Bevacizumab ELISA
(mAb-based)

Enzyme immunoassay for the specific determination of free Bevacizumab in serum and plasma

REF: IG-AB104

For Research Use Only - Not for Use in Diagnostic Procedures

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Contents

1. Intended Use 2
2. Summary and Explanation 2
3. Principle of the Test 2
4. Warnings and Precautions 2
5. Storage and Stability of the Kit 3
6. Specimen Collection, Handling and Storage 3
7. Contents of the Kit 4
8. Materials Required but not Supplied 4
9. Procedure Notes 4
10. Pre-Test Setup Instructions 5
   10.1. Preparation of Components 5
   10.2. Dilution of Standards and Samples 6
11. Test Procedure 6
   11.1. General Remarks 6
   11.2. Assay Procedure 7
   11.3. Quality Control 7
   11.4. Calculation of Results 8
12. Assay characteristics 9
   12.1. Specificity 9
   12.2. Sensitivity 9
   12.3. Precision 9
   12.4. Recovery 9
13. Automation 9
14. References 10
1. INTENDED USE

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2. SUMMARY AND EXPLANATION

The drug Bevacizumab (trade name Avastin®) is a recombinant human IgG1:k monoclonal antibody specific for all human vascular endothelial growth factor-A (VEGF-A) isoforms and it 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 specificity of this test system is achieved by using a monoclonal antibody named "MAY-2B5" for the coating of the microtiter plate. This antibody is specific for Bevacizumab only and does not cross react with other VEGF catchers.

3. PRINCIPLE OF THE TEST

This ELISA is based on Bevacizumab-specific mouse monoclonal antibody (catcher Ab, ImmunoGuide clone MAY-2B5). Standards and diluted samples are incubated in the microtiter plate coated with IG-MAY-2B5 mAb. After incubation, the wells are washed. A horseradish peroxidase (HRP)-conjugated anti-human IgG monoclonal antibody is added and binds to the Fc part of Bevacizumab. Following incubation, wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of Bevacizumab in the sample or standard. Results of samples can be determined by using the standard curve. Binding of Bevacizumab to the solid phase, pre-coated with MAY-2B5, is inhibited by human VEGF-A in a concentration dependent manner. Therefore, the ImmunoGuide Bevacizumab ELISA (mAb-Based) measures the free form of Bevacizumab.

4. WARNINGS AND PRECAUTIONS

1. 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.

2. In case of severe damage of the kit package, please contact Eagle Biosciences or your supplier in writing, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.

3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
6. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
7. Avoid contact with Stop solution. It may cause skin irritations and burns.
8. If any component of this kit contains human serum or plasma it is indicated and if so, it have been tested and were found to be negative for HIV I/II, HBsAg and HCV. However, the 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.
9. 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 water to avoid azide build-up.

5. STORAGE AND STABILITY OF THE KIT
The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters. The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

6. SPECIMEN COLLECTION, HANDLING AND STORAGE
Serum, Plasma (EDTA, Heparin)

| Storage: | 2-8°C | ≤-20°C (Aliquots) | Keep away from heat or direct sun light
| Stability: | 3 d | 6 mon | Avoid repeated freeze-thaw cycles |

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.
7. CONTENTS OF THE KIT

<table>
<thead>
<tr>
<th>QUANTITY</th>
<th>COMPONENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 12 x 8</td>
<td><strong>Microtiter ELISA Plate</strong>&lt;br&gt;BREAK APART STRIPS COATED WITH ANTI-BEVAZICUMAB MONOCLONAL ANTIBODY.</td>
</tr>
<tr>
<td>5 x 0.3 mL</td>
<td><strong>Bevacizumab Standards A-E, Concentrate (1000X)</strong>&lt;br&gt;200; 60; 20; 6; and 0 µg/mL&lt;br&gt;Used for construction of the standard curve. Contains Bevacizumab, proteins, stabilizer and &lt;15 mM NaN₃.</td>
</tr>
<tr>
<td>1 x 12 mL</td>
<td><strong>Assay Buffer</strong>&lt;br&gt;Blue colored. Ready to use. Contains proteins and &lt;15 mM NaN₃.</td>
</tr>
<tr>
<td>1 x 60 mL</td>
<td><strong>Dilution Buffer, Concentrate (5X)</strong>&lt;br&gt;Contains proteins and &lt;15 mM NaN₃.</td>
</tr>
<tr>
<td>1 x 12 mL</td>
<td><strong>Enzyme Conjugate</strong>&lt;br&gt;Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated anti-human IgG mouse monoclonal antibody, Proclin® and stabilizers.</td>
</tr>
<tr>
<td>1 x 12 mL</td>
<td><strong>TMB Substrate Solution</strong>&lt;br&gt;Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).</td>
</tr>
<tr>
<td>1 x 12 mL</td>
<td><strong>Stop Solution</strong>&lt;br&gt;Ready to use. 1 N Hydrochloric acid (HCl).</td>
</tr>
<tr>
<td>1 x 50 mL</td>
<td><strong>Wash Buffer, Concentrate (20x)</strong>&lt;br&gt;Contains buffer, Tween® 20 and Kathon™.</td>
</tr>
<tr>
<td>2 x 1</td>
<td><strong>Adhesive Seal</strong>&lt;br&gt;For sealing microtiter plate during incubation.</td>
</tr>
</tbody>
</table>

8. MATERIALS REQUIRED BUT NOT SUPPLIED
1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Bidistilled or deionised water and calibrated glasswares (e.g. flasks or cylinders).
3. Wash bottle, automated or semi-automated microtiter plate washing system.
4. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
5. Absorbent paper towels, standard laboratory glass or plastic vials, and a timer.

9. 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 pre-treatment 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 readily at the appropriate time. Allow all reagents and specimens to reach room temperature (20-25 °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 the caps of vials. Always cap not used vials. Do not reuse wells 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. Do not open the pouch until it reaches room temperature. Unused wells should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of Components*

<table>
<thead>
<tr>
<th>Dilute/ dissolve</th>
<th>Component</th>
<th>Diluent</th>
<th>Relation</th>
<th>Remarks</th>
<th>Storage</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL</td>
<td>Wash Buffer</td>
<td>up to 200 mL</td>
<td>1:20</td>
<td>Warm up at 37°C to dissolve crystals. Mix vigorously.</td>
<td>2-8 °C</td>
<td>4 w</td>
</tr>
<tr>
<td>10 mL</td>
<td>Dilution Buffer</td>
<td>up to 50 mL</td>
<td>1:5</td>
<td></td>
<td>2-8 °C</td>
<td>4 w</td>
</tr>
</tbody>
</table>

* Prepare Wash and Dilution Buffers before starting the assay procedure.
10.2. Dilution of Standards and Samples

The dilutions depicted below are examples of how to obtain final 1:1000 dilution. Standards and samples should be properly diluted as homogenous mixture before starting the assay procedure. It is recommended mixing the standards and samples well to homogenous solution at each dilution step. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

1. 10 µL of standard or sample added to 90 µL of 1X dilution buffer, giving the total volume of 100 µL and a dilution of 1:10.
2. 10 µL of 1:10 diluted standard or sample added to 990 µL of 1X dilution buffer, giving the total volume of 1000 µL and a dilution of 1:1000.
3. Samples with a drug concentration above the measuring range should be rated as “>highest standard”. The result should not be extrapolated. The sample in question should be further diluted with 1X Dilution Buffer and then retested.

11. TEST PROCEDURE
11.1. GENERAL REMARKS
11.1.1. Before performing the assay, samples and assay kit should be brought to room temperature (about 30 minutes beforehand) and ensure the homogeneity of the solution.
11.1.2. All Standards should be run with each series of unknown samples.
11.1.3. Standards should be subject to the same manipulations and incubation times as the samples being tested.
11.1.4. All steps of the test should be completed without interruption.
11.1.5. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.
**11.2. ASSAY PROCEDURE**

1. Pipette 100 µl of **Assay Buffer** into each of the wells to be used.

2. Pipette 50 µL of each **1:1000 Diluted Standard, and 1:1000 Diluted Samples** into the respective wells of the microtiter plate.
   - Wells
     - A1: Standard A
     - B1: Standard B
     - C1: Standard C
     - D1: Standard D
     - E1: Standard E
     - F1 and so on: Samples (Serum/Plasma)

3. Cover the plate with adhesive seal. Shake plate carefully. **Incubate 60 min at room temperature (RT, 20-25°C).**

4. Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 µL of **Diluted Wash Buffer** per well. Remove excess solution by tapping the inverted plate on a paper towel.

5. Pipette 100 µL of **Enzyme Conjugate** (HRP-anti human IgG mAb) into each well.

6. Cover plate with adhesive seal. Shake plate carefully. **Incubate 30 min at RT.**

7. Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 µL of **Diluted Wash Buffer** per well. Remove excess solution by tapping the inverted plate on a paper towel.

8. Pipette 100 µL of Ready-to-Use **TMB Substrate Solution** into each well.

9. **Incubate 10 min** at RT. Avoid exposure to direct sunlight.

10. 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.

11. Measure optical density (OD) with a photometer at 450 nm (Reference at OD620 nm is optional) within 15 min after pipetting the Stop Solution.

**11.3. QUALITY CONTROL**
The test results are only valid 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/controls must be found within the acceptable ranges. 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 reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.
11. 4. CALCULATION OF RESULTS
A standard curve should be calculated using the standard concentration (X-axis) versus the OD450 (or OD450/620) values (Y-axis). This can be done manually using graph paper or with a computer program. Concerning the data regression by computer, it is recommended to primarily use the “4 Parameter Logistic (4PL)” or alternatively the “point-to-point calculation”. In case of manual plot there are 2 options: Semilog graph (see Fig. A) or linear graph (see Fig. B). Semilog graph paper is available at [http://www.papersnake.com/logarithmic/semilogarithmic/](http://www.papersnake.com/logarithmic/semilogarithmic/).

The concentration of the samples can be read from the standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 1000). If any diluted sample is reading greater than the highest standard, it should be further diluted appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation the final result. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

Typical Calibration Curve
(All steps were performed at 23°C. Just an example. Do not use it for calculation!)

<table>
<thead>
<tr>
<th>1:1000 Diluted Standard</th>
<th>A (ng/mL)</th>
<th>B (ng/mL)</th>
<th>C (ng/mL)</th>
<th>D (ng/mL)</th>
<th>E (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>200</td>
<td>60</td>
<td>20</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Mean OD450/620 nm</td>
<td>2.565</td>
<td>0.998</td>
<td>0.382</td>
<td>0.132</td>
<td>0.013</td>
</tr>
</tbody>
</table>

![Fig. A](Fig.A) ![Fig. B](Fig.B)
12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY
There is no cross reaction with any other proteins present in native human serum. A screening test was performed with 48 different native human sera. All produced OD450/620 nm values (ranged from 0.022 to 0.042) less than the mean OD (0.132) of standard D (6 ng/mL). In addition, binding of Bevacizumab is inhibited by recombinant human VEGF-A in a concentration dependent manner. Therefore, the ImmunoGuide Bevacizumab ELISA (mAb-Based) measures the biologically active free form of Bevacizumab, i.e. not pre-occupied by VEGF. No cross reaction was observed with sera spiked with the other therapeutic antibodies including Infliximab, Adalimumab, Etanercept, Rituximab, Tocilizumab, Trastuzumab, Aflibercept and Golimumab at concentrations up to 2 mg/mL. All produced mean OD450/620 nm values ranged from 0.009 to 0.022. In addition, there is no cross reaction with Ranibizumab as well. Because when anti-human kappa monoclonal antibody was used as the conjugate instead, Ranibizumab did not bind to MAY-2B5 mAb-coated plate.

12.2. SENSITIVITY
The lowest detectable level that can be clearly distinguished from the zero standard is 2 ng/mL (zero standard +2SD read from the curve) under the above-described conditions.

12.3. PRECISION
Intra-assay CV: <10%.
Inter-assay CV: <10%.

12.4. RECOVERY
Recovery rate was found to be >95% with native human serum and plasma samples when spiked with exogenous Bevacizumab at 200 µg/mL, 60 µg/mL, 20 µg/mL or 6 µg/mL.

13. AUTOMATION
The ImmunoGuide Bevacizumab ELISA (mAb-based) is suitable also for being used by an automated ELISA processor.

14. REFERENCES


Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.