Cellworks

A Caltag Medsystems Company

Kit Protocol

Product Code: **ZHA-4000**

From Vasculogenesis to Angiogenesis

NOTE:

The cells in this kit require immediate attention

Take care to follow the protocol instructions carefully

Kit Contents

In this box:

- 24 well tissue culture plate
- 25ml V2a Seeding Medium
- 125ml V2a Growth Medium

Shipped separately:

- Ampoule of cryopreserved V2a co-culture cells
- V2a Seeding Medium Supplement
- V2a Growth Medium Supplement
- Control compounds (VEGF and Suramin)
- Mouse anti-human CD31 Primary Antibody
- Goat anti-mouse IgG Alkaline Phosphatase Secondary Antibody
- BCIP/NBT Substrate

For research use only
Not for diagnostic or therapeutic use

Contents

Please read the entire protocol before opening the kit or proceeding with an experiment.

Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a series of YouTube videos which will assist you with the V2a Kit.

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 patented Cellworks **V2a Kit**, 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 1 - 2 weeks) to form a network of anastomosing tubules which closely resembles the capillary bed found in the CAM assay[14]. The tubules stain positive for von Willebrand's Factor, PECAM 1 (CD31) and ICAM-2. They are also shown to secrete a matrix containing type IV collagen.

The **V2a Kit** assay is responsive to known micro and macro molecular 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.

In addition to primary cells, seeding and growth media (with associated supplements), the **V2a Kit** also includes:

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

2. V2a Kit Format

The **V2a Kit** contains all of the cells and reagents necessary to successfully complete a 24 well angiogenesis assay. The kit contains a cryogenically preserved ampoule of matched cells, 24 well tissue culture plate, medium, supplements, control compounds and staining reagents.

The **V2a Kit** is designed so that test compounds, conditioned media or tissue explants can be added to the culture at any time from the onset of vasculogenesis continuing through to advanced angiogenesis. The resulting effect on tubule formation can then be measured using Cellworks **Image Analysis Software, AngioSys 2.0.**

Control wells that receive no treatment other than medium changes form extensive networks of branching tubules over a period of one to two weeks. This allows both angiogenic inhibitors and angiogenic stimulators to be assessed. The necessary medium changes are included in the kit.

Positive and negative control compounds are included in the **V2a Kit** which consist of validated concentrated stock solutions of VEGF (2µg/ml, positive control) and Suramin (1mM, negative control) ready to add to medium.

BIOHAZARD NOTE

The ZHA-4000 V2a Kit 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.

3. Storage Temperature for Kit Components:

24 well tissue culture plate	Room Temperature
25ml V2a Seeding Medium	2 - 8ºC
125ml V2a Growth Medium	2 - 8ºC
Goat anti-mouse IgG-AP Secondary Antibody	2-8°C or -20ºC*
V2a Seeding Medium Supplement	-20ºC
V2a Growth Medium Supplement	-20ºC
Suramin Control Compound (1mM)	2-8°C or -20ºC*
VEGF Control Compound (2ug/ml)	2-8°C or -20ºC*
Mouse anti-human CD31 Primary Antibody	2-8°C or -20ºC*
BCIP/NBT Substrate Tablet	-20ºC
Cryopreserved V2a Co-Culture Cells	-196ºC (liquid nitrogen)

* 2-8°C storage for up to 2 weeks or -20°C for long term storage.

Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a YouTube video that details how to correctly unpack the V2a Kit

Ensure kit contents are stored at the indicated temperatures immediately

4. Additional Equipment and Reagents Required:

Class 2 laminar flow hood
Test compounds to assay
Sterile serological pipettes
Automated pipette pump set to slow speed setting
Sterile containers for making up test compound dilutions
Sterile aspirating pipettes
Disposable syringe with 0.2µm filter disc
Micropipettes and sterile pipette tips
Incubator set to 37°C with 5% CO_2 humidified atmosphere
70% ethanol
Phosphate Buffered Saline (see section 9.1.1)
Bovine Serum Albumin (BSA)
Relevant personal protective equipment

5. V2a Protocol Summary

Unpack kit and store reagents according to section 3

5.1. Day 1
Prepare V2a Seeding Medium and pre-equilibrate
Add V2a Co-Culture Cells to Seeding Medium
Add Cells and Medium to 24 well plate cell culture plate
Incubate

5.2. Day 2
Prepare V2a Growth Medium
Dilute control and test compounds as required*
Pre-equilibrate V2a Growth Medium and control/test dilutions
Examine cultures microscopically
Change medium into pre-equilibrated V2a Growth Medium (+/- control or test compounds) as required
Incubate

5.3. Day 4
Examine cultures microscopically
Change medium into pre-equilibrated V2a Growth medium (+/- control or test compounds)

5.4. Day 6
Repeat **Day 4** procedure

5.5. Day 8
Repeat **Day 4** procedure

5.6. Day 10
Repeat **Day 4** procedure

5.7. Day 12
Repeat **Day 4** procedure

5.8. Day 14
Examine cultures microscopically
Fix cells and stain tubules
Analyse results either manually or using Cellworks **Image Analysis Software, AngioSys 2.0**.

* To determine effects of test compounds on vasculogenesis, add test compounds on day 2. Alternatively, test compounds may be added at later time points to determine effects on angiogenesis.

6. Guidelines for Test Material Preparation

6.1. Test Compounds

Dissolve the compounds to be tested directly into V2a Growth Medium supplied whenever possible.

If necessary, compounds may be dissolved in other solvents such as DSMO 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 concentrations 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 DSMO and 0.1% (v/v) for ethanol. **N.B.** Ethanol is known to have an angiogenic effect at 1-5% (v/v)[14].

6.2. Explants

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

Small explants of approximately 2-3mm^2 should be placed in a well at the Day 2 or Day 4 medium change. The plate should then not be disturbed until the next 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.

6.3. Conditioned Media

Conditioned medium from other cell cultures may be diluted in fresh V2a Growth Medium supplied with this kit and added directly to the plate. It is recommended that conditioned medium should be diluted at least 1:1 in fresh medium. This guideline must be verified by experiments for each conditioned medium tested.

7. General Tips

Ensure all components that require storage are maintained at the correct temperature, see section 3 for storage conditions. Check kit and component expiry dates before use.

Prepare your tissue culture laminar flow hood and all necessary equipment prior to starting the assay.

Pre-equilibrate medium before adding to cultures. Equilibration with respect to CO_2 is as important as temperature equilibration.

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

8. V2a Protocol

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

8.1. Day 1

8.1.1. Thaw Seeding Medium Supplement and add to the 25ml bottle of V2a Seeding Medium. Mix well.

8.1.2. Add 0.5ml V2a Seeding Medium to each well of the 24 well plate. Incubate the plate at 37°C with 5% CO_2 in a humidified environment for 30 minutes to pre-equilibrate.

8.1.3. Accurately pipette 12ml V2a Seeding Medium into a universal container. Rapidly thaw the ampoule of cryopreserved cells in a 37ºC water bath until just a few ice crystals are remaining. Wipe the outside of the vial with 70% ethanol or isopropanol.

8.1.4. Aseptically open the ampoule and add all the cells to the 12ml V2a Seeding Medium.

8.1.5. Mix cells and V2a Seeding Medium with a serological pipette before adding 0.5ml of cell suspension to each well of the 24 well plate to give a final well volume of 1ml per well. Ensure that the cell mixture remains evenly mixed during this process.

8.1.6. After addition of cell suspension to all wells make sure that the cells are evenly dispersed in the wells. This is important to avoid cell clumping and subsequent over confluence in part of a well.

8.1.7. Place the 24 well plate in an incubator at 37ºC with 5% CO_2 humidified atmosphere.

Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a YouTube video that details how to correctly set up the V2a Kit.

8.2. Day 2

8.2.1. Thaw Growth Medium Supplement and add it to 125ml V2a Growth Medium.

8.2.2. Mix well and pre-equilibrate the V2a Growth Medium by opening lid 1/4 of a turn and placing in an incubator for 30 minutes at 37ºC with 5% CO_2 humidified atmosphere.

8.2.3. Prepare dilutions of control components as follows:

- Thaw VEGF positive control solution and add 11µl to 10.989ml pre-equilibrated V2a Growth Medium.
- Thaw Suramin negative control and add 220µl to 10.780ml pre-equilibrated V2a Growth Medium.
- For the untreated control, aliquot 11ml of pre-equilibrated V2a Growth Medium.

For each of the three controls (VEGF, Suramin and untreated), there is sufficient volume of reagent to complete all medium changes for each well. This is assuming triplicate

wells are assayed. Store solutions at 2-8ºC when not in use; always pre-equilibrate before use.

8.2.4. Prepare dilutions of test compounds in pre-equilibrated V2a Growth Medium and mix well (see section 6).*

8.2.5. Remove 24 well plate from the incubator and examine cultures microscopically for cell morphology and signs of growth.

8.2.6. Carefully aspirate medium from wells without touching the cell layer. To avoid possible desiccation of cells, we recommend replacing medium 4 wells at a time rather than aspirating medium from all 24 wells in a single operation.

8.2.7. Using a serological pipette, gently, down the side of the well, add 0.5ml pre-equilibrated V2a Growth Medium containing test or control materials as required per well (see example plate layout in section 8.5). Do not use micropipettes (e.g. Gilson pipettes) as they will 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.

8.2.8. Incubate the plate at 37ºC with 5% CO_2 humidified atmosphere.

*In house experiments conducted to determine the optimal day for adding the pro- (VEGF) and anti- (suramin) angiogenic factors showed that by adding VEGF and suramin on day 3 there was the largest difference in the number of junctions and that by adding the factors on day 2 there was the largest difference in the number of tubules and total tubule length. This suggested that VEGF and suramin should be added at day 2 or 3 in order to have the greatest effect on angiogenesis. Therefore, it is advisable to introduce test compounds at this stage of the assay, but the user must verify this in their own laboratory.

8.3. Days 4, 6, 8, 10 and 12**

8.3.1. Pre-equilibrate V2a Growth Medium, VEGF control, Suramin controls and any test compounds according to section 8.2.2.

8.3.2. Prepare dilutions of test compounds as described in the day 2 procedure if required (section 8.2.4.).

8.3.3. Carefully examine cultures microscopically for cell morphology and signs of growth. Proceed when you are satisfied that cultures appear normal.

8.3.4. Aspirate and replace media with V2a Growth Media containing control or test compounds as required, according to the day 2 procedure (section 8.2.6. and 8.2.7.).

Incubate the plate at 37ºC with 5% CO_2 humidified atmosphere.

***Although we recommend media changes every 2 days, it is usually possible to leave cultures over a weekend and change media after 3 days without any adverse effect on the cells. This only applies to cultures from the end of Day 2 onwards. Each user must verify this in their own laboratory.**

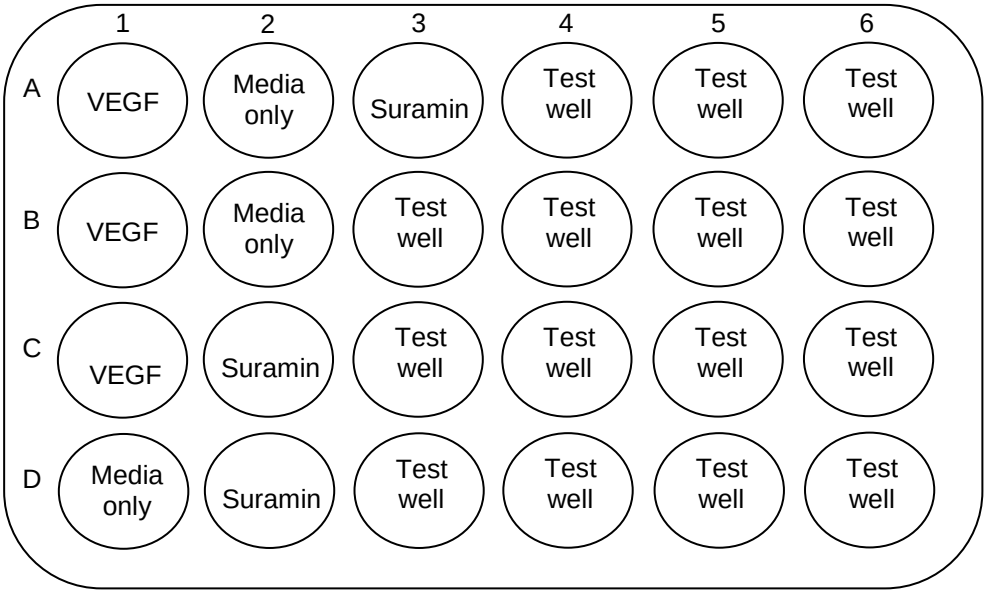
Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a YouTube video that details how to correctly monitor the assay.

8.4. Day 14

8.4.1. After examining the cultures to monitor tubule development, fix them prior to visualisation. See Section 9 for detailed protocols.

The formation of tubules can be monitored by light microscopy. Tubule lumens are visible if phase contrast microscopy is utilised. The extent of tubule formation however, can be difficult to judge in unstained plates and requires a reasonable level of experience.

8.5. Example Plate Layout



VEGF: Positive control
Media only: Untreated control well.
Suramin: Negative control

9. Fixing and Staining Protocols

Wear gloves throughout the fixing and staining procedures.

Visualisation of tubules is more difficult in cultures that 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 Cellworks **AngioSys 2.0.** A demonstration version of this software is available free of charge. See section 12 for details.

The **V2a Kit** contains reagents to stain for CD31 with a permanent insoluble BCIP/NBT chromatogenic substrate for image analysis and/or archiving of the plate.

9.1. Culture Fixation Protocol

Prepare the necessary reagents as described below:

9.1.1. Wash Buffer (Phosphate-Buffered Saline)

Per litre:	KCl	0.20g
	KH_2PO_4	0.20g
	NaCl	8.00g
	Na_2HPO_4	1.15g

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

9.1.2. Blocking Buffer

Prepare 150ml of Wash Buffer (see section 9.1.1.) and supplement with 1% BSA.
Store at 4°C. Pre-equilibrate to room temperature before use.

9.1.3. Fixative

70% ethanol.
Store at –20°C. Use immediately from freezer.

9.1.4. Fixation Protocol

9.1.4.1. Aspirate medium from cells very carefully.

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

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

9.1.4.3. Incubate at room temperature for 30 minutes.

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

9.1.4.5. 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.

Please visit the "Resources" section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a YouTube video that details how to correctly stain the V2a Kit

9.2. Staining for CD31 (PECAM-1)

Only proceed with staining after cultures have been fixed as described above. Centrifuge vials of antibody before opening.

9.2.1. Dilute primary antibody (mouse anti-human CD31) 1:400 in Blocking Buffer (35µl antibody in 14ml Blocking Buffer), mix well.

9.2.2. Add 0.5ml diluted primary antibody per well, incubate for 60 minutes at 37°C.

9.2.3. Immediately prior to washing off the primary antibody, prepare the secondary antibody (goat anti-mouse IgG AP conjugate). Dilute the secondary antibody 1:500 in Blocking Buffer (28µl in 14ml Blocking Buffer), mix well.

9.2.4. Remove the primary antibody solution.

9.2.5. Wash each well with 0.5ml Blocking Buffer and incubate each wash at room temperature for 5 minutes. Decant.

9.2.6. Repeat the wash step 9.2.1.5. two more times. Decant final wash prior to adding secondary antibody.

9.2.7. Add 0.5 ml diluted secondary antibody conjugate per well. Incubate for 60 minutes at 37°C.

9.2.8. Wash wells three times with 0.5ml dH_2O, following the procedure for washing off the primary antibody.

9.3. Insoluble Substrate (BCIP/NBT)

9.3.1. Prepare insoluble (BCIP/NBT) substrate immediately prior to addition to the **V2a Kit**: dissolve two BCIP/NBT tablets in 20ml dH_2O.

9.3.2. Filter substrate using disposable syringe and 0.2µm filter disc. Use within 1 hour.

9.3.3. Add 0.5ml substrate to each **V2a Kit** 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.

9.3.4. Wash wells carefully three times with 0.5ml dH_2O. Discard final wash and leave to air dry.

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

10. Analysing Results

10.1. Tubule Quantification and Analysis Following Histochemical Staining

For analysing plates stained per section 9.

Tubule formation can be analysed either manually (section 10.2), with the aid of Cellworks **Image Analysis Software, AngioSys 2.0** (section 10.3) or using the **Angiogenesis Image Analysis Service** (section 10.4). 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.

10.2. Manual Analysis

10.2.1. 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.

10.2.2. 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.

10.2.3. 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.

10.3. Analysis using AngioSys 2.0 Image Analysis Software

10.3.1 Cellworks **AngioSys 2.0 Image Analysis Software** (Product Code ZHA-5000), can be used for semi-automated analysis of angiogenesis by measuring the number of tubules, the number of junctions, the total tubule length, and the mean tubule length for each image.

Image files can be grouped and processed to provide quantitative and repeatable measurements as a part of rapid analysis. The resulting data is saved in a text format that can easily be read by third party packages such as Excel.

10.3.2 Further information including details of a demonstration version can be viewed at: cellworks.co.uk/angiogenesis_image_analysis.php

Other image analysis software may be adapted to analyse the V2a assay.

Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a YouTube video that details how to correctly analyse the results using AngioSys software.

10.4 Angiogenesis Image Analysis Service

10.4.1 Cellworks offer an Angiogenesis Image Analysis Service (Product Code ZHA-6000). Customer reports include the number of tubules, the number of junctions, the total tubule length, and the mean tubule length for each image.

10.4.2 Further information can be found at:cellworks.co.uk/angiogenesis_image_analysis_service.php

11. Trouble Shooting

Symptom	Possible Reasons/Solutions
No tubule formation	i. High concentrations of angiogenic inhibitors have prevented tubule formation. Check control wells. ii. Solvent used to dissolve test compounds may be at a toxic concentration. Run one or more solvent control wells. iii. Cultures have died. May be due to a number of reasons: 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 V2a medium before aspirating the next wells. 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. c. Ensure all incubations are at 37°C in a 5% CO_2 humidified atmosphere.
Cell sheet detaching from the well surface	i. Cultures have been left to grow for too long. Try to fix and stain cultures before this occurs. Contact Caltag Medsystems Ltd if this occurs before Day 14. If the sheet is still partially attached to the well, attempt to gently fix and stain the tubules.
Cells floating in well	i. Some floating cells is expected and usually nothing to worry about. ii. Floating cells may be the result of adding fresh medium too fast. Always add fresh medium very gently to prevent dislodging cells. Never use micropipettes for adding medium. iii. If all the cells are floating, this indicates that the cultures have died. See above for possible reasons.
Faint Staining of tubules	i. Antibodies not added at the correct concentration. See section 9. ii. Antibody incubation was too short or was performed at the incorrect temperature. See section 9. iii. Cultures left to grow for too long. The matrix is preventing adequate penetration of the primary antibody.
A small number of wells are contaminated	i. Act **promptly** to contain the contamination. Use your preferred method to achieve this. One possible method to use is given in Appendix I.

12. Other Angiogenesis Related Products from the Cellworks Range

Cells

Human Umbilical Vein Endothelial Cells **ZHC-2102**
Angiogenesis tested, pooled donor, cryopreserved, 0.5 million/vial

Human Dermal Fibroblasts **ZHC-5102**
Angiogenesis tested, adult, cryopreserved, 1 million/vial

Early Passage Human Umbilical Endothelial Cells **ZHC-2301**
Pooled donor, cryopreserved, 0.5 million/vial

Reagents

Angiogenesis Control Reagent Kit **ZHA-1300**
(contains VEGF and Suramin)

CD31 Tubule Staining Kit **ZHA-1225**
(contains Anti-CD31, secondary antibody and insoluble substrate)

Media and Supplements

Angiogenesis Seeding Medium Package **ZHA-1960**
(contains Angiogenesis Basal Medium, Angiogenesis Seeding Supplement and Antibiotic Supplement). Suitable for use in the V2a Kit.

Angiogenesis Growth Medium Package **ZHA-1970**
(contains Angiogenesis Basal Medium, Angiogenesis Growth Supplement and Antibiotic Supplement). Suitable for use in the V2a Kit

Software and Services

AngioSys 2.0 Image Analysis Software **ZHA-5000**
Software (Full Licence)
AngioSys 2.0 Image Analysis Software - Demonstration Version **ZHA-5000D**

Software (21 day Free of charge demonstration licence)

Angiogenesis Image Analysis Service - First 24 Images **ZHA-6000**

Angiogenesis Image Analysis Service - Additional Images **ZHA-6001**

Angiogenesis Image Analysis Service Set Up **ZHA-6002**

Angiogenesis Image Analysis Service – Image Capture **ZHA-6003**

Angiogenesis Complete Assay Service **ZHA-7000**

13. References

13.1. General Angiogenesis References

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Visit cellworks.co.uk/bibliography.php for the current list of V2a Kit – Vasculogenesis to Angiogenesis references

14. Technical Assistance

For technical assistance please contact:

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Website: www.cellworks.co.uk

Please visit the “Resources” section of the Cellworks website (www.cellworks.co.uk/videos.php) to view a series of YouTube videos which will assist you with the V2a Kit.

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.

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Intellectual Property Protection

Note that the V2a Kit is protected by US patent 6225118, GB patent 2331763 and European patent 1023599.
Other patents are pending.

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