

INTENDED USE

The Human Anti-Avastin (Bevacizumab) ELISA Kit is an immunoassay suitable for detecting and quantifying human antibody (Ig) activity specific for Avastin, of any isotype, in serum or plasma of human or other species, including monkey, rat, rabbit and pig. *For research use only.*

GENERAL INFORMATION

VEGF (Vascular Epidermal Growth Factor) is a dimeric (kDa 42) signal glycoprotein that stimulates endothelial cell proliferation and new blood vessel formation. VEGF is the target of the monoclonal antibody bevacizumab (Avastin: by Roche). Avastin is a recombinant, humanized monoclonal antibody (IgG1 kappa) containing human framework regions and CDR regions from a mouse antibody that binds to VEGF. In humans, Avastin is used for the treatment of metastatic colorectal cancer and renal cell carcinoma, non-squamous non-small cell lung cancer and glioblastoma.

Avastin is a fully 'humanized' antibody without significant 'mouse' derived sequences that would be recognized by the injected patient as a foreign antigen; thus, immunological response to Avastin is minimal. However, when large amounts of a monoclonal antibody are continually encountered in circulation, the host may mount significant 'anti-idiotypic' response = anti-Avastin antibodies. Such antibodies might be expected to diminish the effectiveness of Avastin as a drug, and perhaps have other metabolic consequences. Like many humanized antibodies, Avastin can induce antibodies (human anti-Avastin or anti-drug antibodies, HAHA/ADA) in patients receiving Avastin. Lucentis also induced anti-drug antibodies in 1-9% patients. However, this is highly dependent upon the sensitivity of the assay. ADI has developed ELISA kits to detect antibodies to Avastin and Lucentis in patients receiving long-term treatments.

PRINCIPLE OF THE TEST

The Human Anti-Avastin ELISA kit is an antigen sandwich ELISA based on the binding of anti-Avastin antibodies (any isotype or species) in samples to Avastin immobilized on the plate; bound anti-Avastin antibody is detected by simultaneously binding to Avastin-HRP, forming the Avastin antigen sandwich. After a washing step, chromogenic substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of anti-Avastin antibody present in the sample. Stop Solution is added to terminate the reaction, and yellow color is then measured using an ELISA reader at 450nm. Anti-Avastin antibody is calculated from standard curve.

PRODUCT SPECIFICATIONS

Specificity

Purified Avastin (Bevacizumab) is used to coat the microwells; thus the assay is specific for antibodies directed to Avastin or other similar human IgG. The Avastin-HRP conjugate reacts with divalent or multivalent antibodies of any isotype (IgG, IgM, IgA, IgE) that are specific to Avastin, and have bound to the Avastin on the plate. Anti-Avastin antibodies from any species may be detected in the assay.

Assay Sensitivity

The Avastin antigen coating level and HRP conjugate are optimized to differentiate anti-Avastin from background (non-antibody) signal with serum samples diluted 1:20 or more.

KIT CONTENTS

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.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. 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.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as Working Sample/Conjugate Diluent and store at 2-8°C until the kit lot expires or is used up.
Avastin- HRP Conjugate Concentrate (100x) Part: 200-814, 0.15ml	Peroxidase conjugated Avastin in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of Working Sample/Conjugate Diluent is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Avastin Microwell Strip Plate	200-811	8-well strips (12)	Coated with Avastin and post-coated with stabilizers.
Anti-Avastin Calibrators			
3 U/ml	200-812B	0.65 ml	Four (4) vials, each containing anti-Avastin antibodies; in buffer with protein, detergents and antimicrobial as stabilizers.
7.5 U/ml	200-812C	0.65 ml	
15 U/ml	200-812D	0.65 ml	
30 U/ml	200-812E	0.65 ml	
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.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Avastin HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

LIMITATIONS OF THE ASSAY

Quantitation of Antibody in a Sample

The ELISA measures anti-Avastin activity, a combination of antibody concentration and avidity for the Avastin antigen. Antibodies with substantially different total Ig concentrations may display similar anti-Avastin activities, due to differences in avidity. The quantitation or activity of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units of Ig (e.g., ug/ml).

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, clarify the sample by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Antibody Stability

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen.

Assay Design

Review Calculation of Results (p5-7) and Limits of the Assay (above) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **3 U/ml Calibrator**. This is usually 1/10 or greater dilution for human sera with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run a set of Calibrators. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, **Method A**, has limitations. See Limits of the Assay (above).

Plate Set-up

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample and internal 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.

ASSAY DESIGN AND SET-UP (continued)

- Add 200-300ul 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.

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 Avastin HRP 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.

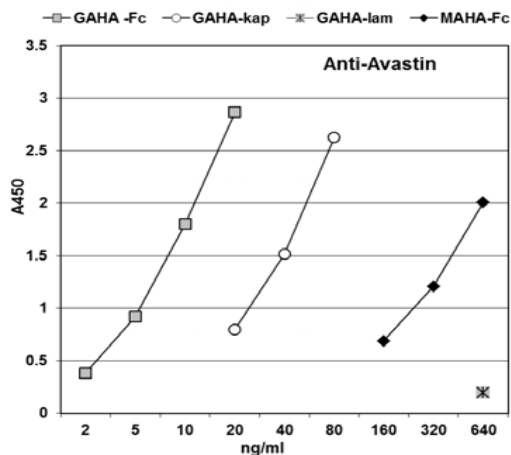
ASSAY PERFORMANCE

Detection Range and Specificity

The Antigen Sandwich ELISA format allows for the detection and quantitation of 'bridging' bivalent and/or multi-valent antibodies of any animal species or of any immunoglobulin isotype and/or subclass – IgG, IgM, IgA or IgE.

This graph shows dilution curves of affinity-purified antibodies reactive with Avastin as antigen, as follows:

- **GAHA-Fc** – goat polyclonal antibodies specific for the Fc region of Avastin; affinity-purified.
- **MAHA-Fc** – mouse monoclonal antibody specific for the Fc region of Avastin; affinity-purified.
- **GAHA-kap** – goat polyclonal antibodies specific for the kappa light chain of Avastin; affinity-purified.
- **GAHA-lam** – goat polyclonal antibodies specific for human lambda light chain; Avastin has no lambda light chain.



Results

- The data demonstrate measuring antibodies of different species.
- This assay, as with all other assays that measure antibody activity, produces a) **different** signal levels with **equivalent** amounts of each antibody, or b) the same signal level with different amounts of each antibody. This means that an individual antibody, calibrated in mass units (e.g., ng/ml) cannot serve as a standard curve to quantify other antibodies in mass units.
- The values for the GAHA-kap antibody were consistent when read from different regions of the GAHA-Fc curve – a measure of parallelism; values for MAHA-Fc were not. When parallelism does not occur, e.g., when antibodies differ significantly in avidity for the Avastin as antigen, use a different method for quantitation (e.g., Method B or C, page 6,7).

INTERPRETATION OF RESULTS

Calculation of Results

Consider several data reduction methods to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune** or **Pre-immune**, and to **Quantitate** positive antibody levels.

Method A. Use of a Calibrator Curve

When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay, page 3, and Assay Performance, page 5), the anti-Avastin activity units may be determined by interpolation from the Calibrator curve.

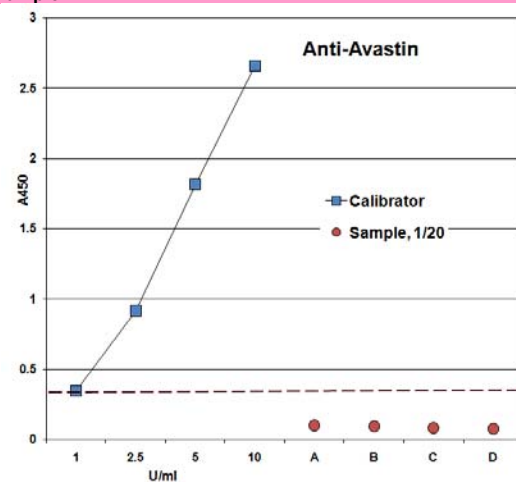
Sample values = curve value, U/ml x 1/sample dilution

Method B. Antibody Activity Threshold Index

Compare Samples to **3 U/ml Calibrator** or **Internal Control**

= **Positive/Negative Cut-off.**

Example:



Results

The **sensitivity** of the assay to detect anti-Avastin, native level or from drug administration, is controlled so that the **3 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted in the Sample Diluent at 1:20 or greater.

* * * *

The **3 U/ml Calibrator** can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative, as follows:

- ❖ Divide each Sample net OD by the **3 U/ml Calibrator** net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

INTERPRETATION OF RESULTS (cont)

C. Positive Index

Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

Immunogenicity of Avastin

As with all therapeutic proteins, there is a potential for an immune response to Avastin. In clinical trials of adjuvant colon carcinoma, 14 of 2233 evaluable patients (0.63%) tested positive for treatment-emergent anti-bevacizumab antibodies detected by an electrochemiluminescent (ECL) based assay. Among these 14 patients, three tested positive for neutralizing antibodies against bevacizumab using an enzyme-linked immunosorbent assay (ELISA). The clinical significance of these anti-product antibody responses to bevacizumab is unknown. Immunogenicity assay results are highly dependent on the sensitivity and specificity of the test method and may be influenced by several factors, including sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to Avastin with the incidence of antibodies to other products may be misleading.

Refs: Avastin Insert (Genentech/Roche).

PRECAUTIONS AND SAFETY INSTRUCTIONS

Controls, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute 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

Human Anti-Avastin (Bevacizumab) ELISA Kit

Cat # 200-810-ADG

For Quantitation of Anti-Avastin Antibodies
in Serum or Plasma
(Human or other species)

For research use only, not for diagnostic or therapeutic use.



ALPHA DIAGNOSTIC
INTERNATIONAL

India Contact:

LIFE TECH
Life Technologies™
Your Molecular & Cell Technology Partner

Life Technologies (India) Pvt. Ltd.
306, Aggarwal City Mall, Opposite M2K Pitampura,
Delhi – 110034 (INDIA).
Ph: +91-11-42208000, 42208111, 42208222
Mobile: +91-9810521400
Fax: +91-11-42208444
Email: customerservice@lifetechindia.com
Web: www.lifetechindia.com

ELISA Kit Components	Amount	Part
Avastin Coated Microwell Strip Plate	8-well strips (12)	200-811
Anti- Avastin Calibrator 3 U/ml	0.65 ml	200-812B
Anti- Avastin Calibrator 7.5 U/ml	0.65 ml	200-812C
Anti- Avastin Calibrator 15 U/ml	0.65 ml	200-812D
Anti- Avastin Calibrator 30 U/ml	0.65 ml	200-812E
Avastin HRP Conjugate (100X)	0.15 ml	200-814
Sample Diluent (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-200-810-ADG