DIVAA™

Directed *In Vivo* Angiogenesis Assay Inhibition Kit

Catalog Number: 3450-048-IK

48 samples

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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BACKGROUND

R&D Systems® 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, called Angioreactors, are filled with 20 µL of Cultrex 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 Cultrex 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 Cultrex BME by the mouse. The Angioreactor uses only a fraction of the materials conserving both Cultrex BME and test compounds used, and up to four Angioreactors may be implanted in each mouse, giving more data for analysis. DIVAA has been used in evaluating the inhibition of angiogenesis by TIMP-2 (2), to study angiogenesis in matrix metalloprotease (MMP)-2-deficient mice and enhancement of angiogenesis associated with adrenomedullin (3) and CD97 (4).

DIVAA Inhibition Kit was developed for screening candidate angiogenesis inhibitors; to this end, ample growth factors are provided for all 48 Angioreactors.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Angioreactors	3450-048-01	48 units	Store at 2-8 °C.
10X Wash Buffer	3450-048-03	25 mL	
200X FITC-Lectin	3450-048-06	250 µg/50 µL	
25X FITC-Lectin Diluent	3450-048-07	400 µL	
Heparin Solution	3450-048-08	10 µL: 2 mg/mL	
Cultrex™ BME, Reduced Growth Factor	3450-048-02	6 x 200 µL	Store at ≤ -20 °C.
DIVAA FGF-2(1.8 µg)/VEGF(600 ng)	3450-048-B10	60 µL	
CellSperse™	3450-048-05	15 mL	

OTHER MATERIALS REQUIRED

Reagents

- Nude Mice
- Deionized water
- DMEM, 10% FBS
- 100 mg/mL Ketamine HCL (anesthesia)
- 20 mg/mL Xylazine (analgesic)
- Angiogenic-modulating factors (except FGF-2)
- Sterile, distilled water
- Sterile Phosphate Buffered Saline (PBS)

Equipment

- Mouse Cages/Facility
- Laminar Flow Hood or Clean Room
- Pipette helper
- Micropipettor
- CO_2 incubator
- Fluorescent plate reader or microscope equipped with fluorescein long pass filter
- 500 mL graduated cylinder
- Fine-point forceps
- Fine-point cartilage forceps
- Dissection scissors
- Surgical scissors
- Skin stapler
- Scalpel

Disposables

- Black 96 well fluorescence assay plate
- Serological pipettes
- Microscope slides and coverslips
- Micropipettor tips

REAGENT PREPARATION

1X Wash Buffer - Dilute 25 mL of 10X Wash Buffer in 225 mL of sterile, distilled water.

Working FGF-2/VEGF Solution - Add 6 µL of Heparin Solution to 60 µL of DIVAA FGF-2(1.8 µg)/VEGF(600 ng), and gently pipette up and down to mix immediately before addition to Cultrex BME.

1X FITC-Lectin Diluent - Dilute 400 µL of FITC-Lectin Diluent in 9.6 mL of sterile, distilled water.

1X FITC-Lectin - Dilute 50 µL of 200X FITC-Lectin in 10 mL of 1X FITC-Lectin Diluent.

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.*

Preparing Angioreactors for Implantation

1. Thaw Cultrex BME, Reduced Growth Factor, at 2-8 °C, on ice, overnight prior to assay. Cultrex BME is to be kept on ice until gelling in Step 6.
2. Pre-chill all pipette tips, Angioreactors, AngioRack, and angiogenesis modulating factors at 2-8 °C, and keep Cultrex BME on ice.
3. Working on ice, add angiogenic factors to one tube (200 µL) of Cultrex BME. Each tube of Cultrex BME is sufficient for eight Angioreactors. Add 10 µL of Working FGF-2 Solution or Working FGF-2/VEGF Solution, and 1 µL of Heparin Solution per 200 µL of Cultrex BME to use for the positive control Angioreactors. Add 11 µL of sterile PBS, or test solvent per 200 µL Cultrex BME to use for the negative control Angioreactors.
4. Still working on ice, add test angiogenesis modulating factors to the remaining microtubes of Cultrex BME; do not add more than 10% total volume (over-diluting Cultrex BME may compromise polymerization). Gently pipette up and down to mix, be careful not to introduce bubbles. Bubbles may be eliminated by centrifuging 250 x g for 5 minutes at 2-8 °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 Cultrex BME with or without modulating factors to each Angioreactor using a pre-chilled, sterile gel-loading tip. Be careful not to introduce bubbles into the Angioreactor. One tube will fill eight angioreactors.

6. Once the eight Angioreactors are filled, immediately invert angioreactors 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.

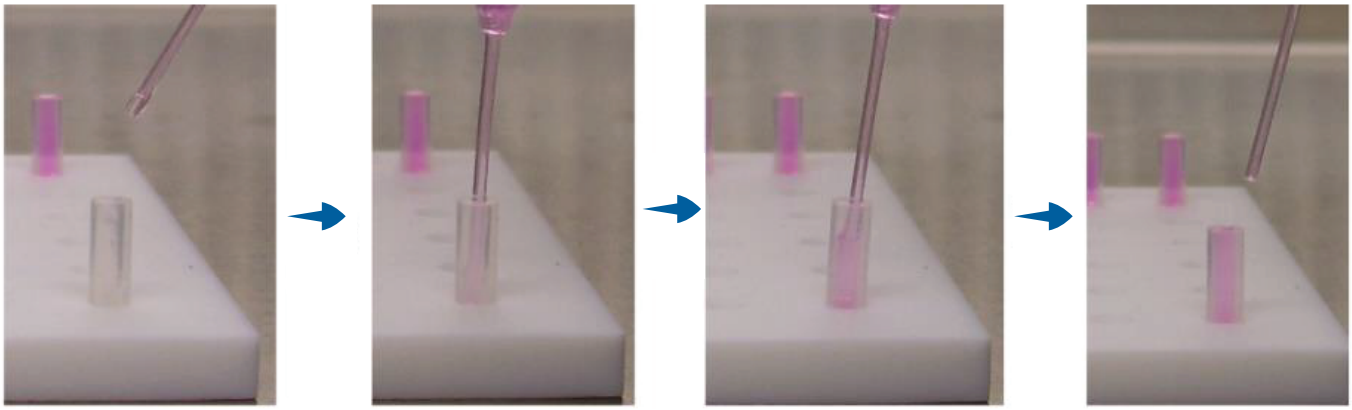


Figure 1. Filled chilled Angioreactors using a chilled gel-loading tip from the bottom up. Start with excess reagent (25 µL) to prevent the introduction of bubbles, insert capillary top completely, and Cultrex BME and slowly withdraw pipet tip from Angioreactor, and fill to the top. Fill eight Angioreactors at a time, and proceed to the next step to prevent premature gelling.

Implanting Angioreactors

Figure 2. AngioRack containing filled Angioreactors.

1. 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.

2. In a laminar flow hood using forceps, remove Angioreactor from micro-tube; cap and save microtube for implant preparation.

3. Incision should be made on the dorsal-lateral surface of a nude mouse, approximately 1 cm above the hip-socket. 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.

4. Implant Angioreactors into dorsal flank of mouse with open end opposite incision; up to 2 Angioreactors may be planted on each side for a total of four Angioreactors per mouse. For implantation procedure and closure of the incision. Distribute Angioreactors with like pairs in each mouse for recommended distribution.

5. Maintain mice for 9-15 days; this step requires optimization. Longer maintenance periods result in more vascularization.

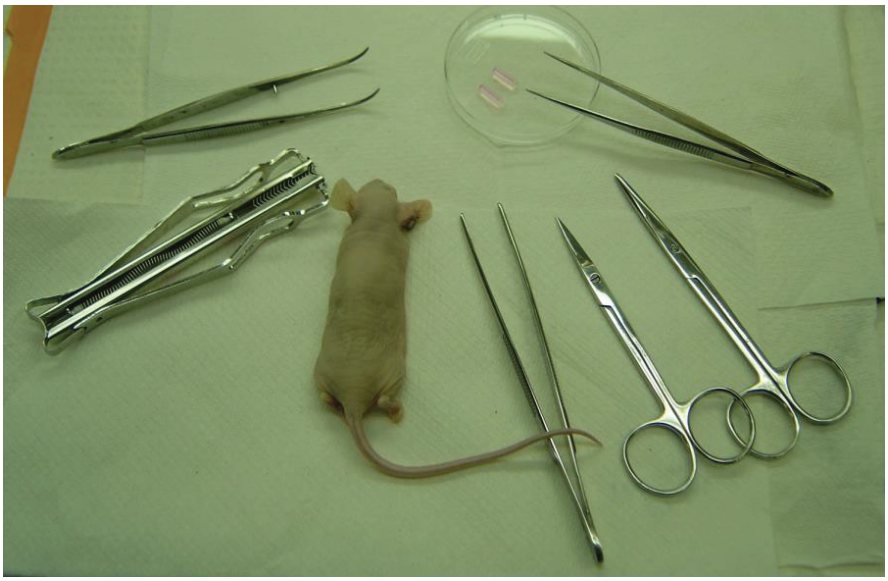


Figure 3. Preparing for implantation. Arrange sterile instrumentation and anesthetize mouse.

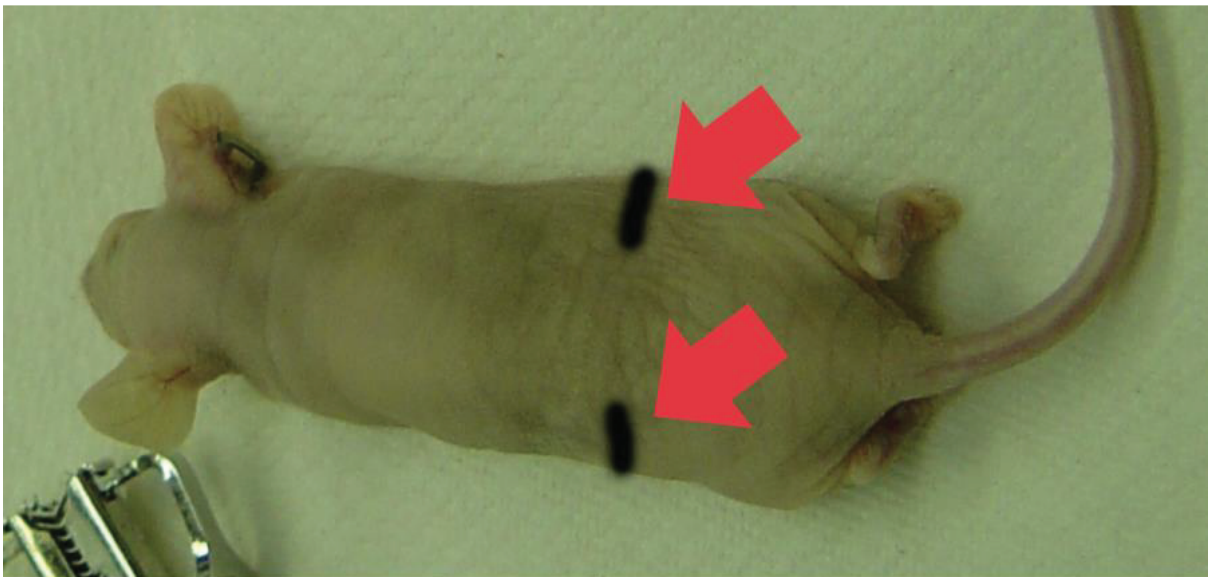


Figure 4. Location of incision.

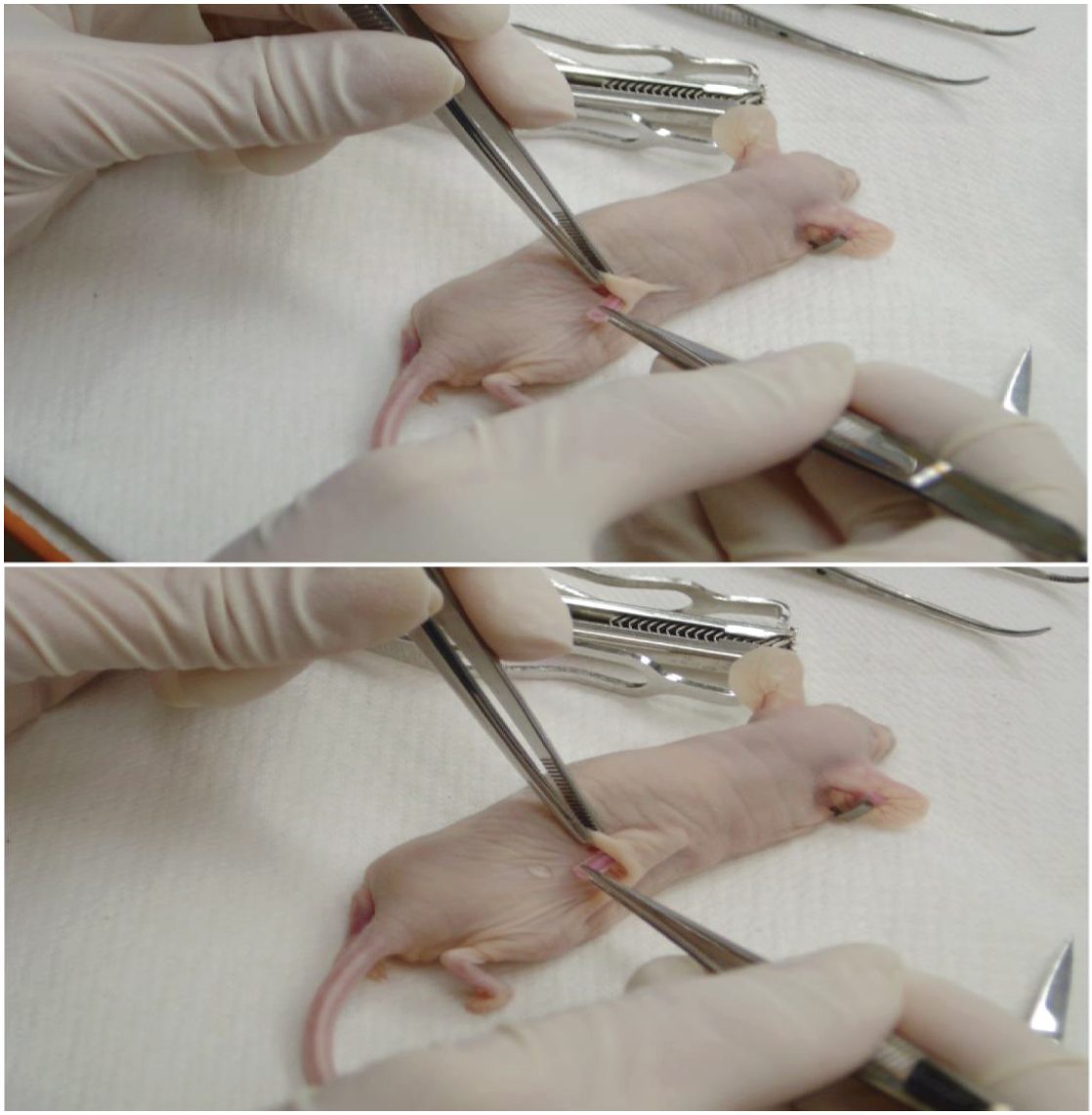


Figure 5. Implanting Angioreactors. For each mouse, make a 5 mm incision on the posterior dorsal flank (left and right), and carefully insert surgical scissors to make a subcutaneous pocket. Using forceps, wet filled Angioreactor in sterile PBS to lubricate, and insert Angioreactor open end first into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

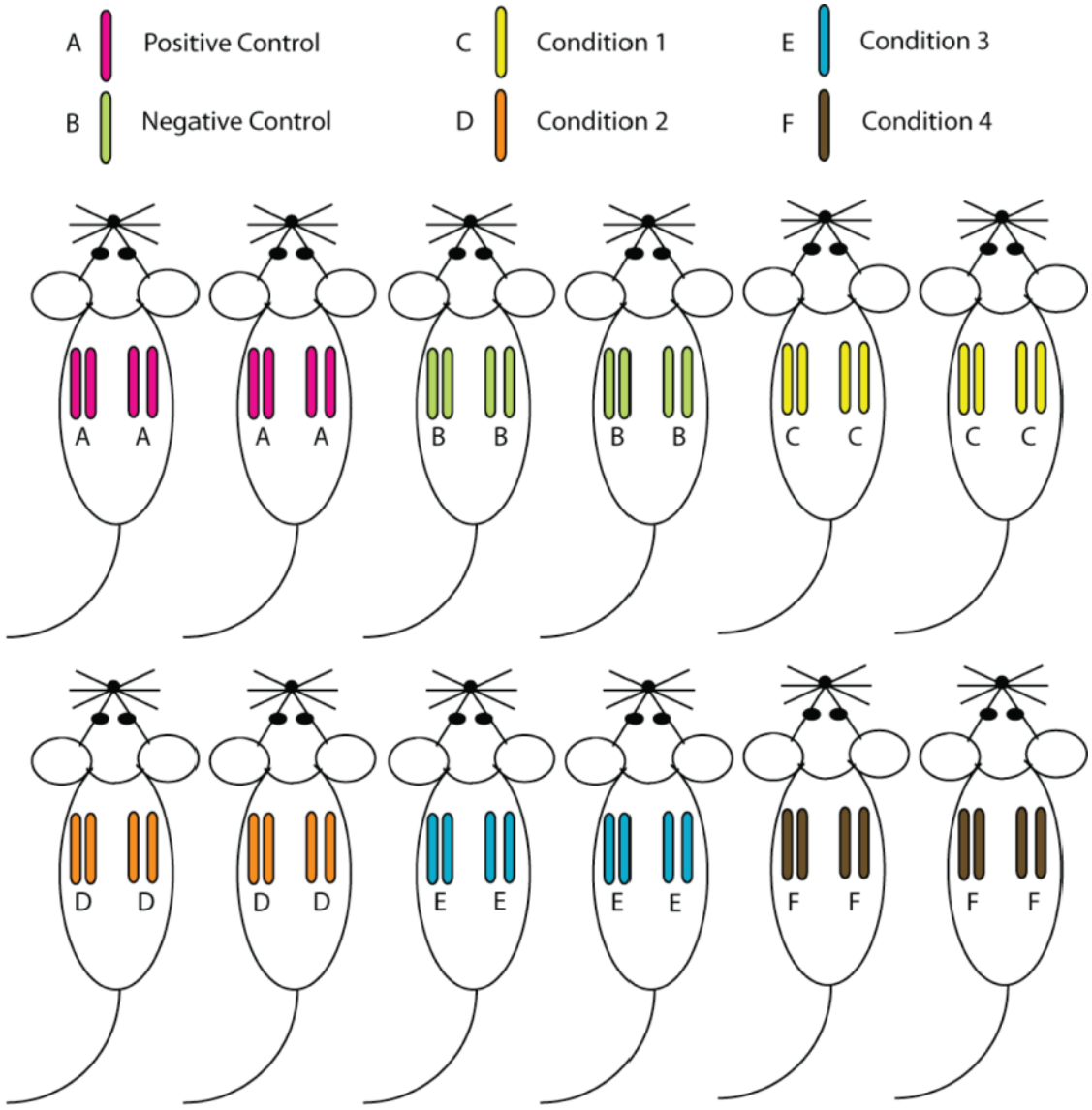


Figure 6. Recommended distribution of Angioreactors in mice.

FITC-Lectin Detection

1. After maintenance period, humanely euthanize mice. Exposure to CO_2 levels greater than 70% for 5 minutes should be adequate.
2. 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 sterile razor blade, and using a sterile 200 µL pipette tip, push vessel complex out of Angioreactor into the sterile microtube. See Figure 7 for examples of vascularization within Angioreactors.

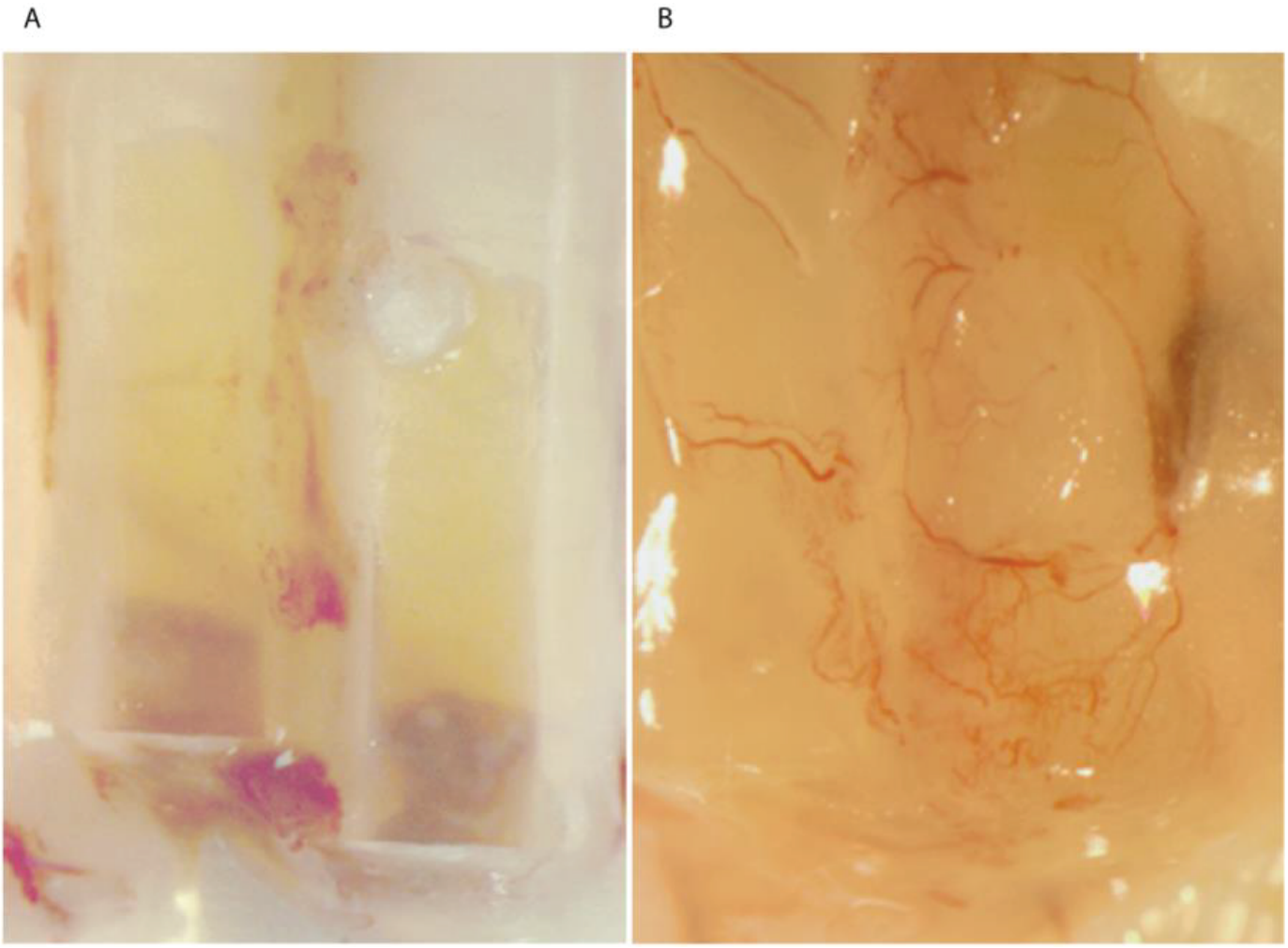


Figure 7. Vascularization within an Angioreactor. New vessel formation is apparent in the angioreactor prior to incision **(A)**, and after harvest from the Angioreactor **(B)**.

4. Rinse inside of each angioreactor with 300 µL of CellSperse™ and transfer solution into a microtube. Dispose of empty Angioreactors. Cap mircotube, and incubate at 37 °C to digest Cultrex BME and create a single cell suspension. This may take 1-3 hours.
5. Prepare 1X Wash Buffer as indicated in the Reagent Preparation section.
6. Centrifuge digested Cultrex 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 containing 10% FBS to allow for cell surface receptor recovery, and incubate at 37 °C for one hour.

7. Centrifuge cells at 250 x g for 10 minutes at room temperature to collect cell pellets. Resuspend pellet in 500 µL of 1X Wash Buffer to wash cells, and centrifuge again. Discard supernatant and repeat wash two more times.
8. Prepare 1X Wash Buffer as indicated in the Reagent Preparation section.
9. Prepare 1X FITC-Lectin as indicated in the Reagent Preparation section. 200 µL of 1X FITC-Lectin is required for each Angioreactor.
10. Resuspend pellet in 200 µL of 1X FITC-Lectin, and incubate at 2-8 °C overnight.
11. Centrifuge at 250 x g, and remove supernatant. Wash pellet three times in 1X Wash Buffer.
12. Suspend pellet in 100 µL of 1X Wash Buffer for fluorometric determination.
13. 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 (consult your equipment user manual).

Optional Protocol for Calcein AM Detection (not included in DIVAA™ kit).

1. After the 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 vessel complex out of Angioreactor into the sterile microtube. See Figure 6 for an example of vascularization.
4. Rinse inside of Angioreactors with 300 µL of CellSperse™ into microtube. Dispose of empty Angioreactors. Cap tube, and incubate at 37 °C to digest Cultrex BME and create a single cell suspension. This may take 1-3 hours.
5. Prepare 1X Wash Buffer as indicated in the Reagent Preparation section.
6. Centrifuge digested Angioreactor contents 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 1X 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 (R&D Systems®, Catalog # 4892-010-01) (in 1X Wash Buffer), and incubate at 37 °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 (consult your equipment user manual).

ASSAY PROTOCOL *CONTINUED*

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

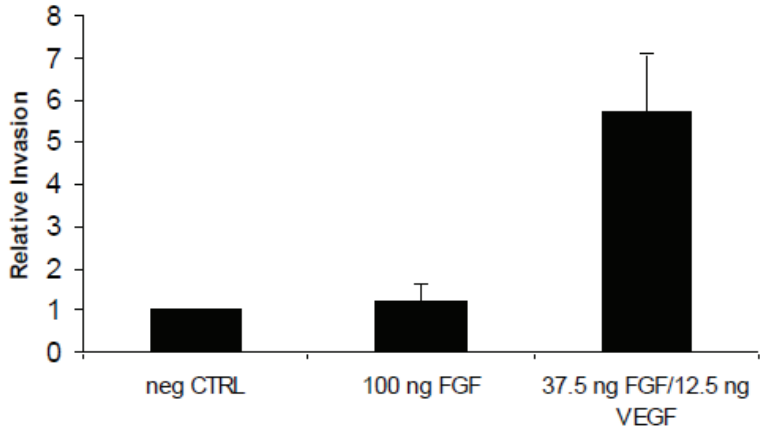
1. After the maintenance period, inject 100 µL of 25 mg/mL Dextran-FITC in 1X Wash Buffer via tail vein, and after 20 minutes, humanely euthanize mice. Exposure to CO_2 levels greater than 70% for 5 minutes should be adequate.
2. Harvest Angioreactors. Remove a 2 cm perimeter of skin surrounding angioreactors 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 vessel complex out of Angioreactor into the sterile microtube. See Figure 7 for vascularization.
4. Rinse inside of Angioreactors with 300 µL of CellSperse into microtube. Dispose of empty Angioreactors. Cap tube, and incubate for 1-3 hr at 37 °C.
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 (consult your equipment user manual).

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 interassay comparison, it may be more practical to compare relative invasion:

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

Data is usually plotted (e.g. below) and thereafter test inhibition can be expressed as a percent inhibition of the positive control.



**Amounts shown per reactor.*

***Data kindly provided by John Basile*

TROUBLESHOOTING

Problem	Potential Cause	Action
Cultrex BME does not gel in Angioreactor	Cultrex BME has been over diluted.	Use a more concentrated compound formulation (do not dilute Cultrex BME more than 10%).
	Cultrex BME integrity has been compromised by inappropriate storage or contamination.	Use new Cultrex BME.
Variability in Assay	Inadequate mixing of Cultrex BME and test compound.	Mix Cultrex 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 two Angioreactors 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 fluorometric plate reader within optimal range.
No or low signal in positive control	Inadequate mixing of Cultrex BME and test compound.	Mix Cultrex 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 two Angioreactors 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 Cultrex 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.

REFERENCES

1. Guedez, L. *et al*. (2003) American J. Pathol. **162**:1431.
2. Seo, D. *et al*. (2003) Cell **114**:171.
3. Martinez, A. *et al*. (2002) J. Natl. Cancer Inst. **94**:1227.