third wash solution and add 0.5ml primary antibody per well
- Incubate at 37°C for 60 min
- Aspirate or decant the primary antibody and wash each well three times with 0.5ml 1% BSA/PBS
- Dilute goat anti-mouse IgG1-alkaline phosphatase conjugate 1:500 in 1% BSA/PBS (e.g. 26µl antibody in 13ml 1% BSA/PBS) and mix thoroughly
- Aspirate or decant the third wash solution and add 0.5ml secondary antibody per well
- Incubate at 37°C for 60 min
- Aspirate or decant the secondary antibody and wash each well three times with 0.5ml deionised water
- Add two BCIP/NBT tablets to 20ml deionised water in a tube, vortex thoroughly until dissolved and use a syringe to pass through a 0.22µm filter
  Take care to handle and dispose of BCIP/NBT tablets appropriately (see MSDS for details)
  Add BCIP/NBT solution to wells within 60 min of preparation (store in the dark prior to use)
- Aspirate or decant the third wash solution and add 0.5ml BCIP/NBT solution per well
- Incubate at room temperature until tubules develop adequate purple colour (usually 7 min ± 3 min)
  Staining should be continually monitored and should not exceed 20 min – the tubules will become slightly darker upon drying the plate
- Aspirate or decant the BCIP/NBT solution and wash each well three times with 0.5ml deionised water
- Aspirate or decant the third wash solution and invert the plate to air-dry overnight at room temperature

Day 12 – plate storage

- Store the plate in the dark at room temperature
  For best results, capture images as required as soon as possible after staining (colour intensity may fade over time)

6. Data Analysis

An appropriate method of analysis should be determined by the end-user. Below are some suggestions by which tubule formation can be quantified.

AngioSys 2.0
Cellworks Image Analysis Software, AngioSys 2.0 (Product Code ZHA-5000), can be used for semi-automated analysis of tubule networks. Measurable parameters include tubule number, tubule branching (number of junctions) and tubule length (mean and total tubule length per image).

Image files can be conveniently grouped and processed to provide quantitative and repeatable measurements. Repetitive image processing sequences can be specified, saved and applied to groups of images to enable rapid analysis. The resulting data is saved in a text format that can easily be read by third party software such as Microsoft Excel.

Further information including details of a free demonstration version can be viewed at:
https://www.cellworks.co.uk/angiogenesis_image_analysis_software.php

Chalkley Graticule
Tubules can be manually measured using a 25-point Chalkley point eyepiece graticule (see Fox, S.B., *et al*, 1995 J Pathol. **177** 275-283). Tubule density can be assessed in a chosen area, following low power scanning. This method does not require storing of images and analysis takes approximately three minutes per field assessed.

Map Reader
The total length of tubules can be determined accurately by tracing photographs using a map reading distance wheel. As above, multiple fields, covering the maximum area practically possible for each well, should be photographed to maximise precision of the measurements taken.

7. Troubleshooting

Issues	Causes/Solutions
Poor cell growth and/or little to no tubule formation	Inconsistent cell culture conditions – ensure the incubator is maintained at 37°C/5% CO_2 humidified atmosphere throughout the assay Incorrect storage or formulation of medium (AGM) – ensure medium components are stored at the appropriate temperatures, added as described and that AGM is stored at 2-8°C (do not freeze) Cell desiccation upon change of medium – replace medium in fewer wells at a time Toxic test compound – prepare stock solution of test compound at a lower concentration Toxic concentration of solvent in test compound conditions – prepare stock solutions of test compounds at a higher concentration and include solvent only control wells
Floating cells and/or cell debris	A small number of floating cells and/or a small amount of debris is typical of cell culture Failure of cells to adhere – inconsistent cell culture conditions or incorrect storage or formulation of medium (as above) Accumulation of floating cells and/or debris during the assay – inconsistent cell culture conditions, cell desiccation or toxic test conditions (as above)
Cell sheet detachment	Inadequate care taken when aspirating and/or dispensing – avoid contacting the well base when aspirating and be sure to gently dispense medium or reagent down the side of each well (cell sheet disruption is more likely with use of a single or multi-channel pipette than with use of a serological pipette) Uneven dispersion of cells upon seeding, leading to uneven cell sheet thickness – after adding cells to the wells, rotate the plate as described and incubate on the bench for 20-30 min before placing in the incubator
Contamination of one or more wells	Contamination arises from inadequate sterile technique and is more likely to occur if the antibiotic supplement is omitted from the medium Contain contamination by treating affected wells with 1M NaOH for 2-3 hours then aspirating and leaving empty for the remainder of the assay
Faint staining of tubules	Over-dilution, decreased incubation time and/or incorrect incubation temperature of primary and/or secondary antibody – ensure antibodies are prepared and added as described Over-dilution and/or premature preparation of BCIP/NBT tablets – ensure BCIP/NBT tablets are dissolved and filtered as described and used within one hour Inadequate incubation time with BCIP/NBT solution – closely monitor cultures during BCIP/NBT solution incubation until tubules are clearly identifiable
Intense staining of tubules and/or staining of non-tubule areas	Under-dilution, increased incubation time and/or incorrect incubation temperature of primary and/or secondary antibody – ensure antibodies are prepared and added as described Under-dilution and/or no filtering of BCIP/NBT tablets – ensure BCIP/NBT tablets are dissolved and filtered as described Excessive incubation time and/or increased incubation temperature of BCIP/NBT solution – closely monitor cultures at ambient temperature and decant BCIP/NBT solution as soon as tubules are clearly identifiable (staining will darken slightly upon plate drying) Cell death or accumulation of debris (as described above) can cause ‘stain trapping’

Appearance of HUVEC 'islands'	Uneven dispersion of cells upon seeding, leading to HUVEC clumps – after adding cells, rotate the plate as described and incubate on the bench for 20-30 min before placing in the incubator

8. Related Cellworks Products

Product type	Product code	Name	Description
Cellworks Cells	ZHC-2301	Early Passage Human Umbilical Vein Endothelial Cells (HUVEC)	Passage 2, pooled donor, cryopreserved, $0.5x10^6$ cells/ml/vial
	ZHC-2102	HUVEC (angiogenesis co-culture validated)	Passage 2, pooled donor, cryopreserved, $0.5x10^6$ cells/ml/vial
	ZHC-2402	GFP-HUVEC (angiogenesis co-culture validated)	Stably expressing TagGFP2, passage 2, pooled donor, cryopreserved, $0.5x10^6$ cells/ml/vial
	ZHC-5102	Human Dermal Fibroblasts (HDF) (angiogenesis co-culture validated)	Passage 8, single adult donor, cryopreserved, $1x10^6$ cells/ml/vial
	ZHC-3311	Early Passage Human Coronary Artery Smooth Muscle Cells (HCASMC)	Passage 4, single adult donor, cryopreserved, $0.5x10^6$ cells/ml/vial
AngioCo Angiogenesis Co-Culture Assay Kits	ZHA-4000-24	Angiogenesis Co-Culture Assay (HUVEC/HDF) 24wp	Angiogenesis assay kit for immunohistochemical staining of tubules (24 well plate format)
	ZHA-4100-24	Angiogenesis Co-Culture Assay (GFP-HUVEC/HDF) 24wp	Angiogenesis assay kit for green fluorescent detection of tubules (TagGFP2-expressing HUVEC) (24 well plate format)
	ZHA-4100-96	Angiogenesis Co-Culture Assay (GFP-HUVEC/HDF) 96wp	Angiogenesis assay kit for green fluorescent detection of tubules (TagGFP2-expressing HUVEC) (96 well plate format)
AngioCo Angiogenesis Co-Culture Assay Reagents	ZHA-1300	Angiogenesis Control Reagent Kit	VEGF positive control and Suramin negative control
	ZHA-1225	CD31 Tubule Staining Kit	Mouse anti-human CD31 primary antibody, goat anti-mouse IgG1-Alk. Phos. secondary antibody and BCIP/NBT tablets
	ZHA-1970	Angiogenesis Growth Medium Package	Angiogenesis basal medium, growth supplement and antibiotic/antimycotic supplement
Software	ZHA-5000	AngioSys 2.0	Cellworks Image Analysis Software (full licence)
	ZHA-5000D	AngioSys 2.0 (demonstration version)	Cellworks Image Analysis Software (free 21-day demonstration licence)

9. References

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10. Technical Support

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