The Avastin or Bevacizumab (humanized Anti-VEGF IgG1) ELISA Kit is an immunoassay for quantifying active avastin (VEGF binding) in human serum/plasma or in other appropriately prepared samples. The kit is validated in human but it may be suitable for mouse, rat, rabbit, monkey or other species as well. The presence of endogenous human or animal IgG1 or IgG2 may interfere in the assay.

Vascular endothelial growth factors (VEGF-VEGF-A, isoforms; VEGF-A120, VEGF-A165, and VEGF-A186) mediate their actions by binding to the cell surface receptors VEGFR1 (FL1) and VEGFR2 (KDR), which are receptors localized to the intima of the cardiovascular system. VEGFA is essential for adults during organ remodeling and diseases that involve blood vessel formation, wound healing, tumor angiogenesis, diabetic retinopathy, and age-related muscular degeneration. Anti-VEGF therapy can be used to treat treat proliferative diabetic retinopathy and vascular leakage in cancer and eye diseases.

Avastin (Bevacizumab; Roche) is a humanized monoclonal antibody (lgG1/149 kDa/CHO produced; 93% human, 7% mouse; original mouse clone A4.6.1) that inhibits all forms of VEGF's. Avastin-VEGF complex is both metabolized and excreted directly. Many diseases of the eye, such as age-related macular degeneration (AMD) and diabetic retinopathy. Ranibizumab (Lucentis, Genentech) Fab fragment of avastin is approved for intraocular use. Like many humanized antibodies, avastin may induce anti-avastin antibodies (IADIAHADA). ADA also has ELISA kits to detect ADA to avastin.

**PRINCIPLE OF THE TEST**

Avastin ELISA kit is based upon capture of active avastin from samples to the VEGF antigen coated on the plate. Bound avastin is then detected by antibody- HRP conjugate. After a washing step, substrate is added and color (blue/yellow) is developed which is directly proportional to the amount of avastin present in the sample. Yellow color is measured at 450um using an ELISA reader. The concentration of avastin in samples and control is calculated from a curve of standards containing known concentrations of avastin.

**STORAGE AND STABILITY**

The microtiter well plate and all other reagents, if unopened, are stable at 2-8°C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

**INTENDED USE**

**KIT CONTENTS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Diluent Concentrate (20x) Cat. # SD-201, 10mL</td>
<td>Dilute with distilled or deionized water to a clean stock bottle. Label as Working Sample Diluent until store at 2-8°C until the kit lot expires or is used up.</td>
</tr>
<tr>
<td>Wash Solution (100x) Cat. # WB-100, 10mL</td>
<td>Dilute the entire vial stock with 100mL distilled or deionized water into a clean stock bottle. Label as Working Wash Solution until store at 2-8°C until the kit lot expires or is used up.</td>
</tr>
</tbody>
</table>

**PRECAUTIONS AND SAFETY INSTRUCTIONS**

Standards, Sample Diluent, and Antibody HRP contain bromonitromethane (BND; 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. Use disposable TMB, sulfuric acid and BND can be requested or obtained from the ADA website: [http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10](http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10)

**ASSAY DESIGN AND SET-UP**

**Sample Collection and Handling**

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For serum, collect least by venipuncture without clotting, and separate the serum by centrifugation at room temperature. For all samples, clarify by centrifugation and/or filtration. If samples will not be assayed immediately, store frozen for long-term storage.

**Dilution standards** in Working Sample Diluent. Dilutions of 1:5–1:500 may be appropriate for standard drug treatment regimens. For accuracy, at least 3 dilution steps are recommended, as follows:

1. 100 ng/ml diluent [1:100].
2. 10 ng/ml diluent [1:100].
3. 1 ng/ml diluent [1:100].
4. 0.1 ng/ml diluent [1:100].
5. 0.01 ng/ml diluent [1:100].
6. 0.001 ng/ml diluent [1:100].

**Ready For Use:** Store as indicated on labels.

**ASSAY PROCEDURE**

**Steps**

1. **1st Incubation** [100ul – 60 min; 4 washes]
   - Add 100ul of calibrators, samples and controls each to pre-determined wells.
   - Tap the plate gently to mix reagents and incubate for 60 minutes.
   - Wash 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. **2nd Incubation** [100ul – 30 min; 5 washes]
   - Add 10ul of diluted Anti-Human IgG HRP Conjugate to each well.
   - Incubate for 30 minutes.
   - Wash wells 5 times as in step 2.

3. **Substrate Incubation** [10ul – 15 min]
   - Add 10ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
   - Incubate for 15 minutes in the dark, e.g. place in a drawer or closed.

**Results:** If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. **Stop Step** [Stop: 100ul]
   - Add 100ul of Stop Solution to each well.
   - Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

**Absorbance Reading**

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations are available.

- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

**Materials Required But Not Provided:**

- Plate reader and spectrophotometer capable of reading 100ul and 1-10ml. A multi-channel pipette is recommended.

- Prepare assay plate reader at 450 nm wavelength (results are valid).

- Absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

- Microtiter plate reader at 450 nm wavelength (results are valid).

- Bevacizumab (humanized Anti-VEGF IgG1) ELISA Kit is an immunoassay for quantifying active avastin (VEGF binding) in human serum/plasma or in other appropriately prepared samples. The kit is validated in human but it may be suitable for mouse, rat, rabbit, monkey or other species as well. The presence of endogenous human or animal IgG1 or IgG2 may interfere in the assay.

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CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Avastin concentrations may be determined as follows:

   a. Calculate the mean OD of duplicate samples.
   b. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of Avastin (x-axis). Draw the best-fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
   c. The Avastin concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
   d. Multiply the values obtained for the sample by the dilution factor of each sample.
   e. Samples producing signals higher than the 100 ng/ml standard should be further diluted and re-assayed.

2. The assay measures Avastin activity, i.e., antibody that actually binds to the VEGF-antigen coated plate, relative to Avastin standards that are presumed to be 100% active antibody. Factors in the sample that diminish Avastin binding, e.g., VEGF antigen or other Avastin-binding molecules, may reduce apparent Avastin concentration in the assay (Recovery).

3. The recovery (accuracy of Avastin measurement in stored serum) may be determined if not diluted at least 1/500 in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual human or mouse serum or plasma samples may differ, and has not been determined.

4. Avastin dose, route, frequency will affect the concentration on the drug in serum or eye. The ELISA assay detection range is 0.5 – 100 ng/ml. So samples must be tested at several dilution to determine the optimal dilution for the ELISA.

PERFORMANCE CHARACTERISTICS

Specificity
   The plate is coated with recombinant purified VEGF-A antigen to which only biologically active avastin binds with high affinity. Inactive avastin or other IgGs will not bind to the coated antigen. Therefore, the assay is highly specific for measuring avastin activity only.

Precision
   Samples containing low, medium and high concentrations of Avastin were assayed as duplicates in multiple assays (r=5) to obtain between-assay reproducibility. Coefficients of variation were calculated for the concentrations using a point-to-point curve-fitting program.

Avastin concentrations were measured with good between-assay (2.1 to 3.5 %CV) reproducibility.

QUALITY CONTROL

Reagents
   Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls
   A Positive Serum Control is provided with the kit, assigned with an Avastin concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab’s expected sample population and that are maintained stabilized. A Sample Blank should also be run; OD should be <0.3 and lower than 0.5 ng/ml Standard OD.

Standard Curve
   The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique
   Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment
   Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

LIMITS OF THE ASSAY

1. The assay measures Avastin activity, i.e., antibody that actually binds to the VEGF-antigen coated plate, relative to Avastin standards that are presumed to be 100% active antibody. Factors in the sample that diminish Avastin binding, e.g., VEGF antigen or other Avastin-binding molecules, may reduce apparent Avastin concentration in the assay (Recovery).

2. Assays that measure Avastin mass concentration may not have a tight correlation with the Avastin activity assay, e.g., full Avastin recovery may be determined by different factors.

3. The recovery (accuracy of Avastin measurement in stored serum) may be determined if not diluted at least 1/500 in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual human or mouse serum or plasma samples may differ, and has not been determined.

4. Avastin dose, route, frequency will affect the concentration on the drug in serum or eye. The ELISA assay detection range is 0.5 – 100 ng/ml. So samples must be tested at several dilution to determine the optimal dilution for the ELISA.

Avastin (Bevacizumab/anti-VEGF-A humanized IgG1)

ELISA Kit # 200-800-AVG

For Quantitation of Active Avastin in animals or Human Serum/Plasma

The assay measures Avastin activity, i.e., antibody that actually binds to the VEGF-antigen coated plate, relative to Avastin standards that are presumed to be 100% active antibody. Factors in the sample that diminish Avastin binding, e.g., VEGF antigen or other Avastin-binding molecules, may reduce apparent Avastin concentration in the assay (Recovery).