ImmunoGuide®

Instructions for Use

Antibody to Bevacizumab ELISA

**Enzyme immunoassay for the determination
of free antibodies to Bevacizumab in serum and plasma**

REF: IG-BB104

12X8   **2-8°C**

CE   IVD

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1

Contents   **Page**

1. INTENDED USE

Enzyme immunoassay for the determination of free antibodies to Bevacizumab in serum and plasma.

2. SUMMARY AND EXPLANATION

The drug Bevacizumab (trade name Avastin$^{®}$) is a recombinant human IgG1:κ 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. As with all therapeutic proteins, there is a potential for immunogenicity. According to the manufacturer's product insert; the incidence of antibody development in patients receiving Bevacizumab has not been adequately determined because the assay sensitivity was inadequate to reliably detect lower titers. The ImmunoGuide 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.

3. PRINCIPLE OF THE TEST

This *ImmunoGuide* anti-drug antibody(ies) (ADA) kit is a bridging type ELISA for the determination of free antibodies against the drug Bevacizumab in serum and plasma samples. During the first incubation period, ADA in serum or plasma samples are captured by the drug coated on the microtiter wells. After washing away the unbound components from samples, a peroxidase-labelled drug conjugate is added and then incubated. ADA, if present in sample, will make a bridge, with its identical Fab arms, between the drug coated on the well and the other drug molecule labelled with peroxidase. After a second washing step, the bound enzymatic activity is detected by addition of tetramethylbenzidine (TMB) chromogen-substrate. Finally, the reaction is terminated with stop solution. The positive reaction is expected to be related to the presence of ADA in the sample.

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 *AybayTech* 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 has 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 preservatives. In case of contact with eyes or skin, flush immediately with water.

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)*

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 (Aliquots)	Keep away from heat or direct sun light
Stability:	3 d	6 mon	Avoid repeated freeze-thaw cycles

* Drug administration/infusion may camouflage/mask the presence of anti-drug antibodies (ADA) in serum/plasma samples. Therefore, blood sampling time is also critical for detection of ADA. It is proposed to obtain blood sample just before administration of the drug.

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	**Microtiter Plate** Break apart strips pre-coated with the drug Bevacizumab.
5 x 1 mL	**Anti-Drug Antibody (ADA) Standards** Standard A-B-C-D-E Lyophilized. Used for construction of the standard curve. Contains anti-drug antibody, preservative and stabilizer. The quantity of anti-drug is indicated on the vial label and/or CoA datasheet. The reconstituted standards can be reused several times.
1 x 1 mL	**Positive Control.** Lyophilized.Green colored. Contains antibody against drug, preservative and stabilizer. The quantity of anti-drug is indicated on the CoA datasheet. The reconstituted Positive Control can be reused several times.
1 x 2 mL	**Calibrator** Yellow colored. Ready to use. Contains proteins and preservative.
1 x 50 mL	**Dilution Buffer** Orange colored. Ready to use. Contains proteins and preservative.
1 x 12 mL	**Assay Buffer** Blue colored. Ready to use. Contains proteins and preservative.
1 x 12 mL	**Enzyme Conjugate** Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated Bevacizumab, Proclin® and stabilizers.
1 x 12 mL	**TMB Substrate Solution** Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).
1 x 12 mL	**Stop Solution** Ready to use. 1 N Hydrochloric acid (HCl).
1 x 50 mL	**Wash Buffer**, Concentrate (20x) Contains buffer, Tween® 20 and Kathon™.
3 x 1	**Adhesive Seal** For sealing microtiter plate during incubation.

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

*ImmunoGuide recommends the use of ultrapure water (water type 1; ISO3696), which is free of undissolved and colloidal ions and organic molecules (free of particles >0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25°C ($\geq$ 18.2 MΩ cm).

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*

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

* Prepare Wash Buffer before starting the assay procedure.

10.2. Reconstitution of Lyophilized Reagents (Standards and Positive Control) and their Stability/Storage.

10.2.1. The lyophilized powder (non-reconstituted) reagents are stable at 2-8°C until the expiry date indicated on the label. Before use, the lyophilized Standards and Positive Control have to be reconstituted with 1 mL of ultrapure water. After addition of ultrapure water, recap vials and allow the vial content to stand for 5 minutes at room temperature (RT) before mixing. After 5 minutes, the vial contents should be mixed by gentle shaking several times to ensure complete reconstitution. Again, as a second time, the vial content should be allowed to dissolve for a further 15 minutes at RT and then mixed thoroughly before use.

10.2.2. The whole plate can be used at once or part by part within a week period of time after reconstitution of standards and positive controls.

10.2.3. If the whole 96-well plate will be not used at once or in parts within a one week time period after reconstitution of lyophilized reagent, then follow the instructions indicated below.

10.2.3.1. Reconstituted Reagents are stable for one week when stored at 2-8°C.

10.2.3.2. If a longer period of stability (up to 12 months) is desired, then the reconstituted reagents should firstly be alliquoted (e.g.each one could be 120 microliter per polypropylen vial or eppendorf with the cap closed) and then stored at temperatures less than -50°C.

10.2.3.3. Repeated freze-thaw-cycles must be avoided.

10.2.3.4. It is highly recomended to perform each run with a freshly thawed set of standard controls that are frozen in alliquotes after reconstitution.

10.2.3.5. Calibrator is a ready to use liquid and should NOT be frozen, instead must be stored at 2-8°C.

10.3. Dilution of Samples*.

Sample	To be diluted	With	Remarks
Serum/ Plasma	1:2	Dilution Buffer	50 μL Serum/Plasma + 50 μL Dilution Buffer

*. Transfer 50 μL of sample and 50 μL of Dilution Buffer into each reaction tube and then mix thoroughly.

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/Controls should be run with each series of unknown samples.

11.1.3. Standards/Controls 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.

11.1.6. The total pipetting time needed for dispensing all samples into the wells should not exceed 5 minutes. If this is difficult to achieve the samples should be pre-dispensed in a separate neutral polypropylene microplate and then transferred into the reaction ELISA plate by a multi channel pipette.

11.2. ASSAY PROCEDURE

1.	Pipette **100 µl** of **Assay Buffer** into each of the wells to be used.
2.	Pipette **50 µL** of **Reconstituted Standards, Positive Control (Reconstitution of these reagents is described in Setion 10.2), Ready-to-Use Calibrator and 1:2 Diluted Samples (Dilution of sample is described in section 10.3)** 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: Positive Control G1: Calibrator H1: Calibrator A2 and so on: 1:2 Diluted Samples (Serum/Plasma)
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. **Incubate the plate on a bench top for 60 min** at **room temperature (RT, 20-25°C)**.
4.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate **4 X 350 µL** of **Diluted Wash Buffer** per well. Remove excess solution by tapping the inverted plate gently on a paper towel.
5.	Pipette **100 µL** of **Enzyme Conjugate** (HRP-drug) into each well.
6.	Cover plate with adhesive seal. Shake plate carefully. **Incubate 60 min** at RT.
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate **4 X 350 µL** of **Diluted Wash Buffer** per well. Remove excess solution by tapping the inverted plate gently on a paper towel.
8.	Pipette **100 µL** of Ready-to-Use **TMB** Substrate Solution into each well.
9.	**Incubate 15 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 only** 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 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 reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

11. 4. CALCULATION OF RESULTS
11.4.1. QUALITATIVE INTERPRETATION

The qualitative analysis is performed based on the Cut-Off value which is determined by the mean OD value of Calibrators studied duplicate and then followed by multiplication with a predefined constant value. The Cut-Off value is compatible with the "Limit of Detection (LoD)". And the LoD-compatible Cut-Off is estimated on 120 samples and evaluated according to NCCLS publication EP17-A, Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline and as briefly outlined below.

• $LoD = LoB + 1.645\ \sigma s$.
Where LoB and σS are the value of Limit of Blank and standard deviation of the population of the low sample measurements, respectively.
• $LoB = \mu B + 1.645\ \sigma B$
Where μB and σB are the mean and standard deviation of the blank measurements, respectively

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The results are evaluated by dividing each individual Optical Density (OD) result of 1:2 diluted patient's sample by the Cut-Off Value. The Cut-Off Value is determined as described below:

Mean OD of Calibrator (Mean OD value of two wells used for Calibrator) = (The OD of the first well used for Calibrator + The OD of the second well used for Calibrator) / 2
(i.e. The sum OD450nm is divided by 2)
Cut-Off Value =Mean OD of Calibrator multiplied by a pre-determined constant value

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Cut-Off Value = Mean OD of Calibrator X 1.8

11.4.1.2. If the "1:2 diluted sample OD450nm" is less than (<) the "Cut-Off Value", the sample is regarded as NEGATIVE for Anti-Drug-Antibody (ADA) specific for the drug in concern.

11.4.1.3. If the "1:2 diluted sample OD450nm" is equal and higher than ($\geq$) the "Cut-Off Value", the sample is regarded as POSITIVE for Anti-Drug-Antibody (ADA) specific for the drug in concern.

And if required, the OD of 1:2 pre-diluted positive samples may be plotted on the standard curve for quantitative analysis.

OD450nm of 1:2 Diluted Patient Sample	Interpretation
$\geq$ Cut-Off Value	POSITIVE
< Cut-Off Value	NEGATIVE

11.4.2 QUANTITATIVE INTERPRETATION

11.4.2.1. For the calculation of the positive sample concentration, polynomial regression is recommended. Standard curve is constructed by plotting the units of the 5 standard points (ng/mL) along the abscissa (X axis) and the corresponding OD450nm values along the ordinate (Y axis).

11.4.2.2. The positive control value should be comprised into the range indicated on the vial label.

11.4.2.3. The Anti-Drug Antibody (ADA) concentration of positive samples can be reported based on the principles indicated below.

11.4.2.4. The ADA concentration of pre-diluted (at the ratio of 1:2) positive samples can be directly read on the standard curve. In order to report the ADA concentration for the corresponding undiluted positive sample, the value must be multiplied by the factor of 2. Example: If a 1:2 pre-diluted positive sample results in an ADA concentration of 100ng/mL on the standard curve, then its undiluted sample value corresponds to 200ng/mL (100ng/mL x 2 = 200ng/mL).

11.4.2.5. The 1:2 diluted positive samples with OD450nm value higher than that of the highest standard supplied could be further diluted, e.g. 1:20, using Dilution Buffer and then retested to obtain more precise result (see below). In such a situation, the number of units should be multiplied by the selected dilution factor.

Dilution of a high positive sample at 1:20:

| Sample	To be diluted	With	Remarks
Serum/ Plasma	1:20	Dilution Buffer	10 μL Serum/Plasma + 190 μL Dilution Buffer

11.4.2.6. If any diluted positive sample is still reading OD450nm higher than that of highest standard supplied, it should be further diluted (in such a case 1:200 dilution of the sample is proposed) appropriately with Dilution Buffer and then retested. Also this second dilution has to be used for calculation of the final result. It is recommended that each laboratory determines the best dilution ratio for their samples in order to minimize retesting.

11.4.2.7. Rarely some 1:2 diluted positive samples may produce a value less than the sensitivity level of the assay. In such a situation, it is highly recommended to repeat the test with a new patient's sample obtained at another time but just before the time of drug administration.

Typical Calibration Curve
(Example. Do not use it for calculation!)

Standard	[C] (ng/mL)	Mean OD450nm
A	1000	1,209
B	300	0,646
C	100	0,286
D	30	0,120
E	0	0,052
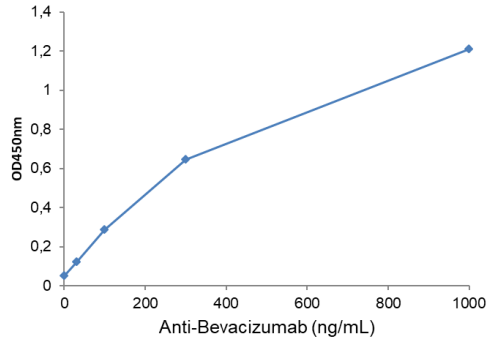
12. ASSAY CHARACTERISTICS

12.1. ANALYTICAL SENSITIVITY-THRESHOLD VALUE
•The Detection Threshold for the assay is 20 ng/mL.
•The assay sensitivity for undiluted clinical samples corresponds to 40 ng/mL Because the serum or plasma samples are instructed to be diluted at two-fold (1:2) before starting the assay.

12.2. ASSAY RANGE
For anti-Bevacizumab antibodies in serum and plasma, the method has been demonstrated to be highly linear.

12.3. ASSAY PRECISION
Intra-assay CV:

Mean	CV (%)
1000	<10
300	<10
100	<10

Inter-assay CV: <10%.

12.4. ASSAY RECOVERY
Recovery rate was found to be 85-115% using native serum and plasma samples spiked with exogenous Anti Drug Antibody (ADA) positive samples.

13. AUTOMATION
The *ImmunoGuide* ADA ELISA is suitable also for being used by an automated ELISA processor.

14. SPECIAL NOTES ABOUT ANTI DRUG ANTIBODY (ADA) & ELISA

The detection of ADA formation is highly dependent on the sensitivity and specificity of the assay used. The initial screening assay should be sensitive to low and high-affinity ADA. Endogenous and exogenous components in serum or plasma may influence assay results. Measuring immune responses to therapeutic protein products that possess Ig tails, such as mAb and Fc-fusion proteins, may be particularly difficult when RF is present in serum or plasma.

If the serum/plasma sample of a patient, a candidate for a specific monoclonal drug treatment, is negative at the time before starting drug therapy and become positive during treatment, it could be concluded that the induction of the specific ADA is suspected in this individual. As shown in Figure 1, the true positive reaction of the sample is inhibited by pre-incubation of the sample with the specific drug itself (samples are spiked with certain amount of drug). However, inhibition was not observed in false positive reaction related with other components in sample (Figure 1).

If it is desired to further confirm the specificity of the ADA positive signal, ImmunoGuide suggests a "Confirmatory Assay (competitive drug inhibition test)": Confirmatory Assay is performed by retesting the positive sample after dilution (at 1:2-1:10) with Dilution Buffer, 0.9% NaCl solution or phosphate buffered saline containing the drug in concern at a concentration of 50µg/mL that is exogenously added.

Figure 1.
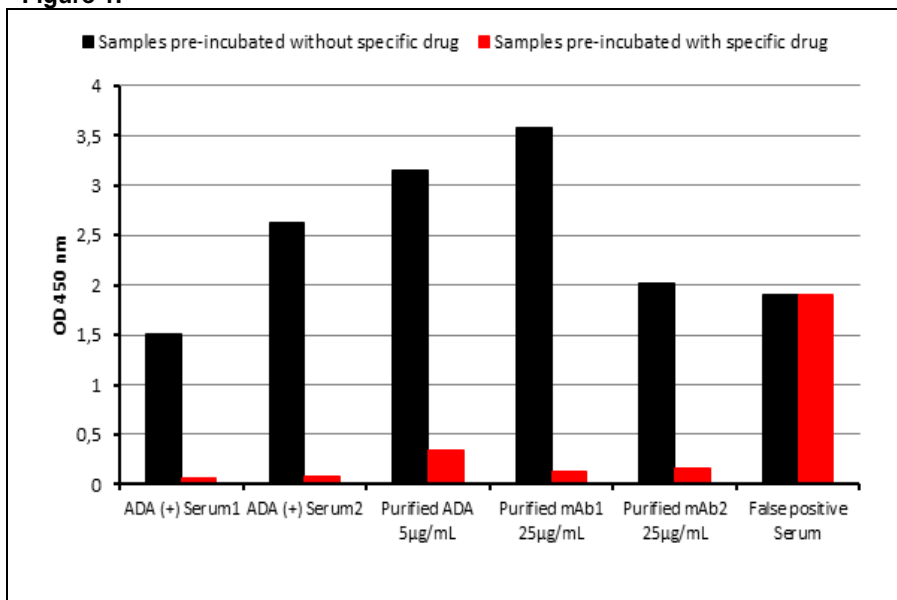
Generally, the sensitivity of the ADA assay is calculated as the lowest concentration of the positive control that can consistently generate a positive signal. However, as shown in Figure 2 below, data obtained at the *ImmunoGuide* Laboratory, the assay sensitivity also differs significantly depending on the high vs low affinity antibody used for the construction of the standard curve in the assay system. For example, when an immunoaffinity-purified ADA was used, it was observed that the lowest detectable level that can be clearly distinguished from the negative control value is somewhere around 10ng/mL. It is much more lower when mAb2 is used instead.

In any case this number is highly depending on the characteristics of the ADA under investigation. Therefore this number may be significantly different for each individual sample under investigation. Therefore, it is best to keep up using the same ADA for constructing the standard curve.

Figure 2.
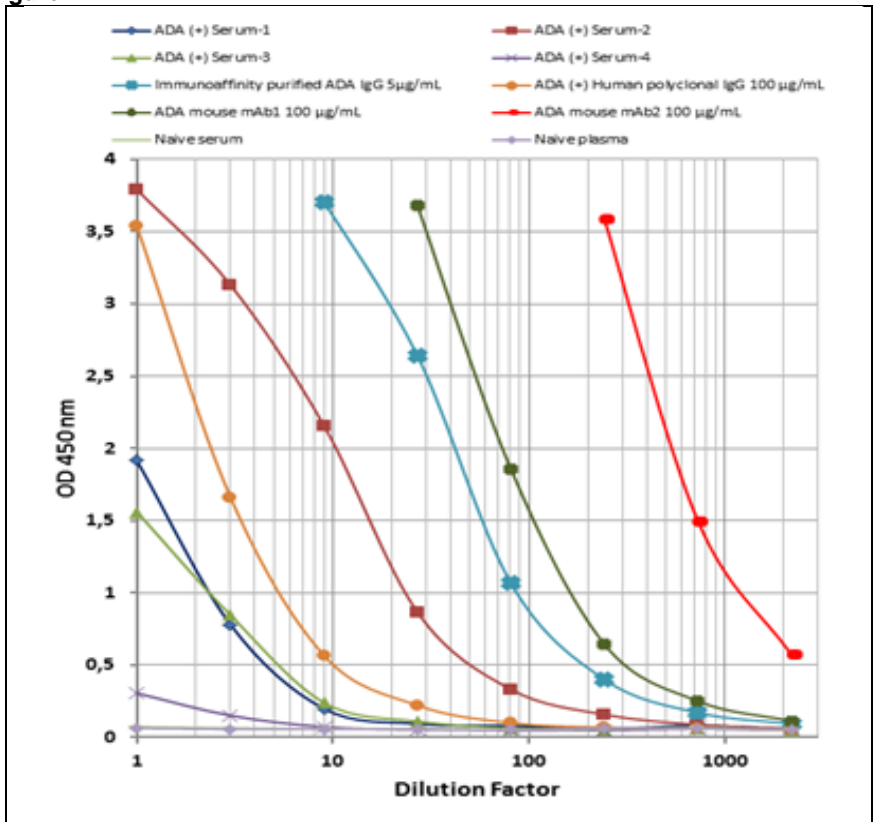
15. REFERENCES

1. Han K, Peyret T, Quartino A, Gosselin NH, Gururangan S, Daw NC, Navid F, Jin J, Allison DE. Bevacizumab dosing strategy in pediatric cancer patients based on population pharmacokinetic analysis with external validation. Br J Clin Pharmacol. 2015 Sep 7. doi: 10.1111/bcp.12778.

2. Avery RL, Castellarin AA, Steinle NC, Dhoot DS, Pieramici DJ, See R, Couvillion S, Nasir MA, Rabena MD, Le K, Maia M, Visich JE. Systemic pharmacokinetics following intravitreal injections of ranibizumab, bevacizumab or aflibercept in patients with neovascular AMD. Br J Ophthalmol. 2014 Dec;98(12):1636-41.

3. Bunni J, Shelley-Fraser G, Stevenson K, Oltean S, Salmon A, Harper SJ, Carter JG, Bates DO. Circulating levels of anti-angiogenic VEGF-A isoform (VEGF-Axxxb) in colorectal cancer patients predicts tumour VEGF-A ratios. Am J Cancer Res. 2015 May 15;5(6):2083-9.

4. Imbs DC, Négrier S, Cassier P, Hollebecque A, Varga A, Blanc E, Lafont T, Escudier B, Soria JC, Pérol D, Chatelut E. Pharmacokinetics of pazopanib administered in combination with bevacizumab. Cancer Chemother Pharmacol. 2014 Jun;73(6):1189-96.

5. Stewart MW, Rosenfeld PJ, Penha FM, Wang F, Yehoshua Z, Bueno-Lopez E, Lopez PF. Pharmacokinetic rationale for dosing every 2 weeks versus 4 weeks with intravitreal ranibizumab, bevacizumab, and aflibercept (vascular endothelial growth factor Trap-eye). Retina. 2012 Mar;32(3):434-57.

6. Shields LB, Shelton BJ, Shearer AJ, Chen L, Sun DA, Parsons S, Bourne TD, LaRocca R, Spalding AC. Dexamethasone administration during definitive radiation and temozolomide renders a poor prognosis in a retrospective analysis of newly diagnosed glioblastoma patients. Radiat Oncol. 2015 Oct 31;10(1):222. doi: 10.1186/s13014-015-0527-0.

7. Li Q, Yan H, Zhao P, Yang Y, Cao B. Efficacy and Safety of Bevacizumab Combined with Chemotherapy for Managing Metastatic Breast Cancer: A Meta-Analysis of Randomized Controlled Trials. Sci Rep. 2015 Oct 27;5:15746. doi: 10.1038/srep15746.

8. Li E, Subramanian J, Anderson S, Thomas D, McKinley J, Jacobs IA. Development of biosimilars in an era of oncologic drug shortages. Drug Des Devel Ther. 2015;9:3247-55.

9. Tang N, Guo J, Zhang Q, Wang Y, Wang Z. Greater efficacy of chemotherapy plus bevacizumab compared to chemo- and targeted therapy alone on non-small cell lung cancer patients with brain metastasis. Oncotarget. 2015 Oct 20. doi: 10.18632/oncotarget.6184.

10. Ogata H, Kikuchi Y, Natori K, Shiraga N, Kobayashi M, Magoshi S, Saito F, Osaku T, Kanazawa S, Kubota Y, Murakami Y, Kaneko H. Liver Metastasis of a Triple-Negative Breast Cancer and Complete Remission for 5 Years After Treatment With Combined Bevacizumab/Paclitaxel/Carboplatin: Case Report and Review of the Literature. Medicine (Baltimore). 2015 Oct;94(42):e1756. doi: 10.1097/MD.0000000000001756.

15