Version 10-04/24

User's Manual

Bevacizumab ELISA Kit

REF **DEIABL222**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Enzyme Immunoassay has been developed for the quantitative analysis of biologically active form of free bevacizumab (Avastin®)* in serum and plasma samples.

General Description

Bevacizumab (Avastin®) is a recombinant human IgG1:k 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 chemotherapyassociated 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.

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Serum through levels might be related to predict some clinical outcome during maintenance therapy. It was also possible that the surveillance of circulating concentration during maintenance therapy represents a direct and/or indirect factor for some other side effects. In this context, identification of biomarkers for (non-) response and risk factors for adverse drug reactions that might be related to serum drug levels and maintaining the effective minimum concentration in order to potentially avoid some side effects with a reliable method might be beneficial.

Principles of Testing

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the double antigen assay principle. Diluted standards and samples (serum or plasma) are incubated in the microtitre plate coated with human vascular endothelial growth factor (VEGF). After incubation, the wells are washed. A biotin conjugated human VEGF is added and binds to bevacizumab (Avastin®) captured by the reactant on the surface of the wells. Following incubation, wells are washed and then HRP conjugated probe (HRP) is added. After incubation, the wells are washed and the bound enzymatic activity is detected by addition of chromogen- substrate. The colour developed is proportional to the amount of bevacizumab in the sample or standard. Results of samples can be determined directly using the standard curve.

Reagents And Materials Provided

1. Microtiter Plate (1 x 12 x 8)

Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with reactant.

2. Bevacizumab Standards A-E (100X), High Level Control (100x), Low Level Control (100x) (7 x 0.3 mL)

100 ; 30; 10; 3; 0 µg/mL Ready to use. Used for construction of the standard curve. Contains human serum, bevacizumab (Avastin®) and <0.1% NaN_3.

3. Assay Buffer (2 x 50 mL)

Blue colored. Ready to use. Contains proteins and <0.1% NaN_3.

4. HRP Conjugate (1 x 12 mL)

Red colored. Ready to use. Contains HRP conjugated probe (HRP) and stabilizers.

5. TMB Substrate Solution (1 x 12 mL)

Ready to use. Contains TMB

6. TMB Stop Solution (1 x 12 mL)

Ready to use. 1N HCl.

7. Wash Buffer, Concentrate (20x) (1 x 50 mL)

Contains Buffer with Tween 20.

8. Adhesive Film (2 x 1)

For covering of Microtiter Plate

Materials Required But Not Supplied

1. Micropipettes (Multipipette Eppendorf or similar devices, < 3% CV).
2. Calibrated measures.
3. Tubes (1 mL) for sample dilution.
4. Wash bottle, automated or semi-automated microtiter plate washing system
5. Microtiter plate reader capable of reading absorbance at 450/650 nm.
6. Bidistilled or deionised water, paper towels, pipette tips and timer

Storage

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 Preparation

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 Avastin. The Laboratories suggests to obtain blood sample just before the infusion of bevacizumab (Avastin®) or at least 2 weeks after the infusion of bevacizumab (Avastin®).

Reagent Preparation

Wash Buffer - Dilute the concentrate wash buffer with distilled water at the ratio of 1/20 before starting assay procedure. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. The diluted wash buffer can stable for 2 weeks at 2-8°C.

Standards and Serum/Plasma - Dilute the concentrate standards and samples with assay buffer at the ratio of 1/100 before starting assay procedure. E.g., 10 µL standard/sample + 990 µL assay buffer.

Patient samples with a concentration of bevacizumab above the measuring range are to be rated as> "Highest Standard (Standard A)". The result must not be extrapolated. The patient sample in question should be further diluted with Assay Buffer and retested.

Assay Procedure

1. Dilute each of the standards and samples (serum/plasma) at 1:100 using Assay Buffer as described in "Reagent Preparation" section.
2. Pipette 100µl of Assay Buffer non-exceptionally into each of the wells to be used.
3. Pipette 25 µL of Diluted Standards, High Level Control, Low Level control and Samples into the respective wells of microtiter plate.

Wells:

A1: Standard A

B1: Standard B

C1: Standard C

D1: Standard D

E1: Standard E

F1: Standard F

G1: Standard G

H1 and on: Sample (Serum/Plasma)

4. Cover the plate with adhesive film. Incubate 30 min at room temperature (18- 25°C).
5. 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.
6. Pipette 100 µL of ready-to use HRP Conjugate into each well.
7. Cover the plate with a new adhesive film. Incubate 30 min at room temperature.
8. Remove adhesive foil. 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.
9. Pipette 100 µL of TMB Substrate Solution into each well.
10. Incubate 10 min (without adhesive film) at room temperature (18-25°C) in the dark.
11. 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.
12. Measure optical density with a photometer at 450/650 nm within 30 min after pipetting of the Stop Solution.

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 and/or label. 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.

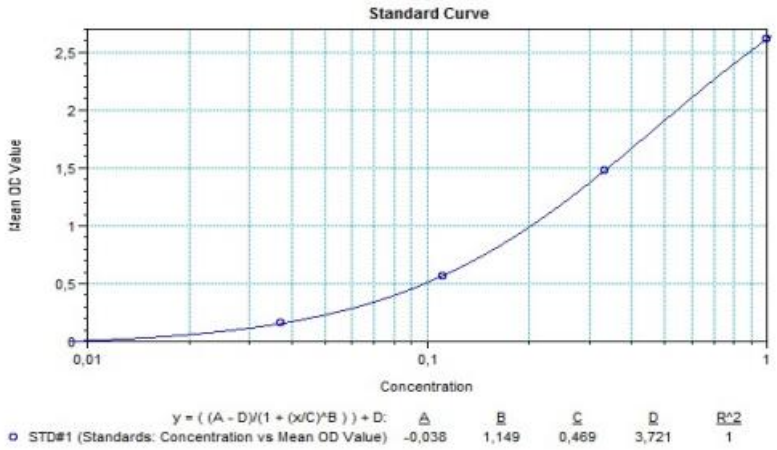
Calculation

1. Using the diluted standards (1000; 300; 100; 30; 0 ng/mL) disregarding zero standard, construct a standard

curve by plotting the OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding Bevacizumab 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 thisstandard curve. Using the absorbance value for each sample, determine the corresponding concentration of bevacizumab 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 bevacizumab 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. To obtain the exact values of the samples, the concentration determined from the standard-curve must be multiplied by the dilution factor (100x). Any sample reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. Therefore, if the pre-diluted samples have been further diluted, the concentration determind from the standard curve must be multiplied by the further dilution factor. E.g.; If the pre-diluted sample further diluted in a ratio of 1:10 then results should be multiplied by 100.
5. Automated method: Computer programs can also generally give a good fit.

Typical Standard Curve



Standard	Concentration (μg/mL)	Mean OD450/650
A	100	2,611
B	30	1,478
C	10	0,565
D	3	0,155
E	0	0,032

Precision

Intra-assay CV: <15% for bevacizumab range 30-1000 ng/mL.

Inter-assay CV: <15% for bevacizumab range 30-1000 ng/mL.

Sensitivity

The lowest detectable level that can be distinguished from the zero standard is less than 30 ng/mL.

Specificity

There is no cross reaction with native serum immunoglobulins.

Recovery

Recovery rate was found to be between 85-115% with normal human serum samples with known concentrations.

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 CD 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 biohazardsin use and for disposal.
10. Some reagents contain sodium azide (NaN3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of waterto avoid azide build-up.