



Human angiogenesis model for analysing the pro-angiogenic or anti-angiogenic properties of test compounds

**NOTE:**

**The kit must be processed immediately on receipt as detailed in this protocol**

**Take care to follow protocol instructions carefully**

## Kit Contents

- 24 well pre-seeded tissue culture plate containing early stage co-cultures
- Extra sterile plate (use lid only)
- 5 x 25ml single use bottles of Optimised Growth Medium (store at 2-8°C) (one extra bottle to requirements is included for convenience)
- Additional reagents can be purchased separately: angiogenesis control reagent kit, staining kits and extra medium. See section 11 of this protocol.

**For research use only  
Not for diagnostic or therapeutic use**

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**Please read the entire protocol before opening the kit or proceeding with an experiment. Do this straight away.**

## 1. Introduction

Angiogenesis is the multistep process whereby new blood vessels develop from pre-existing vasculature. This derives from the key role of angiogenesis 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 would 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 patented TCS Cellworks **AngioKit** model, human endothelial cells are co-cultured with other human cells in a specially designed 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 (by 8-11 days) to form a network of anastomosing tubules which closely resembles the capillary bed found in the CAM assay (14-24). The tubules stain positive for von Willebrand's Factor, PECAM 1 and ICAM-2. They are also shown to secrete a matrix containing type IV collagen.

The **AngioKit** assay is responsive to known small molecule and macromolecular inhibitors and stimulators of angiogenesis and so, unlike some other models, measures both positive and negative effects on angiogenesis. It yields reproducible dose response curves permitting comparison of different treatment regimes and product concentrations. The basic TCS **AngioKit** is complemented by fully validated antibodies for tubule visualisation and by positive and negative angiogenesis control preparations.

## 2. AngioKit Format

The **AngioKit** is supplied as growing cultures at the earliest stages of tubule formation in a 24 well plate format. It is designed so that test compounds, conditioned media or tissue explants can be added to the cultures within individual wells. The resulting effect on tubule formation can then be measured.

Control wells that receive no treatment other than medium changes, form extensive networks of branching tubules over a period of 8-11 days. This allows both angiogenic inhibitors and angiogenic stimulators to be assessed. The necessary reagents for media changes are included in the **AngioKit**. Examples of images showing angiogenic cultures in various stages of development are included in Appendix II.

Positive and negative control compounds are available in the TCS Angiogenesis Control Reagent Kit (product code ZHA-1300). This kit contains **AngioKit**-validated concentrated stock solutions of VEGF (2µg/ml positive controls) and Suramin (1mM negative controls) which require diluting in the medium supplied in the kit before use.

A range of validated **AngioKit** staining kits and reagents are also available (see section 11).

To run a complete **AngioKit** assay requires only a basic **AngioKit**, control reagents (ZHA-1300) and one of the available staining kits (see section 11).

**The kit must be processed immediately on receipt as detailed in this protocol.**

### 3. Additional Equipment and Reagents Required

Angiogenesis Control Reagent Kit (product code ZHA-1300)  
Class 2 laminar flow hood  
Test materials to assay  
Sterile serological pipettes  
Automated pipette pump set to slow speed setting  
70% Ethanol  
Sterile blunt ended forceps  
Sterile hypodermic needle  
Sterile containers for making up test compound dilutions  
Sterile aspirating pipettes  
Incubator set to 37°C with 5% CO<sub>2</sub> humidified atmosphere  
Micropipettes and pipette tips  
Relevant personal protective equipment

#### *Fixing and Staining reagents:*

Staining kit (see section 11)  
Disposable syringe with 0.2µm filter disc  
Dulbecco's Phosphate-Buffered Saline (PBS)  
Bovine Serum Albumin (BSA)  
3M NaOH  
A 96 well microtitre (only required if performing the '**AngioKit** ELISA' staining procedure)  
96 well plate reader set to measure absorbance at 405nm (only required if performing the '**AngioKit** ELISA' staining procedure)

#### **BIOHAZARD NOTE**

**The ZHA-1000 AngioKit contains cells of human origin. Although the cells test negative for HIV-1, Hepatitis B, Hepatitis C, mycoplasma, bacteria and fungi, no test procedure can guarantee the absence of known and unknown infectious agents. Consequently, all products of human origin should always be considered potentially biohazardous and appropriate precautions should be taken. Use good laboratory practice and aseptic technique at all times.**

See for example Grizzle, W.E. and Potts, S.S. (1988) Guidelines to avoid personnel contamination by infectious agents in research laboratories, *J. Tissue Culture Methods* **11**; 4.

## 4. AngioKit Protocol Summary

### 4.1. Day 1 (Tuesday)

Unpack the kit and pre-equilibrate one bottle of Optimised Growth Media  
Remove silicone rubber seals from the plate following specific instructions detailed in the protocol  
Cover plate with new lid and examine cultures microscopically  
Place culture plate in incubator and prepare required medium and treatments  
Change medium  
Incubate plate

### 4.2. Day 4 (Friday)

Prepare required medium and treatments (if necessary) and pre-equilibrate  
Examine cultures microscopically  
Change medium  
Incubate plate

### 4.3. Day 7 (Monday)

Repeat **Day 4** procedure

### 4.4. Day 9 (Wednesday)

Repeat **Day 4** procedure

### 4.5. Day 11 (Friday)

Examine cultures microscopically  
Fix cells  
Stain tubules  
Score results either manually, colourimetrically or using the TCS Cellworks AngioSys Image Analysis software.

## 5. Guidelines for Test Material Preparation

### 5.1. Test compounds

Dissolve the compound to be tested directly in the Optimised Growth Medium supplied whenever possible.

If necessary, compounds may be dissolved in other solvents such as DMSO or ethanol. In this case a concentrated stock solution should be prepared and diluted with medium to give the required concentration of test compound. Final solvent concentration should be kept constant and the experimental design should include control wells treated with solvent alone.

Final concentration of solvent should not exceed 0.1% (v/v) for DMSO and 0.1% (v/v) for ethanol. **N.B.** Ethanol has an angiogenic effect at 1-5% (v/v) (Jones *et al* 1998, *Biochem Biophys Res Commun* **249:1** 118-123).

### 5.2. Explants

Ensure sterility of tissue explants has been maintained prior to their addition to cultures.

Small explants of approximately 2-3 mm<sup>2</sup> should be placed in a well at the Day 1 medium change. The plate should then not be disturbed until the Day 4 medium change.

Medium aspiration and replacement must be performed with care to ensure that the explant is not dislodged or the cell sheet damaged. Exact protocols must be determined by each researcher as required.

### 5.3. Conditioned media

Conditioned medium from other cell cultures may be diluted in the fresh Optimised Growth Medium supplied with this kit and added directly to the angiogenesis plate. It is recommended that conditioned medium should be diluted at least 1:1 in fresh medium. This guideline, however, must be verified by experiment for each conditioned medium tested.

## 6. General Tips

Ensure that the media contained in this kit is stored at 2-8°C.

Prepare your tissue culture laminar flow hood and all necessary equipment prior to opening the kit.

Pre-equilibrate medium before adding to cultures. Loosen bottle cap a quarter turn and place in an incubator at 37°C, 5% CO<sub>2</sub> for 30 minutes. Equilibration with respect to CO<sub>2</sub> is as important as temperature equilibration.

Have required dilutions of test compound(s) in medium prepared ready for use before starting medium changes.

## 7. AngioKit Protocol

**Use rigorous aseptic technique at all times. Only open the culture plate and medium bottles in a sterile laminar flow hood.**

### 7.1. Day 1

- 7.1.1. Place 4 bottles of Optimised Growth Medium from the kit at 2-8°C.
- 7.1.2. Pre-equilibrate one bottle of Optimised Growth Medium for 30 minutes. Loosen the cap a quarter turn and place in an incubator at 37°C with 5% CO<sub>2</sub> humidified atmosphere.
- 7.1.3. Remove the sealed culture plate from its packaging and wipe the outside with ethanol or isopropanol prior to placing in a flow hood. Remove the lid of the culture plate and discard (use the lid from the extra sterile plate from now on).
- 7.1.4. To remove the silicone well seals:
  - To remove the vacuum created, insert a sterile hypodermic needle through the silicone above the well and gently ease up the edge of the seals using sterile blunt forceps.
  - Repeat the procedure on all wells until the seals have been completely loosened. Carefully remove the silicone seals. Be careful to avoid causing aerosols and make sure that no droplets are left around the well walls.
- 7.1.5. Open the packet containing a new plate in the flow hood. Use the new lid to cover the plate.
- 7.1.6. Examine cultures microscopically for cell morphology and signs of growth. Small islands of endothelial cells should be visible within a matrix (see Appendix II, Figure 1). If the cell morphology differs markedly from Figure 1, contact TCS Cellworks Ltd., **immediately** (see section 13).
- 7.1.7. Incubate the plate at 37°C with 5% CO<sub>2</sub> in a humidified environment. Meanwhile, prepare dilutions of test compounds (or control compounds) in the pre-equilibrated medium. **Do not attempt to mix test compounds directly in the culture plate as this will damage the cell monolayer.** See section 5 for further details on sample preparation.
- 7.1.8. Carefully aspirate medium from the wells **without** touching the cell layer. Make sure that no droplets are left around the well walls. **To avoid possible desiccation of cells, it is recommended that medium is removed from only a few wells at a time (maximum 4) and fresh medium is added before going on to the next wells.**
- 7.1.9. Using a serological pipette, gently, down the side of the well, add 0.5ml of pre-equilibrated Optimised Growth Medium containing test or control materials as required per well (see example plate layout in section 7.4.). **Do not use micropipettes (e.g. Gilson pipettes) as they may cause damage to the culture. Do not attempt to mix test compounds directly in the culture plate as this will also damage the cell monolayer.**
- 7.1.10. Incubate the plate at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere.

## 7.2. Day 4, 7 and 9

- 7.2.1. Pre-equilibrate one bottle of Optimised Growth Medium and dilutions of test or control compounds, according to section 7.1.2.
- 7.2.2. Carefully examine cultures for cell morphology and signs of growth (see Appendix II, Figure 2).
- 7.2.3. Aspirate medium from wells and replace with pre-equilibrated medium containing test or control materials as required, according to the Day 1 procedure (section 7.1.8. – 7.1.9.).
- 7.2.4. Incubate the plate at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere.

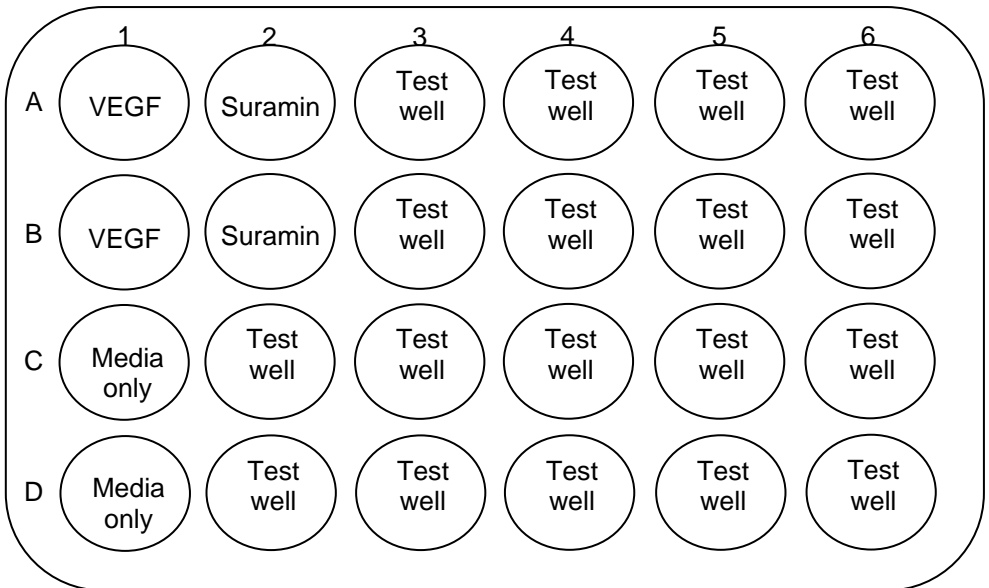
## 7.3. Day 11

- 7.3.1. After examining the cultures to monitor tubule development, fix them prior to visualisation. See section 8 for a detailed protocol.

The formation of tubules can be monitored by light microscopy. Tubule lumens are visible if phase contrast microscopy is utilised. However the extent of tubule formation can be difficult to judge in unstained preparations and requires a reasonable level of experience with **AngioKits**.

#### 7.4. Example Plate Layout

Example plate layout containing potential control wells and test wells.



VEGF: Positive control (from TCS Angiogenesis Control Reagent Kit ZHA-1300)

Media only: Untreated control well.

Suramin: Negative control (from TCS Angiogenesis Control Reagent Kit ZHA-1300)

## 8. Fixing and Staining AngioKit Plates

***Wear gloves throughout the fixing and staining procedures.***

Visualisation of tubules is more difficult in cultures if they are not stained. To facilitate analysis it is best to fix the cells at the end of the experiment and stain for one or more endothelial markers. This is essential if image analysis software is to be used for tubule quantification, for example TCS Cellworks AngioSys. A fully functional 30 day use demonstration version of this software is available free of charge (conditions apply, please contact TCS Cellworks).

Recommended markers are CD31 (PECAM-1) or von Willebrand Factor.

Several kits of validated **AngioKit** staining reagents are available from TCS to suit your specific analytical needs:

- **AngioKit** ELISA (product code ZHA-1100)  
This staining kit uses the ELISA principle to give a rapid overview of tubule development without the need for image analysis. The kit contains our standard anti-CD31 primary antibody and anti-IgG alkaline phosphatase conjugated secondary antibody together with a soluble substrate which permits tubule quantification by simple optical density measurement. (N.B. Plates may subsequently be permanently stained for detailed analysis of tubule morphology and plate archiving as described below).
- Tubule Staining Kit for image analysis (product code ZHA-1225)  
Contains CD31 (PECAM-1) primary antibody, relevant secondary antibody and insoluble substrate to allow tubule visualisation for image analysis and/or plate archiving.
- Combined **AngioKit** ELISA / CD31 tubule staining kit (product code ZHA-1125)  
Contains all reagents to complete the ELISA assay followed by standard CD31 staining for image analysis and/or archiving plates.
- Tubule Staining Kit for image analysis (product code ZHA-1250)  
Contains von Willebrand Factor primary antibody, relevant secondary antibody and substrate to allow tubule visualisation. (Note that ZHA-1225 is normally recommended for image analysis as it gives less background colouration).
- Combined Tubule Staining Kit for image analysis (product code ZHA-1200)  
Contains both CD31 (PECAM-1) and von Willebrand primary antibodies, relevant secondary antibodies and substrates to allow tubule visualisation.

***Do not use micropipettes for any additions to culture plate wells as this will cause damage to the cells.***

## **8.1. Culture Fixation Protocol (identical for all staining kits)**

Prepare the wash buffer, blocking buffer and fixative below.

### **8.1.1. Wash Buffer (Dulbecco's Phosphate-Buffered Saline)**

Per litre:	KCl	0.20g
	$\text{KH}_2\text{PO}_4$	0.20g
	NaCl	8.00g
	$\text{Na}_2\text{HPO}_4$	1.15g

Store at 4°C. Pre-equilibrate to room temperature before use.

### **8.1.2. Blocking Buffer**

1 x wash buffer (section 8.1.1.) supplemented with 1% BSA

Store at 4°C. Pre-equilibrate to room temperature before use.

### **8.1.3. Fixative**

70% ethanol

Store at -20°C. Use immediately from freezer.

### **8.1.4. Fixation Protocol**

8.1.4.1. Aspirate medium from cells very carefully.

8.1.4.2. Wash each well with 0.5ml Washing Buffer. Take care not to disturb the cell sheet in the well when removing the buffer.

8.1.4.3. Add 0.5ml of ice cold fixative carefully to each well.

8.1.4.4. Incubate at room temperature for 30 minutes.

8.1.4.5. Decant the fixative and wash wells 3 times with 0.5ml Blocking Buffer.

8.1.4.6. Decant final wash and add primary staining antibody as detailed below.

***Do not allow the cell sheet to dry out until staining has been completed.***

## 8.2. Staining for CD31 (PECAM-1)

***Only proceed with staining after cultures have been fixed as described in section 8.1. Centrifuge vials of antibody before opening.***

All the CD31 staining kits available from TCS Cellworks use identical primary antibody treatments as detailed below. Different chromogenic substrates are used however, for the ELISA assay (soluble p-NPP) or for tubule staining *in situ* (insoluble BCIP/NBT).

### 8.2.1. Anti-CD31 Primary and Anti-IgG Alkaline Phosphatase Secondary Antibody Treatments

- 8.2.1.1. Dilute primary antibody (mouse anti-human CD31) 1:400 in Blocking Buffer (35µl of antibody in 14ml of Blocking Buffer) and mix well.
- 8.2.1.2. Add 0.5ml diluted primary antibody per well and incubate for 60 minutes at 37°C.
- 8.2.1.3. Meanwhile, prepare the secondary antibody (goat anti-mouse IgG AP conjugate) immediately before washing off the primary antibody. Dilute the secondary antibody 1:500 in Blocking Buffer (28µl in 14ml of Blocking Buffer), mix well.
- 8.2.1.4. Remove the primary antibody solution and wash the wells 3 times with 0.5ml Blocking Buffer, incubate each wash at room temperature for 5 minutes. Decant the final wash prior to adding the secondary antibody.
- 8.2.1.5. Add 0.5ml diluted secondary antibody conjugate per well and incubate for 60 minutes at 37°C.
- 8.2.1.6. Wash wells three times with 0.5ml dH<sub>2</sub>O, following the procedure for washing off the primary antibody.

***To perform the optional ELISA stain, proceed to section 8.2.2 otherwise proceed immediately to section 8.2.3 to permanently stain tubules for image analysis and plate archiving. Do not allow the cell sheet to dry until the staining has been completed.***

### 8.2.2. CD31 ELISA Soluble Substrate (p-NPP) - optional

- 8.2.2.1. Prepare the soluble substrate by dissolving one p-nitrophenol phosphate (p-NPP) tablet and one Tris buffer tablet (gold) in 20ml dH<sub>2</sub>O. Use within 1 hour.
- 8.2.2.2. Prepare a 96 well plate by adding 25µl of 3M NaOH to 50 wells. Add 100µl of p-NPP substrate directly to 2 wells to act as blanks.
- 8.2.2.3. Add 0.3ml p-NPP to each well of the **AngioKit 24 well plate** and incubate at 37°C for exactly 20 minutes.
- 8.2.2.4. Remove two 100µl aliquots from the first well of the **AngioKit** and add each to separate wells of the 96 well plate already containing 3M NaOH.
- 8.2.2.5. Repeat for each AngioKit well. You will have 48 test wells plus 2 p-NPP blank control wells on the 96 well plate.

- 8.2.2.6. Read the 96 well plate on a plate reader at 405nm against the blank wells prepared as indicated above.
- 8.2.2.7. Wash the **AngioKit** wells carefully 3 times with 0.5ml dH<sub>2</sub>O. Discard final wash and continue to tubule staining procedure if required (ZHA-1225).

***Proceed immediately with the insoluble staining procedure below to enable automated scoring and provide a permanent record of tubule development.***

### **8.2.3. CD31 Tubule Staining Insoluble Substrate (BCIP/NBT)**

- 8.2.3.1. Prepare the insoluble substrate immediately prior to addition to the **AngioKit**. Dissolve two BCIP/NBT tablets in 20ml dH<sub>2</sub>O. Filter the substrate using a disposable syringe and 0.2µm filter disc. Use within 1 hour.
- 8.2.3.2. Add 0.5ml substrate per **AngioKit** well. Incubate at room temperature until tubules develop a dark purple colour (usually 3 – 10 minutes). Staining should be continually monitored and should not exceed 20 minutes.
- 8.2.3.3. Wash wells carefully 3 times with 0.5ml dH<sub>2</sub>O. Discard final wash and leave to air dry.

Plates can be stored in the dark but colour intensity will fade with time. For best results, plates should be photographed or an image recorded as soon as possible after staining.

### **8.3. Staining for von Willebrand Factor**

***Only proceed with staining after cultures have been fixed as described in section 8.1. Centrifuge vials of antibody before opening.***

- 8.3.1. Dilute primary antibody (sheep anti-human von Willebrand Factor) 1:200 in Blocking Buffer (70µl or antibody in 14ml of Blocking Buffer) and mix well.
- 8.3.2. Add 0.5ml diluted primary antibody per well and incubate for 60 minutes at 37°C.
- 8.3.3. Meanwhile prepare the secondary antibody (donkey anti-sheep IgG Horseradish Peroxidase conjugate) immediately prior to washing off the primary antibody. Dilute the secondary antibody 1:400 in Blocking Buffer (35µl of antibody in 14ml of Blocking Buffer) and mix well.
- 8.3.4. Decant the primary antibody solution and wash the wells three times with 0.5ml Blocking Buffer, incubate each wash at room temperature for 5 minutes. Decant the final wash prior to adding the secondary antibody.
- 8.3.5. Add 0.5ml diluted secondary antibody conjugate per well and incubate for 60 minutes at 37°C.
- 8.3.6. Wash wells 3 times with 0.5ml dH<sub>2</sub>O, incubate each wash at room temperature for 5 minutes. Decant the final wash.

8.3.7. Prepare DAB substrate during the plate washing. Crush one tablet of DAB and one tablet of Urea buffer. Add 15ml of dH<sub>2</sub>O and mix well.

8.3.8. Add 0.5ml substrate per well. Incubate at 37°C until tubules develop a dark brown colour (usually within a few minutes and no more than 30 minutes). Staining should be continually monitored and development stopped when appropriate level of staining has been achieved.

See staining kit product data sheet for DAB disposal recommendations.

8.3.9. Wash wells carefully three times with 0.5ml dH<sub>2</sub>O, incubate each wash at room temperature for 5 minutes. Discard final wash and leave to air dry.

Plates can be stored in the dark but colour intensity will fade with time. For best results, plates should be photographed or an image recorded as soon as possible after staining.

## 9. Scoring Results

### 9.1. Optical Density Measurement (ELISA)

*For scoring plates stained per sections 8.2.1. and 8.2.2.*

This provides a rapid overview of the extent of tubule development which may be sufficient for comparative evaluation of different angiogenic effector concentrations. However it cannot provide the detailed information on tubule morphology etc that is available when the tubules are stained using histochemical techniques.

### 9.2. Tubule Quantitation and Analysis Following Histochemical Staining

*For scoring plates stained per sections 8.2.1. and 8.2.3.*

Tubule formation can be scored either manually or with the aid of **TCS Cellworks AngioSys Image Analysis Software**. The full extent of tubule development can be difficult to assess in unstained cultures. It is essential that cultures are fixed and stained prior to definitive scoring.

#### 9.2.1. Manual Scoring

##### 9.2.1.1. By eye:

It is possible to estimate the extent of tubule formation by eye, for example using a plus scale to designate tubule density. It is recommended that the maximum area practically possible should be examined in each well (this will probably depend on the optical set up in use). Scoring by eye in this way can only provide semi-quantitative, comparative results.

##### 9.2.1.2. Chalkley graticule:

Another means of manually measuring the tubules uses a 25-point Chalkley point eyepiece graticule (see Fox, S.B., *et al*, 1995 J Pathol. **177** 275-283). Microvessel density is assessed in a chosen area, following low power scanning. This method does not require storing of images and analysis takes approximately 3 minutes per field assessed.

##### 9.2.1.3. By 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.

### 9.2.2. Automated Scoring

A dedicated software package, TCS Cellworks **AngioSys Image Analysis Software** (product code ZHA-1800) has been developed for quantitative measurement of tubule development in the **AngioKit** and is available to purchase. This permits analysis of total tubule number, total tubule length, mean tubule length, number of junctions, or other parameters required by the investigator. A 30 day use demonstration version can be supplied free of charge to score the Angiogenesis assay\*.

\* Conditions apply; please contact TCS Cellworks for further details.

Other image analysis software may be adapted to score the Angiogenesis assay. In this case, it is advised that CD31 (PECAM-1) with BCIP/NBT substrate (ZHA-1225 kit) is used for staining as it gives better contrast.

## 10. Trouble Shooting

Symptom	Possible Reasons/Solutions
No tubule formation	<ul style="list-style-type: none"> <li>i. High concentrations of angiogenic inhibitors have prevented tubule formation. Check control wells.</li> <li>ii. Solvent used to dissolve test compounds may be at a toxic concentration. Run one or more solvent control wells.</li> <li>iii. Cultures have died. May be due to a number of reasons:               <ul style="list-style-type: none"> <li>a. Cells were allowed to become dry between medium changes. Aspirate old medium from only a few wells at a time and replace with fresh optimised medium before aspirating the next wells.</li> <li>b. Final concentration of solvent in which treatment is dissolved, is too high. Make a more concentrated stock solution of test compound and dilute this further in medium.</li> <li>c. Ensure all incubations are at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.</li> </ul> </li> </ul>
Cell sheet detaching from the well surface	<ul style="list-style-type: none"> <li>i. Cultures have been left to grow for too long. Try to fix and stain cultures before this occurs. Contact TCS Cellworks if this occurs before Day 11. If the sheet is still partially attached to the well, attempt to gently fix and stain the tubules.</li> </ul>
Cells floating in well	<ul style="list-style-type: none"> <li>i. This is the result of adding fresh medium too fast. Always add fresh medium very gently to prevent dislodging cells. Never use micropipettes for adding medium.</li> <li>ii. Cultures have died. See above for possible reasons.</li> </ul>
Faint Staining of tubules	<ul style="list-style-type: none"> <li>i. Antibodies not added at the correct concentration. See section 8.</li> <li>ii. Antibody incubation was too short or was performed at the incorrect temperature. See section 8.</li> <li>iii. Cultures left to grow for too long. The matrix is preventing adequate penetration of the primary antibody.</li> </ul>
A small number of wells are contaminated	<ul style="list-style-type: none"> <li>i. Act <b>promptly</b> to contain the contamination. Use your preferred method to achieve this. One possible method to use is given in Appendix I.</li> </ul>

## 11. AngioKit Related Products

<b>New V2a Kit</b> (Vasculogenesis to Angiogenesis) This kit is supplied with cryopreserved co-culture cells, allowing users to begin the experiment at a time to suit them. Treatments can be added at any stage of the tubule development process to determine their effects, early during vasculogenesis or later for angiogenesis.	<b>ZHA-4000</b>
<b>Angiogenesis Control Reagent Kit</b> (contains VEGF and Suramin)	<b>ZHA-1300</b>
<b>Angiogenesis Tubule Staining Kit ELISA</b> (contains Anti-CD31, secondary antibody and soluble substrate only)	<b>ZHA-1100</b>
<b>Angiogenesis Tubule Staining Kit CD31</b> (contains Anti-CD31, secondary antibody and insoluble substrate)	<b>ZHA-1225</b>
<b>Angiogenesis Tubule Staining Kit ELISA and CD31</b> (contains Anti-CD31, secondary antibody with both soluble and insoluble substrate)	<b>ZHA-1125</b>
<b>Angiogenesis Tubule Staining Kit vWF</b> (contains Anti-von Willebrand Factor, secondary antibody and substrate)	<b>ZHA-1250</b>
<b>Angiogenesis Tubule Staining Kit vWF and CD31</b> (contains Anti-CD31, Anti-von Willebrand Factor secondary antibodies and substrates)	<b>ZHA-1200</b>
<b>Optimised Growth Medium (100ml)</b>	<b>ZHA-1400</b>
<b>Anti-von Willebrand Factor polyclonal*</b>	
<b>Anti-PECAM-1 (CD31) monoclonal*</b>	
<b>Goat anti-mouse IgG; AP conjugate*</b> (Secondary for ZIS-3090)	
<b>Donkey anti-sheep IgG; HRP conjugate*</b> (Secondary for ZIS-9906)	
<b>AngioSys Image Analysis software license</b> Free 30 day use demonstration disc available – conditions apply.	<b>ZHA-1800</b>

\* Bulk preparations of antibodies used in the various staining kits. Please contact TCS Cellworks for ordering information.

## 12. References

### General Angiogenesis References

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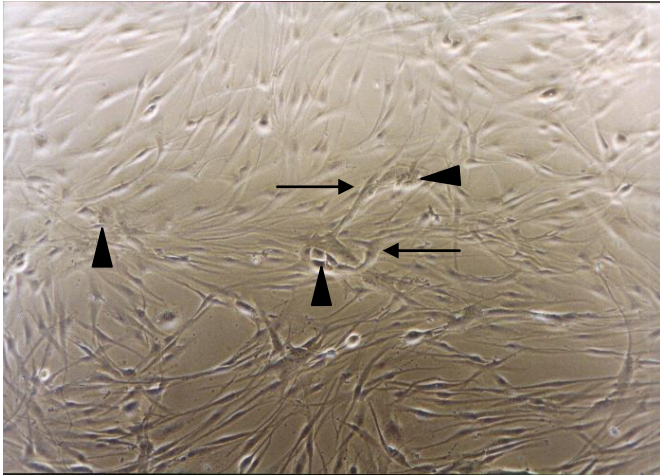
## Appendix I

### *Suggested protocol for containing contamination*

- Fill the infected wells using 1M NaOH. Take care to avoid any spillage into adjacent wells.
- Leave for 2-3 hours.
- Aspirate well taking great care not to cause any aerosols.
- Leave the well empty for the duration of the experiment.

## Appendix II

**Figure 1.**

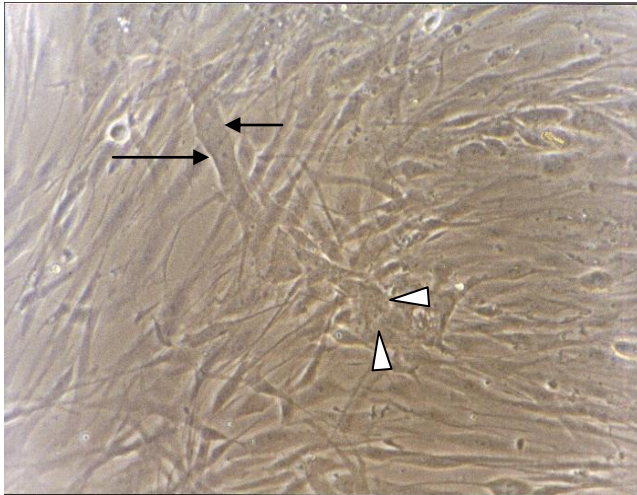


Culture in the earliest stages of angiogenesis. Cultures within this kit should have a similar appearance on day 1.

The arrowheads show small islands of endothelial cells forming.

Arrows point to structures resulting from endothelial cell migration and leading to rudimentary tubule formation.

**Figure 2.**

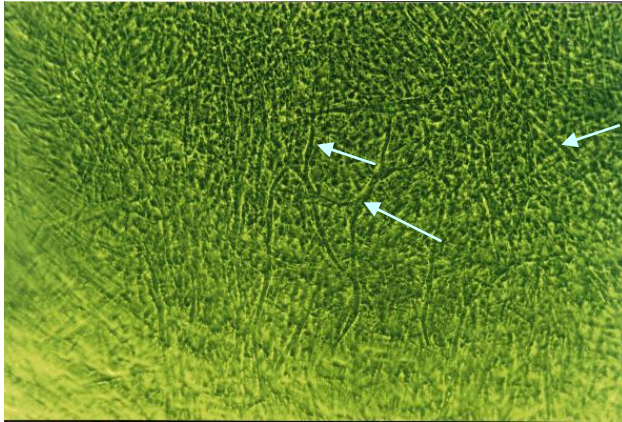


Culture exhibiting further development of the tubules. Cultures from the angiogenesis kit should resemble this at approximately day 4.

Arrows point to a developing tubule. If this is compared to the previous figure it can be seen that the tubule has increased in both length and width.

Arrowheads show island of endothelial cells from which the tubule has developed.

**Figure 3.**



Angiogenesis cultures at day 11.

Arrows indicate an anastomosing network of tubules.

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### Intellectual Property Protection

**Note that the AngioKit is protected by US patent 6225118, GB patent 2331763 and European patent 1023599.  
Other patents are pending.**



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