INTENDED USE

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 qualified 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 animal or human IgG1 do not interfere in the assay.

GENERAL INFORMATION

Vascular endothelial growth factors (**VEGF/VEGF-A;** isoforms; VEGF-A120, VEGF-A165, and VEGF-A188) mediate their actions by binding to the cell surface receptors **VEGFR1 (FLT1)** and **VEGFR2 (KDR)** receptors located in endothelial cells of the cardiovascular system. VEGFA is essential for adults during organ remodeling and diseases that involve blood vessels, for example, in wound healing, tumor angiogenesis, diabetic retinopathy, and age-related muscular degeneration. Anti-VEGFA therapy can be used to treat patients with undesirable angiogenesis and vascular leakage in cancer and eye diseases. Avastin (Bevacizumab; Roche) is a humanized monoclonal antibody (IgG1/149 kda/CHO produced; 93% human, 7% mouse; original mouse clone A4.6.1) that inhibits all forms of VEGFs. 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,** Fab2 fragment of avastin is approved for intraocular use. Like many humanized antibodies, avastin may induce anti-avastin antibodies (ADA/HADA). ADI 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 450nm 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^{O} C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

KIT CONTENTS

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To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Sample Diluent Concentrate (20x) Cat.#. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8^{O}C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. # WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part No. 200-514, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10 ul of concentrate to 1 ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8^{O}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Antigen Coated Strip Plate	200-811	8-well strips (12)	Coated with VEGF-A antigen and post-coated with stabilizers.
Avastin Standards			
1 ng/ml 2.5 ng/ml 5 ng/ml 10 ng/ml 25 ng/ml 50 ng/ml	200-813A 200-813B 200-813C 200-813D 200-813E 200-814F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (6) vials, each containing FDA-approved avastin diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
LOT specific concn are on the vial and may differ from the draft manual.			
Positive Control [Avastin] range on label	200-812	0.65 ml	Avastin of stated concentration range; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (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. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: 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 blood by venipuncture, allow 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.

DILUTE serum samples in Working Sample Diluent. Dilutions of 1:5k–1:500k may be appropriate for standard drug treatment regimens. For accuracy, multiple dilution steps are recommended, as follows:

1) 10ul serum + 990ul diluent = [1:100],
2) 10ul [1:100] + 490ul diluent = **[1:5k].**

Diluted samples are stable for at least a year refrigerated.

Assay Validation
Validate the performance of the avastin sample and matrix in the assay system for recovery (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of Avastin in the sample relative to the Avastin Standards.

Prepare and run a series of dilutions of the Avastin sample (within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. Serum and plasma require greater than 1/400 dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Avastin was spiked into dilutions of human serum & plasma, 1 pool and 9 individual samples, or Sample Diluent (Control), at a final concentration of 4.5 ng/ml.

Results: recovered values ranged from **74** to **102%** of Control with sera diluted 1/500. Recovery was **less** when serum was diluted less than 1/100. Low recovery suggests serum factors that interfere with Avastin binding to the antigen on the plate.

Plate Set-up
Bring all reagents to room temperature (18-30^{O} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200 ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

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Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

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 wells 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 100ul 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 [100ul – 15 min]

- Add 100ul 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 closet.

Note: 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.

5. 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 if 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.

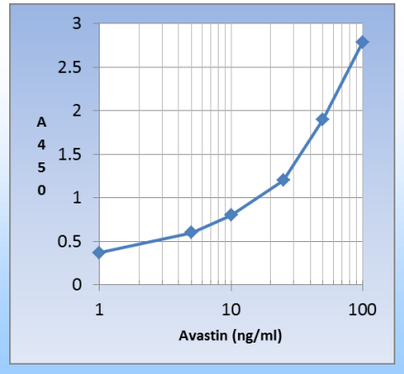
CALCULATION OF RESULTS

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:
2. Calculate the mean OD of duplicate samples.
3. 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.
4. The Avastin concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 100 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators		A450 nm
A1,2	**Negative Diluent Blank**		0.039
B1,2	1 ng/ml	**Standard**	0.37
C1,2	5 ng/ml	**Standard**	0.0.6
D1,2	10 ng/ml	**Standard**	0.86
E1,2	25 ng/ml	**Standard**	1.2
F1,2	50 ng/ml	**Standard**	1.9
F1,2	100 ng/ml	**Standard**	2.78
G1,2	**Positive**	**Control**	1.02
H1,2	Sample	**1:500**	0.51

Sample Result: **0.85** ng/ml x 500 dilution = **50,000** ng/ml



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 (n=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.

Sample	Avastin ng/ml	Inter-assay %CV
High Concn	25.66	**3.1**
Medium Concn	12.78	**3.5**
Low Concn	5.28	**2.1**

Recovery
Avastin was spiked into human serum or plasma diluted 1/500 in Sample Diluent (1 pooled and 9 individual samples), and assayed in duplicate. **Recovery** was calculated comparing the observed (O) values to the expected (E) values for each diluted sample. All serum and plasma samples were 0 Avastin (E).

O/E values ranged from **74%** to **102%**. See Limits of the Assay.

Human Serum & Plasma Samples	Avastin Concn (E) = 4.50 ng/ml	
	Observed (O)	O/E %
BC Pooled Serum	4.60	**102**
Serum, male A	3.71	**82**
Serum, male B	3.33	**74**
Serum, female C	3.93	**87**
Serum, female D	3.66	**81**
Serum, male E	3.82	**85**
Plasma, male F	3.76	**84**
Plasma, male G	3.39	**75**
Plasma, female H	4.13	**92**
Plasma, female I	3.83	**85**

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 Diluent 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 diminished 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 IgG/ml. So samples must be tested at several dilution to determine the optimal dilution for the ELISA.

Instruction Manual No. M-200-800-AVG

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

ELISA Kit # 200-800-AVG

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

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ELISA Kit Components	Amount	Part
VEGF Antigen Coated Microwell Plate	8-well strips (12)	200-811
Avastin Control	0.65 ml	200-812
Avastin Standard 1 ng/ml	0.65 ml	200-813A
Avastin Standard 2.5 ng/ml	0.65 ml	200-813B
Avastin Standard 5 ng/ml	0.65 ml	200-813B
Avastin Standard 10 ng/ml	0.65 ml	200-813C
Avastin Standard 25 ng/ml	0.65 ml	200-813D
Avastin Standard 50 ng/ml	0.65 ml	200-813E
Avastin Standard 100 ng/ml	0.65 ml	200-813G
Anti-Human IgG-HRP Conjugate (100X)	0.15 ml	200-814
Sample Diluent Concentrate (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-200-800-AVG

DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.