MATRIKS BIOTEK
innovation for health & wellness

"trace & catch"

Instructions for Use

Antibody to Bevacizumab (Avastin®) ELISA

SHIKARI®
S-ATB

Enzyme immunoassay for the quantitative determination of Bevacizumab (Avastin®) in serum and plasma

REF **TR-ABEVAv2** 12 x 8 2-8°C

Revision # 2.1 February 2017

CE
IVD

Matriks Biotek® Laboratories
www.matriksbiotek.com

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	SHIKARI S-ATB
	Bevacizumab (Avastin®) antibodies quantitative analyses
Required Volume (µl)	10
Total Time (min)	140
Sample	Serum, plasma
Sample Number	96
Detection Limit (ng/mL)	30
Spike Recovery (%)	%97
Shelf Life (year)	1

Intended Use

The *Matriks Biotek* Antibody to Bevacizumab (Avastin®)* (ATB) Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) Kit is intended for the quantitative determination of antibodies to bevacizumab (Avastin®) in serum and plasma. It is for professional use only.

* Avastin® is a registered trademark of Genentech, Inc.

Summary and Explanation

Bevacizumab (Avastin®) is a recombinant human IgG1:κ monoclonal antibody specific for all human vascular endothelial growth factor-A (VEGF-A) isoforms. In 1997, the humanization of the murine anti-VEGF Mab A.4.6.1. was reported. Like its murine counterpart, bevacizumab binds to and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments, but not mouse or rat VEGF. However, bevacizumab was observed to inhibit the growth of human tumor cell lines in nude mice. In addition, studies have demonstrated that bevacizumab, in combination with chemotherapy, resulted in increased survival in patients with previously untreated metastatic colorectal cancer relative to chemotherapy alone, leading to FDA approval of the first anti-angiogenic agent.

Anti-VEGF monoclonal antibodies and other VEGF inhibitors block the growth of several tumor cell lines in nude mice. Clinical trials with VEGF inhibitors in a variety of malignancies are ongoing. The humanized anti-VEGF monoclonal antibody, bevacizumab, has been approved by the FDA as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy. Furthermore, VEGF is implicated in intraocular neovascularization associated with diabetic retinopathy and age-related macular degeneration. The pharmacokinetic properties of bevacizumab in several species have been previously described and are consistent with a typical humanized monoclonal antibody.

In 1997, Phase I clinical trials with bevacizumab was initiated. These Phase I studies showed that the antibody as a single agent was relatively non-toxic and that adding it to standard chemotherapy regimens did not significantly exacerbate chemotherapy-associated toxicities. In 1998, several Phase II studies were initiated with bevacizumab in different tumor types, either as a single agent or in combination with chemotherapy. Bevacizumab was combined with Standard first-line chemotherapy in metastatic colorectal cancer and stage IIIb/IV non-small cell lung cancer.

The potential clinical utility of VEGF inhibition in oncology is not limited to solid tumors. There is growing evidence that VEGF and VEGF receptors are expressed by a variety of leukemias and other hematologic malignancies, suggesting that inhibition of VEGF or VEGFR signaling may have a role in the treatment of such conditions. Several clinical trials are currently testing these hypotheses.

Although bevacizumab was generally well tolerated, but some serious and unusual toxicities were noted. Some open-label Phase I and II clinical trials had identified a number of adverse events, including thrombosis and bleeding as potential bevacizumab-related toxicities. In addition, most common adverse reactions are epistaxis, headache, hypertension, rhinitis, proteinuria, taste alteration, dry skin, rectal hemorrhage, lacrimation disorder, back pain and exfoliative dermatitis.

Bevacizumab is dosed and administered up to 15 mg/kg (Non- squamous non-small cell lung cancer: 15 mg/kg IV every 3 weeks with carboplatin/paclitaxel) in patients without evidence of doselimiting toxicities. However, in case of overdosage, it is recommended that the patient be monitored for any signs or symptoms of adverse reactions or effects and appropriate treatment instituted immediately.

As with all therapeutic proteins, there is a potential for immunogenicity. Accoring to the manufacturer's product insert; the incidence of antibody development in patients receiving Avastin has not been adequately determined because the assay sensitivity was inadequate to reliably detect lower titers. Enzyme-linked immunosorbent assays (ELISAs) were performed on sera from approximately 500 patients treated with Avastin, primarily in combination with chemotherapy. High titer human anti-Avastin antibodies were not detected. Immunogenicity data are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay 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.

The *Matriks Biotek* Antibody to Bevacizumab ELISA Kit can be used for monitoring anti-bevacizumab antibodies during therapy and offers the clinician a tool for decision on possible preventive measures.

Test Principle

The *Matriks Biotek* Antibody to Bevacizumab (Avastin®) ELISA is a "double antigen sandwich assay" for the determination of antibodies against bevacizumab in serum and plasma samples. During the first incubation period, the drug bevacizumab coated on the wall of the microtiter wells captures the antibodies to bevacizumab in patient serum and plasma samples. After washing away the unbound components from samples, a Peroxidase-labelled bevacizumab conjugate is added to each well and then incubated. After a second washing step, the bound enzymatic activity is detected by addition of tetramethylbenzidine (TMB) chromogen-substrate. Finally, the reaction is terminated with an acidic stop solution. The intensity of the reaction color is proportional to the concentration of antibodies to bevacizumab in sample.

Warnings and Precautions

1. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.
3. In case of severe damage of the kit package please contact Matriks Biotek or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. All reagents of this kit containing human serum or plasma (i.e. standards) have been tested and were found negative for HIV I/II, HBsAg and HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

10. Some reagents contain sodium azide (NaN_3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN_3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin)*

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light
Stability:	2 d	6 mon	Avoid repeated freeze-thaw cycles

*.Bevacizumab (Avastin®) infusion camouflages/masks the presence of antibody to bevacizumab in serum/plasma samples. Therefore, blood sampling time is critical for detection of bevacizumab. Matriks Biotek Laboratories propose to obtain blood sample just before the infusion of Bevacizumab (Avastin®) or at least 2 weeks after the infusion of Bevacizumab (Avastin®) .

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with bevacizumab
5 x 1 mL	STND A-E	**ATB Standards A-E** 500; 250; 125; 62; 0 ng/mL Ready to use. Used for construction of the standard curve. Contains antibody to bevacizumab, human serum and <0,1% NaN_3
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains proteins, RF blockers and <0.1% NaN_3
1 x 12 mL	CONFIRMATION REAGENT	**Confirmation Reagent** Ready to use. Contains optimized concentration of the bevacizumab, proteins, stabilizer, RF blockers and <0,1% NaN_3
1 x 12 mL	POD CONJ	**Peroxidase Conjugate** Red colored. Ready to use. Contains peroxidase (POD) conjugate, RF blockers, stabilizer and preservatives.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer, Concentrate (20x)** Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.
2 x 1	SLGP	**Semi-Log Graph Paper** For constructing standard curve and calculation of results.

Materials Required but not Supplied

1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV).
2. Calibrated measures.
3. Wash bottle, automated or semi-automated microtiter plate washing system
4. Microtiter plate reader capable of reading absorbance at 450 nm.
5. Bidistilled or deionised water, paper towels, pipette tips and timer.

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. ***The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions***. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. ***Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells***.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Preparation Of Component

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	bidist. Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w

Prepare Wash Buffer before starting assay procedure.

2. Dilution of Standards and Samples (serum/plasma)*

Sample	To be diluted	With	Relation	Remarks
Serum/Plasma	1/10	Assay Buffer	1:10 – 1:100	For dilution 1:10 10µl Sample + 90µl Assay Buffer For dilution 1:100 5µl Sample + 495µl Assay Buffer

*. Patient samples with a concentration of ATB above the measuring range are to be rated as "> highest standard". The result must not be extrapolated. The patient sample in question should be diluted with Assay Buffer and then retested.

PREPRATION OF CONFIRMATION TEST MIXTURE

Sample	To be diluted	With	Relation	Remarks
Serum/Plasma	Initially no	Confirmation Reagent*	1:10	For dilution 1:10 10µL Sample + 90µL Confirmation Reagent

Test Procedure

1	**QUANTITATIVE ELISA TEST FORMAT** Pipette 100 µL of ready-to use **Standards, Samples and Confirmation test mixture** into the respective wells of microtiter plate. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E (Negative Control) F1 and on: Sample (Serum/Plasma)
2	Cover the plate with adhesive film. Briefly mix contents by gently shaking the plate. **Incubate 60 min** at room temperature (18-25°C).
3	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
4	Pipette 100 µL of ready-to use **Peroxidase Conjugate** into each well.
5	Cover the plate with adhesive film. **Incubate 60 min** at room temperature (18- 25°C).
6	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
7	Pipette **100 µL** of **TMB Substrate Solution** into each well.
8	**Incubate 20 min** (without adhesive foil.) at room temperature (18-25°C) in the dark.
9	Stop the substrate reaction by adding **100 µL** of **Stop Solution** into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow
10	Measure optical density with a photometer at **450/650 nm** within **30 min** after pipetting of the Stop Solution.

Confirmation Test

	CONFIRMATION TEST FOR POSITIVE SAMPLES
	Incubate positive patient samples and optimized confirmation reagent for **60 minutes in a microtube**. After the incubation proceed the test procedure from step one given above.

QUALITY CONTROL

The test results are only valid only if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards must be found within the acceptable ranges as stated above. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

Calculation & Interpretation of Results

QUANTITATIVE INTERPRETATION

1. Using the standards (500, 250, 125, 62 ng/mL) disregarding zero standard, construct a standard curve by plottingthe OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding ATB concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of ATB from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the ATB concentration for the unknown sample.

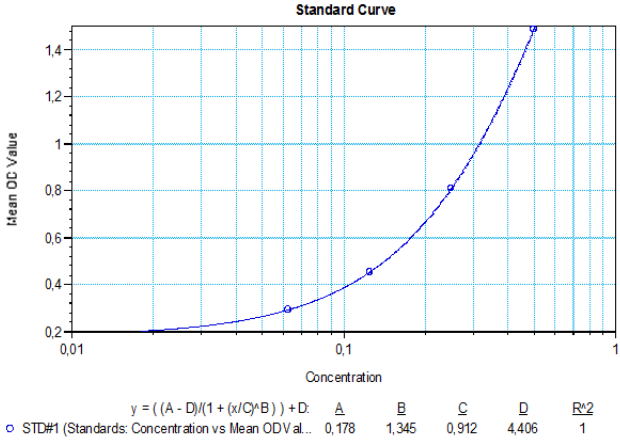
3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

4. Any sample reading greater than the highest standard should be diluted appropriately with Assay Buffer and retested. Therefore, if the samples have been diluted, the concentration determined from the standard-curve must be multiplied by the dilution factor. Because the samples initially have been diluted to 1:10, the concentration determind from the standard curve must be multiplied by the dilution factor.

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve

(Example. Do not use for calculation!)



Standard	Concentration (ng/mL)	Mean $OD_{450/650}$
A	500	1,482
B	250	0,809
C	125	0,452
D	62	0,209
E	0	0,143

Assay Characteristics

1. Specificity: Bevaczumab (Avastin®) infusion camouflages/masks the presence of antibody to bevacizumab (ATB) in serum/plasma samples. Therefore, blood sampling time is critical for detection of ATB. It is convenient to obtain blood sample just before the infusion or at least 2 weeks after the infusion of Bevacizumab (Avastin®).

2. **Sensitivity:**The lowest detectable level that can be distinguished from the zero standard is <30 ng/mL.

3. **Precision of Kit:**
   Intra-assay CV: <20% for the ATB range of 62-500 ng/mL.
   Inter-assay CV: <20% for the ATB range of 62-500 ng/mL.

4. **Recovery:** Recovery rate was found to be higher than 97% when spiked using normal human serum samples with known concentrations.

Automation

Experiments have shown that the Matriks Biotek® S-ATB ELISA is also suitable to run on an automated ELISA processor.

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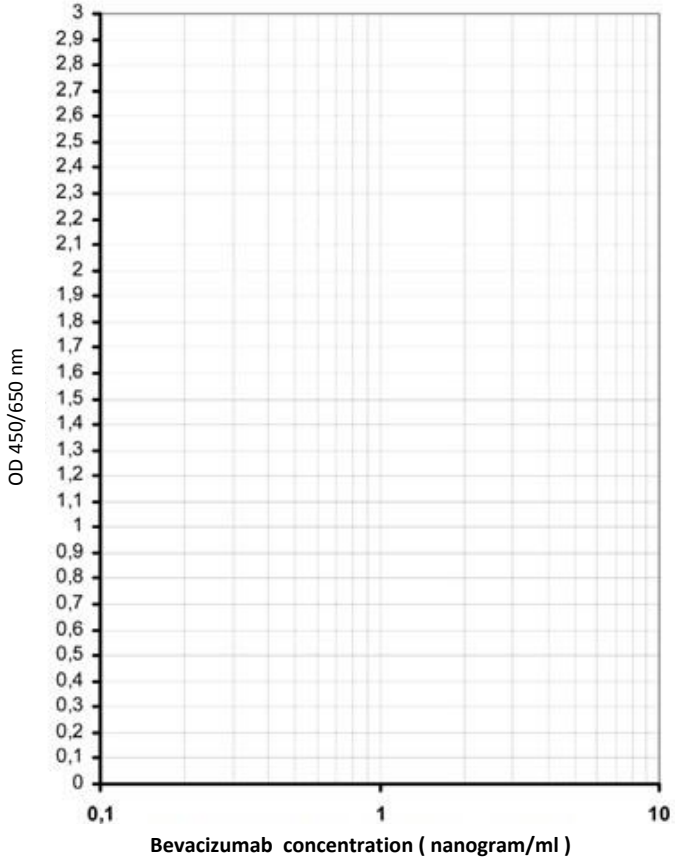
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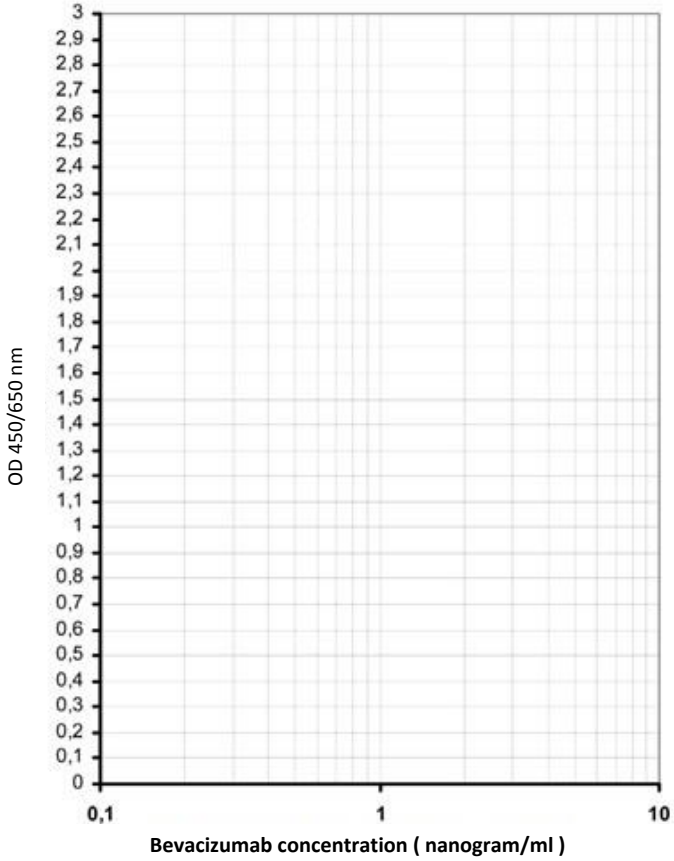
Bevacizumab concentration (nanogram/ml)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Bevacizumab concentration (nanogram/ml)