Directed In Vivo Angiogenesis Assay

DIVAA™
DIVAA™ Background/Significance

- Invented and developed at the National Institute of Health by William Stetler-Stevenson, MD, Ph.D.
- Quantitative Assay to Assess Angiogenesis In Vivo
- Alternative to Plug Assay
**DIVAA™ Background/Significance**

**Angioreactor™**

**Advantages**

- Prevents absorption
- Directed
- Fixed surface area for reaction
- Small size (20 µl)
  - Conserve samples
  - Implant up to 4 reactors per mouse
Institutional Requirements

- Training/certification
- Approved animal handling protocol
  - We were provided with a copy of the protocol used by Dr. Stetler-Stevenson’s lab.
  - Please email for details
    (info@amsbio.com).
- Please consult your organization.
• **Mouse Selection:**
  - Most data has been generated with athymic nude mice, but success has been reported in C57Bl/6 as well.
  - Recommend 6 to 8 weeks old.
  - Studies have been done with both males and females. Females are recommended because males may remove angioreactors from each other via fighting.
Implantation

- Optimal implantation periods may vary depending on experimental design:
  - angiogenic factors
  - doses
  - mice
    - Genetic background
    - Athymic nude mice (9-15 days)
    - C57Bl/6 mice (15 days)

- Increasing implantation period generally improves signal, but it may also increase background.
- Placement of the angioreactors is also critical.
• **Anesthesia**
  
  • Ketamine is recommended
  
  • General Guidelines
    - [http://iacuc.cwru.edu/policy/mouseaa.html](http://iacuc.cwru.edu/policy/mouseaa.html)
    - [http://www.iacuc.ucsf.edu/Proc/awMouseFrm.asp](http://www.iacuc.ucsf.edu/Proc/awMouseFrm.asp)

  • Please check for guidelines and protocols for your organization
Preparation

Plan Distribution of Angioreactors™

Recommended distribution of angioreactors in mice.
**PathClear™ BME is Recommended**

<table>
<thead>
<tr>
<th>Sterility Testing (USP XXIV):</th>
<th>Sterile for Bacteria and Fungi</th>
<th>PASS</th>
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<tbody>
<tr>
<td>Endotoxin:</td>
<td>&lt;20 EU/ml by LAL Assay</td>
<td>0.27 EU/mL</td>
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<tr>
<td>Purity:</td>
<td>&gt; 90% by SDS-PAGE</td>
<td>PASS</td>
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<tr>
<td>Functional Assay:</td>
<td>Promotes tube formation</td>
<td>PASS</td>
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<tr>
<td></td>
<td>by SVEC4-10 cells</td>
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**MAP Tests:**
- **Mycoplasma pulmonis**  PASS
- **E. cuniculi** PASS

**Viruses Tested:**
- MHV, TMEV, MMV, PVM, MVM
- HANTAAN, SENDAI, MTV, MCMV
- ECTROMELIA, LCMV, LDEV
- POLYOMA, K Virus, REO(1,3)
- MPV, EDIM, MAV(1,2)

**PCR Tests:**
- **Mycoplasma spp.** PASS

**Viruses Tested:**
- LDEV, SENDAI, MHV, PVM, MVM
- MPV(1,2,3), NOROVIRUS, REO3
- EDIM, ECTROMELIA, LCMV, K, MTV
- POLYOMA, HANTAAN, MAD(1,2)
- MCMV, KILHAM's, TOOLAN's, PARVO
- RCMV, CORONA, RMV, SEOUL
- SIALODACRYOADENITIS, TMEV, TMELV
DIVAA™ Procedure

- **Preparing PathClear™ BME:**
  - Thaw BME overnight on ice
  - Keep BME on ice
    - Warming promotes gelling
    - Multiple phase transitions may compromise BME integrity

Thaw BME overnight at 4°C on ice.
DIVAA™ Procedure

- Add angiogenic factors (4°C)
  - Keep on ice
  - No more than 10% total volume
  - Gently pipet up and down to mix
  - No bubbles

Add Angiogenic Factors
(Growth Factors, Hormones, Cytokines, Inhibitors, etc.)
Do not add more than 20 μl total (10% volume); this may over-dilute BME and prevent proper gelling. Gently pipet up and down to mix; inadequate mixing may result in sample variation.
**DIVAA™ Procedure**

**Filling Angioreactor™**

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 proceed to next step to prevent premature gelling.
DIVAA™ Procedure

Gelling BME in Angioreactor™

Incubate at 37°C in 5% CO₂ for one hour. Invert filled angioreactors, and place in sterile microtubes. Incubate at 37°C for one hour to promote gelling; not inverting angioreactors may result in the formation of a meniscus at the opening of the angioreactor which could compromise contact and subsequent angiogenesis.
Arragne sterile instrumentation in a biological cabinet, and anesthetize mice.
Making the Incision

- Location -
  - Dorsal-lateral-ventral
  - Approx. 1 cm above hip socket
- Pinch and pull skin
- With surgical scissors, make a small incision, and extend to 1 cm
Creating the Pocket

Open the pocket using forceps, and slowly and carefully insert surgical scissors into pocket while opening scissors to enlarge pocket. The finished pocket should be approximately 1.5 cm deep and 1.5 cm across.

Photo provided by William Stetler-Stevenson
DIVAA™ Procedure

Implanting the Angioreactor™

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 4 angioreactors per mouse). Close incision with skin staple and/or Vetbond™, and tag mouse for identification. Place mice under heat lamp for 15 minutes to aid in recovery.

Vetbond is a trademark of 3M Corporation.

Photo provided by William Stetler-Stevenson
DIVAA™ Procedure

In Vivo Angiogenesis

The basement membrane extract provides the correct microenvironment for vessel formation in response to chemoattractants; FGF-2 and an FGF-2/VEGF mix are provided as a control.
DIVAA™ Procedure

- **Harvesting Angioreactor™**
  - Reddish color indicative of angiogenesis
  - Angioreactors must be excised to prevent loss of contents

Photo provided by William Stetler-Stevenson
DIVAA™ Procedure

Remove Sealed End
DIVAA™ Procedure

Transfer Angioreactor™ Contents to Microtube

Using a 200 μl pipet tip, push angioreactor contents into a microtube.
Contents of Angioreactor™

- Small vessel formation within the BME
- Remodeling of ECM proteins

Photo provided by William Stetler-Stevenson
DIVAA™ Procedure

FITC-Lectin Detection

Cell Dissociation in CellSperse for 1 hour

Receptor recovery in DMEM, 10%FBS 1 hour

WASH

FITC Lectin Binding Overnight

WASH

Fluorescence Analysis
DIVAA™ Data

Time-Course

Figure 2. Kinetics of angiogenesis invasion into FGF-2-containing DIVAA. Angioreactors were prepared and implanted as described. On removal from the mouse, angioreactors are photographed using a Leica MZ125 microscope connected to a digital camera (Spot, Diagnostic Instruments, Sterling Heights, MI). Top: Paired angioreactors recovered at 6 (A), 9 (B), and 15 days (C) after implantation with FGF-2 or without (control) containing Matrigel. Angioreactors are oriented with open end at bottom and sealed end at top, arrow indicates direction of invasion from open end. Progression of angiogenic response is observed with increased vascular tissue within the lumen. Bottom: The relative distance of the extent of invasion of the vascular response from the open end of the assay is plotted versus the day of recovery after implantation. A mild angiogenic response is observed in two of four DIVAA's recovered at days 9 and 15 after implantation into athymic nude mice. We selected day 9 for further characterization of the assay. Original magnifications, ×10.

DIVAA™ Data

Dose-Response

Figure 4. Quantification of DIVAA by FITC-dextran injection and validation of endothelial responses. Angioreactors are prepared and surgically implanted subcutaneously in nude mice as in Materials and Methods. A: Before recovery of the angioreactors each animal received a single tail-vein injection of 100 μl of 25 mg/ml FITC-dextran dissolved in phosphate-buffered saline. Twenty minutes after this injection the animals were sacrificed and the angioreactors recovered. Responses in both FGF-2 (5 to 500 ng/ml)- and VEGF (50 to 1000 ng/ml)-containing Matrigel and Matrigel without angiogenic factors at 9 day after implantation are determined by fluorescence quantitation of FITC-dextran after Dispase digestion, as explained in Materials and Methods. Results are expressed as relative fluorescence units of FITC-dextran. The results demonstrate that the level of angiogenic response is proportional to the dose of angiogenic factor in both FGF-2- and VEGF-containing angioreactors. B: Results of six independent experiments, each using five replicate determinations, comparing response to negative control (white bars) with FGF-2-induced angiogenesis (black bars). Student's t-test comparison of results for FGF-2 and negative controls indicates statistically significant differences (P < 0.01) in five of six experiments (only experiment 2 failed). In addition pooled results from all six experiments were tested for variation around the mean and interassay variation. The two-way coefficient of variation (CV) for the pooled assays is ~10% (95% confidence interval = 8.3 to 13%). This low CV value indicates that DIVAA is suitable for interassay comparison of results. C: Comparison of FITC-lectin (endothelial cell response) and DIVAA in control assays. Angioreactors with (pos) and without (neg) FGF-2 are prepared and implanted for 9 days as before. After recovery of angioreactors, the angiogenic response is quantified by DIVAA or for direct endothelial cell invasion using FITC-lectin staining of Dispase cell pellets, as described in Materials and Methods. Results demonstrate that the FGF-2 (positive) angioreactors contain increased numbers of FITC-lectin-stained endothelial cells and FITC-dextran signal than implants without this well-characterized angiogenic factor. Although the signal intensity is greater in the endothelial invasion assay (FITC-lectin), there are also larger SDs (error bars) and background (greater signal in negative control) associated with this assay.

FITC-Lectin detection 9 days post-implantation in athymic nude mice. Data provided by John Basile.
DIVAA™ Data

Inhibition of Angiogenesis

**Figure 5.** DIVAA determination of EC₅₀ for TNP-470. Angioreactors containing FGF-2 with indicated concentrations of TNP-470 were prepared and assayed at 9 days as before. Results show a dose-response of decrease in the angiogenic response with increasing TNP-470 concentration. DIVAA determination reveals an EC₅₀ = 88 pmol/L with a high correlation for the curve fit analysis (R² = 0.91). Complete inhibition of the angiogenic response in this assay is observed around 10 nmol/L. These results compare favorably with those obtained using the FITC-lectin determination of endothelial cell invasion (bottom). In the FITC-lectin assay the TNP-470 EC₅₀ = 600 pmol/L, also with a high coefficient curve fitting (R² = 0.94), but maximal inhibition was observed at ~100 nmol/L concentration of TNP-470.

FITC-Lectin detection 15 days post implantation.
Data provided by Jennifer Doll
Caldas, et al., Dissecting the role of endothelial SURVIVIN \(\Delta\text{Ex}3\) in angiogenesis *Blood* 109:1479-89’2007

Basile et al., MT1-MMP Controls Tumor-Induced Angiogenesis Through the Release of *Semaphorin 4D* *J Biol Chem.* 275:40974-80’2007

Hayashi et al., Involvement of \(\gamma\)-secretase in postnatal angiogenesis *Biochem Biophys Res Comm* 363:584-90’2007

Zudaire et al., Adrenomedullin is a cross-talk molecule that regulates tumor and mast cell function during human carcinogenesis *Am J Path* 168:280-91’2006

Wang et al., CD97, an adhesion receptor on inflammatory cells, stimulates angiogenesis through binding integrin counter-receptors on endothelial cells *Blood* 105:2836-44’2005

Martínez et al., Gastrin-releasing peptide (GRP) induces angiogenesis and the specific GRP blocker 77427 inhibits tumor growth in vitro and in vivo *Oncogene* 24:4106-113’2005

Bianco et al., Role of Human Cripto-1 in tumor angiogenesis *J NCI* 97:132-41’2005

Special Thanks

- William Stetler-Stevenson, MD, Ph.D.
- John Basile, Ph.D.
- Jennifer Doll, Ph.D.

Product Information:

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalog#</th>
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<td>3450-048-SK</td>
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<tr>
<td>DIVAA™ Activation Assay Kit</td>
<td>3450-048-K</td>
</tr>
<tr>
<td>DIVAA™ Inhibition Assay Kit</td>
<td>3450-096-IK</td>
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