CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

DIVAA™

Directed *In Vivo* Angiogenesis Assay (DIVAA™)

Catalog #: 3450-048-K
48 Samples

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I. Background

Please read the entire *Instructions for Use* prior to performing tests. Trevigen's **Directed *In Vivo* Angiogenesis Assay** (**DIVAA™**), is the first *in vivo* system for the study of angiogenesis that provides quantitative and reproducible results.[1] The DIVAA system was developed for, and qualified using **nude** mice. Therefore, optimization will be necessary for normal mouse strains.

During the course of the assay, implant grade silicone cylinders closed at one end, called angioreactors, are filled with 20 µl of Trevigen's basement membrane extract (BME) premixed with or without angiogenesis modulating factors. These angioreactors are then implanted subcutaneously in the dorsal flanks of **nude** mice. If filled with angiogenic factors, vascular endothelial cells migrate into, and proliferate in the BME to form vessels in the angioreactor. As early as nine days post-implantation, there are enough cells to determine an effective dose response to angiogenic factors. The sleek design of the angioreactor provides a standardized platform for reproducible and quantifiable *in vivo* angiogenesis assays. Compared to the plug assay[5], the angioreactor prevents assay errors due to absorption of BME by the mouse. In addition, the angioreactor uses only a fraction of the materials conserving both BME and test compounds used, and up to four angioreactors may be implanted in each mouse, giving more data for analysis. Trevigen's **DIVAA™** has been used in evaluating the inhibition of angiogenesis by TIMP-2,[2] to study angiogenesis in matrix metalloprotease (MMP)-2-deficient mice[1] and enhancement of angiogenesis associated with adrenomedullin[3] and CD97[4]. Trevigen's **DIVAA™** was designed for assessing angiogenesis activation by test compounds, and sufficient angiogenic factors are provided for 8 FGF-2 controls and 8 positive controls.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the products contained within the Directed *In Vivo* Angiogenesis Assay may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS sheets are available.

III. Materials Supplied

Catalog#	*Description*	*Quantity*	*Storage*
3450-048-01	Angioreactors	48 units	4 °C
3450-048-02	BME, Growth Factor Reduced PathClear®	6 x 200 µl	-20 °C
3450-048-03	10X Wash Buffer	25 ml	4 °C
3450-048-04	FGF-2	100ng/10 µl	-20 °C
3450-048-05	CellSperse™	15 ml	-20 °C
3450-048-06	200X FITC-Lectin	250 µg/50 µl	4 °C
3450-048-07	25X FITC-Lectin Diluent	400 µl	4 °C
3450-048-08	Heparin Solution	10 µl: 2 mg/ml	4 °C
3450-048-B9	FGF-2(300 ng)/VEGF(100 ng)	10 µl	-20 °C

IV. Materials/Equipment Required But Not Supplied

Equipment

1. Mouse Cages/Facility
2. Laminar Flow Hood or Clean Room
3. Pipette helper
4. Micropipettor
5. CO_2 incubator
6. Fluorescent plate reader or microscope equipped with fluorescein long pass filter
7. 500 ml graduated cylinder
8. Fine-point forceps
10. Fine-point cartilage forceps
11. Dissection scissors
12. Surgical scissors
13. Skin stapler
14. Scalpel
15. AngioRack™ (Catalog# 3450-048-09; sold separately)

Reagents

1. **Nude** Mice
2. Deionized water
3. DMEM, 10% FBS
4. 100 mg/ml Ketamine HCL (anesthesia)
5. 20 mg/ml Xylazine (analgesic)
6. Calcein AM
7. FITC-Dextran
8. Angiogenic-modulating factors (except FGF-2)

Disposables

1. Black 96 well fluorescence assay plate
2. Serological pipettes
3. Microscope slides and coverslips
4. Micropipettor tips

V. Reagent Preparation

1. 10X Wash Buffer

Dilute 25 ml of 10X Wash Buffer in 225 ml of sterile, deionized water.

2. FGF-2 (100 ng)

Add 1 µl of Heparin Solution to 10 µl of FGF-2(100 ng), and gently pipette up and down to mix immediately before addition to BME.

3. FGF-2(300 ng)/VEGF(100 ng)

Add 1 µl of Heparin Solution to 10 µl of FGF-2(300 ng)/VEGF(100 ng), and gently pipette up and down to mix immediately before addition to BME.

4. 25X FITC-Lectin Diluent

Dilute 400 µl of 25X FITC-Lectin Diluent in 9.6 ml of sterile, deionized water.

5. 200X FITC-Lectin

Dilute 50 µl of 200X FITC-Lectin in 10 ml of 1X FITC-Lectin Diluent.

VI. Assay Protocol

Note: The entire procedure must be conducted under sterile conditions using aseptic technique to prevent contamination and subsequent infection in nude mice. The use of normal mice will require optimization.

A. Preparing Angioreactors for Implantation

1. Thaw Growth Factor Reduced BME at 4 °C, on ice, overnight prior to assay. BME is to be kept on ice until gelling in step 6.
2. Pre-chill all pipette tips, angioreactors, AngioRack™ (Catalog# 3450-048-09; sold separately), and angiogenesis modulating factors at 4 °C, and keep BME on ice.
3. Working on ice, add angiogenic factors to one tube (200 µl) of Growth Factor Reduced BME. Each tube of BME is sufficient for 8 angioreactors. Add 10 µl of FGF-2 (100 ng) (Cat# 3450-048-04) **or** 10 µl of FGF-2(300 ng)/VEGF (100 ng) (Cat# 3450-048-B9), and 1 µl of Heparin Solution per 200 µl of BME to use for the positive control angioreactors. Add 11 µL of sterile PBS, or test solvent per 200 µl BME to use for the negative control angioreactors.
4. Still working on ice, add test angiogenesis modulating factors to the remaining microtubes of Growth Factor Reduced BME; do not add more than 10% total volume (over-diluting BME may compromise polymerization). Gently pipette up and down to mix test or control factors and BME; be careful not to introduce bubbles into the BME. Bubbles may be

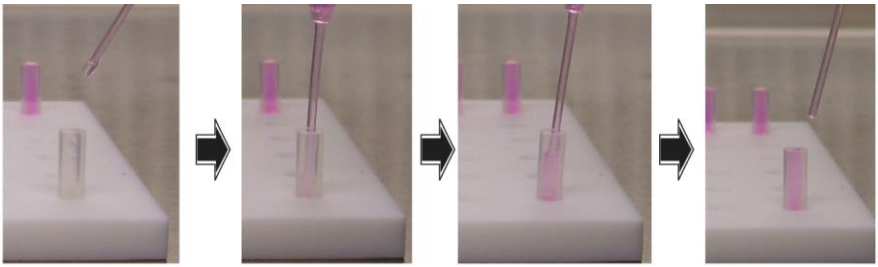


Figure 1. Fill chilled (4˚C) angioreactors using a chilled (4˚C) gel-loading tip from the bottom up. Start with excess reagent (25 µL) to prevent the introduction of bubbles, insert capillary tip completely, add BME and slowly withdraw pipet tip from angioreactor, and fill to the top. Fill 8 angioreactors at a time, and procede to next step to prevent premature gelling.

eliminated by centrifuging 250 x g for 5 minutes at 4 °C.

5. Prepare to fill angioreactors. Angioreactors must be kept chilled on ice prior to filling, whether inside microtubes or situated in an AngioRack™. Place angioreactors in the AngioRack™. Add 20 µl of BME with or without modulating factors to each angioreactor using a pre-chilled, sterile gel-loading tip; see Figure 1. Be careful not to introduce bubbles into the angioreactor. One tube will fill eight angioreactors; see Figure 2.
6. Once the eight angioreactors are filled, immediately invert angrioreactors and transfer to a sterile microtube, and place at 37 °C for 1 hour to promote gelling (inverting angioreactors during gelling prevents the formation of a meniscus at the open end of the angioreactor). Repeat for the remainder of the angioreactors.

Figure 2. AngioRack™ containing filled angioreactors.

B. Implanting Angioreactors

7. Anesthetize each mouse immediately before implantation. Recommended: one part anesthesia, 100 mg/ml Ketamine HCL (not included), to four parts analgesic, 20 mg/ml Xylazine (not included), injected subcutaneously.
8. In a laminar flow hood using forceps, remove angioreactor from microtube; cap and save microtube for step 6. See Figure 3 for implant preparation.
9. Incision should be made on the dorsal-lateral surface of a nude mouse, approximately 1 cm above the hip-socket; see Figure 4. Start by pinching back the skin and making a small cut using dissecting scissors. Then extend cut to 1 cm in length, being careful not to puncture underlying tissues.

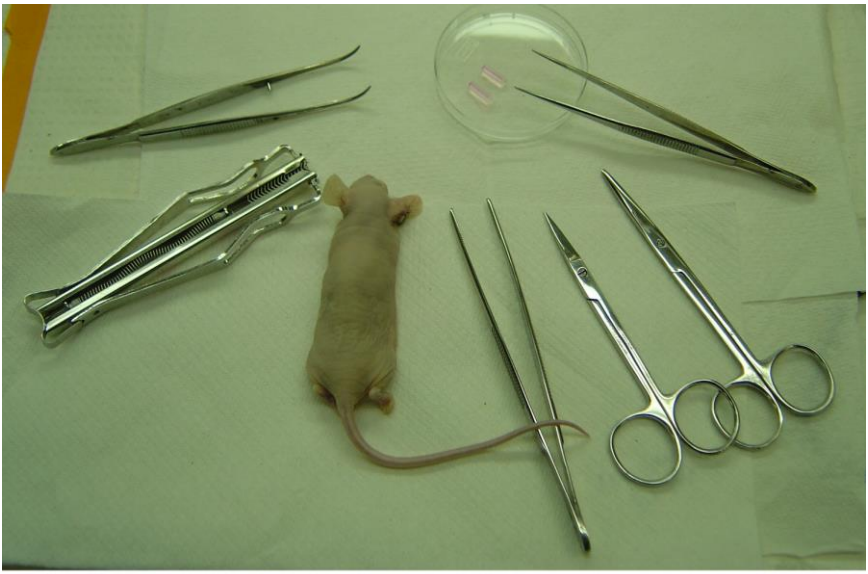


Figure 3. Preparing for implantation. Arrange sterile instrumentation, and anesthetize mouse.

Photo Provided By William Stetler-Stevenson

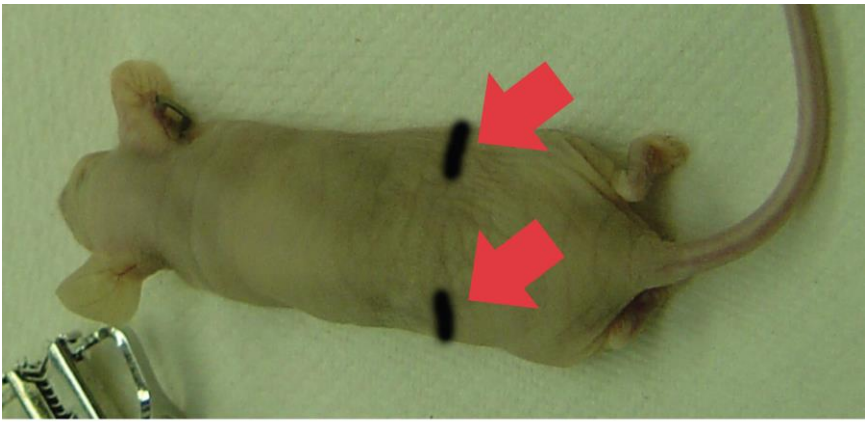


Figure 4. Location of Incision.

Photo Provided By William Stetler-Stevenson

10. Implant angioreactors into the dorsal flank of a mouse with the open end opposite the incision; up to 2 angioreactors may be planted on each side for a total of 4 angioreactors per mouse. See Figure 5 for implantation procedure and closure of the incision. Distribute angioreactors with like pairs in each mouse; see Figure 6 for recommended distribution.
11. Maintain mice for 9 to 15 days; this step requires optimization. Longer maintenance periods result in more vascularization.

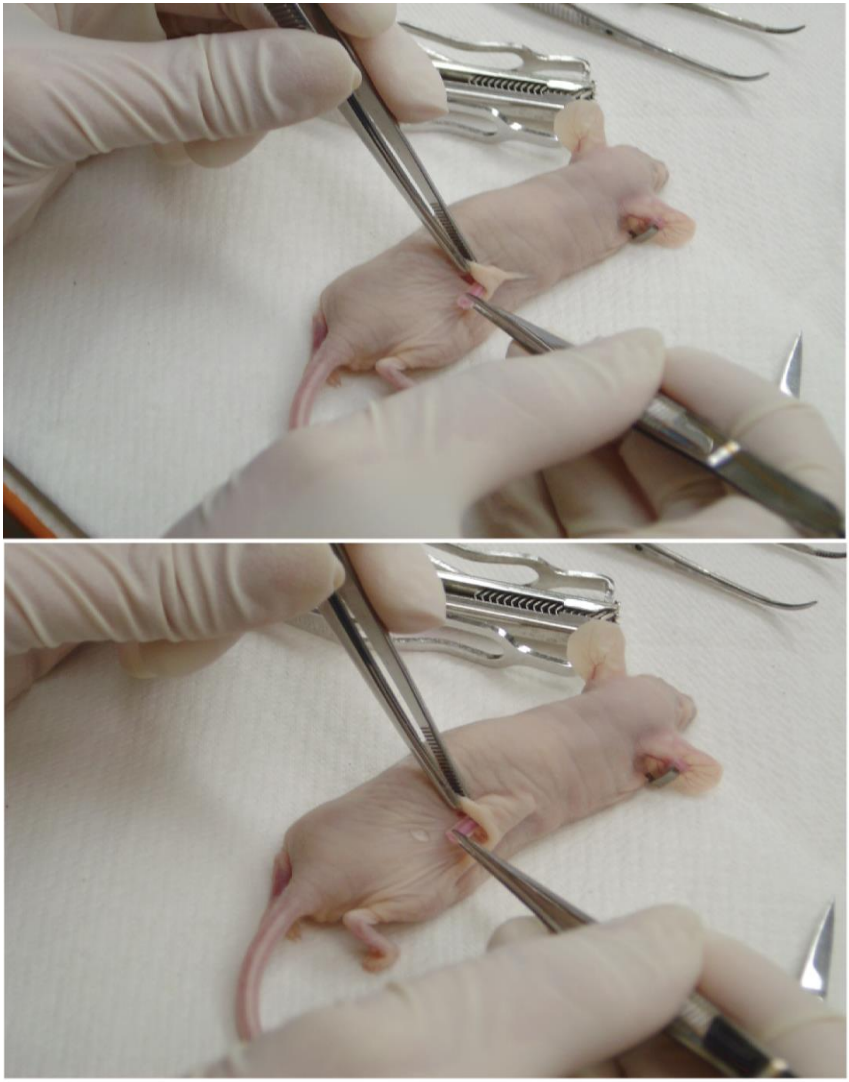


Figure 5. Implanting angioreactors. For each mouse, make a 5 mm incission on the posterior dorsal flank (left and right), and carefully insert surgical scissors to make a subcutaneous pocket. Using forceps, wet filled angioreactor in sterile 1X PBS to lubricate, and insert angioreactor open end first into pocket (up to two angioreactors can be placed in each pocket for a maximum of 4 angioreactors per mouse). Close incission with skin staple, and tag mouse for identification. Place mice under heat lamp for 15 minutes to aid in recovery.

Photo Provided By William Stetler-Stevenson

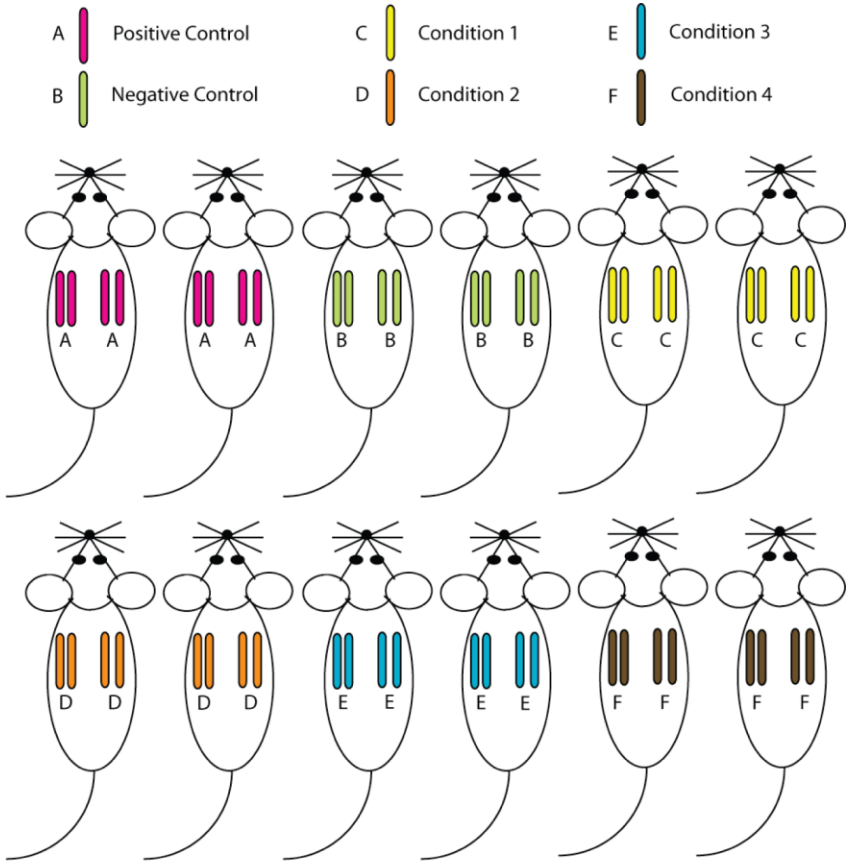


Figure 6. Recommended distribution of angioreacters in mice.

C. FITC-Lectin Detection

12. After maintenance period, humanely euthanize mice. Exposure to CO_2 levels greater than 70% for 5 minutes should be adequate.
13. Remove a 2 cm perimeter of skin surrounding angioreactors using dissection scissors. Using a scalpel, cut along open end of angioreactor to sever any vessels that may be growing into it. Recover angioreactor using dissection forceps.
14. Carefully remove the bottom cap of the angioreactors with a sterile razor blade, and using a sterile 200 µl pipette tip, push BME/vessel complex out of angioreactor into the sterile microtube. See Figure 7 for vascularization in DIVAA™ Reduced Growth Factor BME plus FGF-2/VEGF.

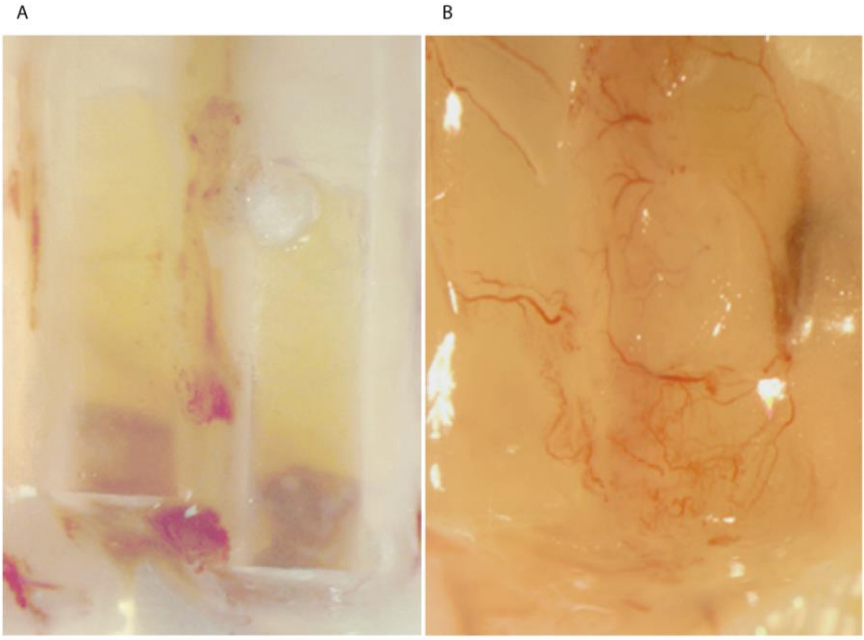


Figure 7. Vascularization in DIVAA™ angioreactor. New vessel formation is appearant in the DIVAA™ RGF BME inside the angioreactor prior to excission (A), and after harvest from the angioreactor (B).

Photo Provided By William Stetler-Stevenson

15. Rinse inside of each angioreactor with 300 µl of CellSperse™ and transfer into a microtube. Dispose of empty angioreactors. Cap tube, and incubate at 37 ºC to digest BME and create a single cell suspension. This may take 1 – 3 hours.
16. Dilute 25 mL DIVAA™ 10X Wash Buffer to 250 mL using deionized water, and label "DIVAA™ Wash Buffer."
17. Centrifuge digested BME at 250 *x g* for 5 minutes at room temperature to collect cell pellets and insoluble fractions, and discard supernatant. Resuspend pellet in 500 µl of DMEM, 10% FBS to allow for cell surface receptor recovery, and incubate at 37 ºC for one hour.
18. Centrifuge cells at 250 *x g* for 10 minutes at room temperature to collect cell pellets. Resuspend pellet in 500 µl of DIVAA™ Wash Buffer to wash cells, and centrifuge again. Discard supernatant and repeat wash two more times.
19. Dilute 400 µl DIVAA™ 25X FITC-Lectin Dilution Buffer to 10 ml using deionized water, and label "DIVAA™ FITC-Lectin Dilution Buffer."
20. For each angioreactor, dilute 1 µl DIVAA™ 200X FITC-Lectin to 200 µl using DIVAA™ FITC-Lectin Dilution Buffer, and label "DIVAA™ FITC-Lectin."
21. Resuspend pellet in 200 µl of DIVAA™ FITC-Lectin, and incubate at 4 ºC overnight.
22. Centrifuge at 250 *x g*, and remove supernatant. Wash pellet three times in DIVAA™ Wash Buffer as indicated in step 12.

23. Suspend pellet in 100 µl of DIVAA™ Wash Buffer for fluorometric determination.
24. Measure fluorescence in 96-well plates (excitation 485 nm, emission 510 nm); some fluorometers may require adjustment of Gain for an optimal range of values (please consult your equipment user manual).

D. Optional Protocol for Calcein-AM Detection (not included in the DIVAA kit).

1. After maintenance period, humanely euthanize mice. Exposure to CO_2 levels greater than 70% for 5 minutes should be adequate.
2. Harvest angioreactors. Remove a 2 cm perimeter of skin surrounding angioreactors using dissection scissors. Using a scalpel, cut along open end of angioreactor to sever any vessels that may be growing into it. Recover angioreactor using dissection forceps.
3. Carefully remove the bottom cap of the angioreactors with a razor blade, and using a sterile 200 µl pipette tip, push BME/vessel complex out of angioreactor into the sterile microtube. See Figure 6 for vascularization in DIVAA™ RGF BME plus angiogenic factors.
4. Rinse inside of angioreactors with 300 µl of CellSperse™ into microtube. Dispose of empty angioreactors. Cap tube, and incubate at 37 ^{o}C to digest BME and create a single cell suspension. This may take 1 – 3 hours.
5. Dilute 25 ml DIVAA™ 10X Wash Buffer to 250 ml using deionized water, and label "DIVAA™ Wash Buffer."
6. Centrifuge digested BME at 250 *x g* for 5 minutes at room temperature to collect cell pellets and insoluble fractions, and discard supernatant. Resuspend pellet in 500 µl of DIVAA™ Wash Buffer to wash cells, and centrifuge again. Discard supernatant and repeat wash two more times.
7. Add 100 µl of 1 µM Calcein AM (in DIVAA™ Wash Buffer), and incubate at 37 ^{o}C for 60 minutes.
8. Measure fluorescence in 96-well plates (excitation 485 nm, emission 510 nm); some fluorometers may require adjustment of Gain for an optimal range of values (please consult your equipment user manual).

E. Optional Protocol for Dextran-FITC Detection (not included in DIVAA™ kit).

1. After maintenance period, inject 100 µl of 25 mg/ml Dextran-FITC in DIVAA™ Wash Buffer via tail vein, and after 20 minutes, humanely euthanize mice. Exposure to CO_2 levels greater than 70% for 5 minutes should be adequate.
2. Harvest angioreactors. Remove a 2 cm perimeter of skin surrounding angioreactors using dissection scissors. Using a scalpel, cut along open end of angioreactor to sever any vessels that may be growing into it. Recover angioreactor using dissection forceps.
3. Carefully remove the bottom cap of the angioreactors with a razor blade, and using a sterile 200 µL pipet tip, push BME/vessel complex out of angioreactor into the sterile microtube. See Figure 7 for vascularization in DIVAA™ RGF BME with angiogenic factors.

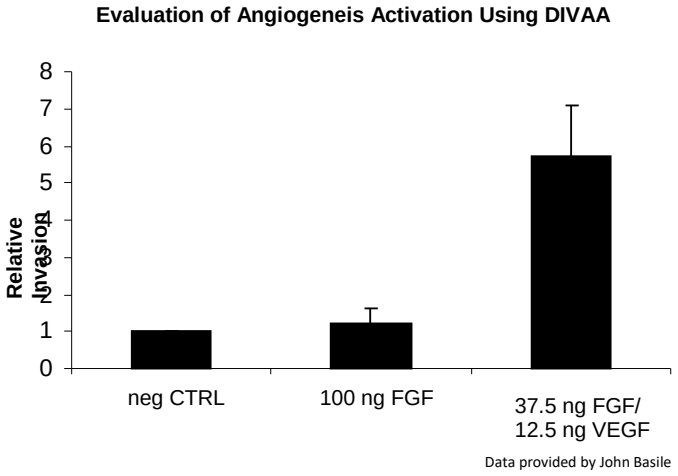
4. Rinse inside of angioreactors with 300 µl of CellSperse™ into microtube. Dispose of empty angioreactors. Cap tube, and incubate for 1 hour at 37 ºC.
5. Clear incubation mix by centrifugation, 15,000 *x g* for 5 minutes at room temperature.
6. Measure fluorescence of supernatant in 96-well plates (excitation 485 nm, emission 510 nm); some fluorometers may require adjustment of Gain for an optimal range of values (please consult your equipment user manual).

VII. Data Interpretation

Values for cell invasion will be expressed in Relative Fluorescent Units (RFUs). Calculate the mean for each condition and its corresponding standard deviation. Differences in conditions may be evaluated using a paired student's t-test. For inter-assay comparison, it may be more practical to compare relative invasion:

Relative invasion = Test sample (RFU) / Negative Control (RFU)

Data is usually plotted in a bar graph as such (amounts shown are per reactor):



VIII. Troubleshooting

Troubleshooting Guide		
Problem	Cause	Solution
BME does not gel in angio-reactor	BME has been over diluted	Use a more concentrated compound formulation (do not dilute BME more than 10%)
	BME integrity has been compromised by inappropriate shipping/storage or contamination	Use new BME
Variability in Assay	Inadequate mixing of BME and test compound	Mix BME and test com-pound thoroughly by gently pipeting up and down
	Air pockets in angioreactor	Do not use angioreactors containing air pockets
		Invert angioreactors when gelling
	Improper implantation	Implant up to 2 angio-reactors in each preformed pocket in dorsal flanks subcutaneously, open end first inside pocket.
	Insufficient receptor recovery after CellSperse™ treatment	Allow cell surface receptors to recover for 1 hour by incubating cell in culture media containing 10% FBS
	Use of C57Bl/6 mice	Use nude mice
High back-ground in negative control	Insufficient washing of cells after FITC-Lectin Staining	Wash cells again in 1X Wash Buffer
	Implantation period is too long	Reduce and optimize implantation period
	Gain is improperly set on fluorometric plate reader	Adjust gain on fluoro-metric plate reader within optimal range

Problem	Cause	Solution
No or low signal in positive control	Inadequate mixing of BME and test compound	Mix BME and test compound thoroughly by gently pipeting up and down
	Air pockets in angioreactor	Do not use angioreactors containing air pockets
		Invert angioreactors when gelling
	Improper implantation	Implant up to 2 angio-reactors in each pre-formed pocket in dorsal flanks subcutaneously, open end first inside pocket.
	Insufficient receptor recovery after CellSperse™ treatment	Allow cell surface receptors to recover for 1 hour by incubating cell in culture media containing 10% FBS
	Omitting or inadequate mixing of Heparin in FGF-2	Add Heparin to FGF-2 and mix well before adding to BME
	Implantation period was not sufficient to elicit angiogenic response	Extend and optimize implantation period
	Gain is improperly set on fluorometric plate reader	Adjust gain on fluorometric plate reader within optimal range

IX. References

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X. Appendices

A. Reagent Composition

1. Angioreactor (Cat# 3450-048-01)
The angioreactor is a one centimeter long cylinder that is sealed on one end and houses 20 µl total volume. It is made of implant-grade silicone and provided sterile. Angiogenesis is directed into the cylinder at the open end in response to angiogenesis modulating factors.

2. Growth Factor Reduced Basement Membrane Extract (BME) (Cat# 3450-048-02) BME is an extract from Engelbreth-Holm-Swarm (EHS) tumor composed primarily of Laminin I, Collagen IV, and Entactin. BME provides an angiogenesis permissive matrix for vessel formation in response to angiogenic factors.

3. 10X Wash Buffer (Cat# 3450-048-03)
Proprietary buffer formulation.

5. CellSperse™ (Cat# 3450-048-05)
A neutral metalloprotease from *Bacillus polymyxa* that provides for BME digestion and gentle cell dissociation.

6. 200X FITC-Lectin (Cat# 3450-048-06)
Fluorescence labeled *Griffonia Simplicifolia* Lectin I binds to alpha-D-galactosyl and N-acetyl galactosaminyl groups on the surface of endothelial cells.

7. 25X FITC-Lectin Diluent (Cat# 3450-048-07)
Proprietary buffer formulation.

8. Heparin Solution (Cat# 3450-048-08)
2 mg/mL Heparin.

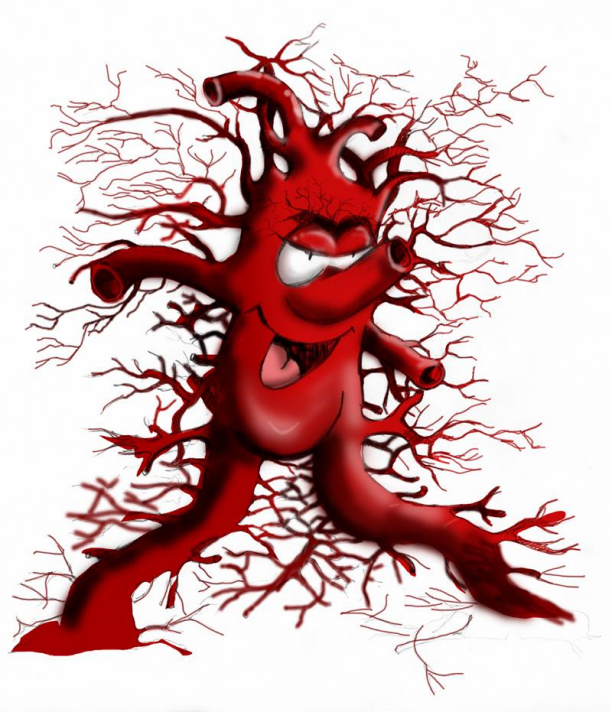
9. **FGF-2(300 ng)/VEGF(100 ng) (Cat# 3450-048-B9)**
   300 ng FGF and 100 ng VEGF

B. Related products available from Trevigen.

Catalog#	Description	Size
3450-048-SK	Cultrex® DIVAA™ Starter	48 samples
3450-048-IK	Cultrex® DIVAA™ Inhibition Kit	48 samples
3471-096-K	Cultrex® In Vitro Angiogenesis Assay Endothelial Cell Invasion Kit	96 tests
3470-096-K	Cultrex® In Vitro Angiogenesis Assay Tube Formation Kit	96 tests
3455-024-K	24 Well BME Cell Invasion Assay	24 inserts
3484-096-K	CultreCoat® 96 well BME-Coated Cell Invasion Optimization Assay	96 samples
3455-096-K	Cultrex® 96 well BME Cell Invasion Assay	96 samples
3456-096-K	Cultrex® Laminin I Cell Invasion Assay	96 samples
3457-096-K	Cultrex® Collagen I Cell Invasion Assay	
3458-096-K	Cultrex® Collagen IV Cell Invasion Assay	96 samples
3465-096-K	Cultrex® 96 Well Cell Migration Assay	96 samples
3465-024-K	Cultrex® 24 Well Cell Migration Assay	12 samples

Accessories:

Catalog#	Description	Size
3400-010-01	Cultrex® Mouse Laminin I	1 mg
3446-005-01	Cultrex® 3-D Culture Matrix™ Laminin I	5 ml
3440-100-01	Cultrex® Rat Collagen I	100 mg
3442-050-01	Cultrex® Bovine Collagen I	50 mg
3447-020-01	Cultrex® 3-D Culture Matrix™ Collagen I	100 mg
3410-010-01	Cultrex® Mouse Collagen IV	1 mg
3420-001-01	Cultrex® Human Fibronectin PathClear®	1 mg
3416-001-01	Cultrex® Bovine Fibronectin	1 mg
3421-001-01	Cultrex® Human Vitronectin PathClear®	50 µg
3417-001-01	Cultrex® Bovine Vitronectin	50 µg
3439-100-01	Cultrex® Poly-D-Lysine	100 ml
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3445-048-01	Cultrex® 3-D Culture Matrix™ BME	15 ml
3430-005-02	Cultrex® BME with Phenol Red, PathClear®	5 ml
3431-005-02	Cultrex® BME with Phenol Red, Growth Factor Reduced, PathClear®	5 ml
3432-005-02	Cultrex® BME, PathClear®	5 ml
3433-005-02	Cultrex® BME Growth Factor Reduced, PathClear®	5 ml
3437-100-K	Cultrex® Cell Staining Kit	100 ml
3450-048-05	CellSperse™	15 ml



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