



**EAGLE**  
BIOSCIENCES

# Aflibercept ELISA Assay Kit

Catalog Number:

**IG-AA115 (1 x 96 wells)**

For Research Use Only. Not for use in diagnostic procedures.

v. 3.0 (15 APR 24)

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Eagle Biosciences, Inc.  
20A Northwest Blvd., Suite 112, Nashua, NH 03063  
Phone: 617-419-2019 Fax: 617-419-1110  
[www.EagleBio.com](http://www.EagleBio.com)



## **INTENDED USE**

The Aflibercept ELISA Assay Kit is an enzyme immunoassay for the quantitative determination of free Aflibercept in serum and plasma. The Aflibercept ELISA Assay Kit is for research use only and not for use in diagnostic procedures.

## **INTRODUCTION**

The drug Aflibercept (trade names Eylea<sup>®</sup>, Zaltrap<sup>®</sup>) is a recombinant fusion protein consisting of VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 fused to the Fc portion of human IgG1. Aflibercept has broad affinity for all ligands that bind to these receptors, including isoforms of VEGF-A, VEGF-B, and placental growth factors. Aflibercept demonstrated antitumor effects and antiangiogenic activity as a single agent and enhanced activity in combination with chemotherapy. Identification of biomarkers for (non-)response and risk factors for adverse drug reactions that might be related to serum concentrations and maintaining the effective concentration of Aflibercept in order to potentially avoid some side effects with a reliable method might be beneficial.

## **ASSAY PRINCIPLE**

This ELISA assay is based on sandwich type ELISA. Diluted standards and samples (serum or plasma) are incubated in the microtiter plate coated with recombinant human vascular endothelial growth factor-A (rhVEGF-A). 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 Aflibercept pre-captured by the rhVEGF-A on the surface of the wells. Following incubation, the wells are washed, and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of Aflibercept in the sample or standard. Results of samples can be determined by using the standard curve.

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

### 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 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.

### 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. Avoid repeated freeze-thaw cycles.
Stability:	3 d	6 mon	

### CONTENTS OF THE KIT

Part	Description	Quantity
Microtiter ELISA Plate	Break apart strips coated with recombinant human vascular endothelial growth factor-A (rhVEGF-A).	1 x 12 x 8
Aflibercept Standards A-E, Concentrate (10X)	2000; 600; 200; 60; and 0 ng/mL Used for construction of the standard curve. Contains Aflibercept, proteins, preservative, and stabilizer.	5 x 0.5 mL
Assay Buffer	Blue colored. Ready to use. Contains proteins and preservative.	1 x 12 mL
Dilution Buffer, Concentrate (5X)	Contains orange dye, proteins, and preservative.	1 x 60 mL
Enzyme Conjugate	Red colored. Ready to use. Contains horseradish peroxidase (HRP)-conjugated anti-human IgG mouse monoclonal antibody, 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 12 mL
Wash Buffer, Concentrate (20x)	Contains buffer, Tween® 20 and Kathon™.	1 x 50 mL
Adhesive Seal	For sealing microtiter plate during incubation.	2 x 1



## MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettes (< 3% CV) and tips to deliver 5-1000  $\mu$ L.
- Bidistilled or deionized water and calibrated glasswares (e.g. flasks or cylinders).
- Wash bottle, automated or semi-automated microtiter plate washing system.
- Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
- Absorbent paper towels, standard laboratory glass or plastic vials, and a 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 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  $^{\circ}$ 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 Micro pipettor 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.

## PRE-TEST SETUP INSTRUCTIONS

### 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
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 C	4 w

\*Prepare Wash and Dilution Buffer before starting the assay procedure.

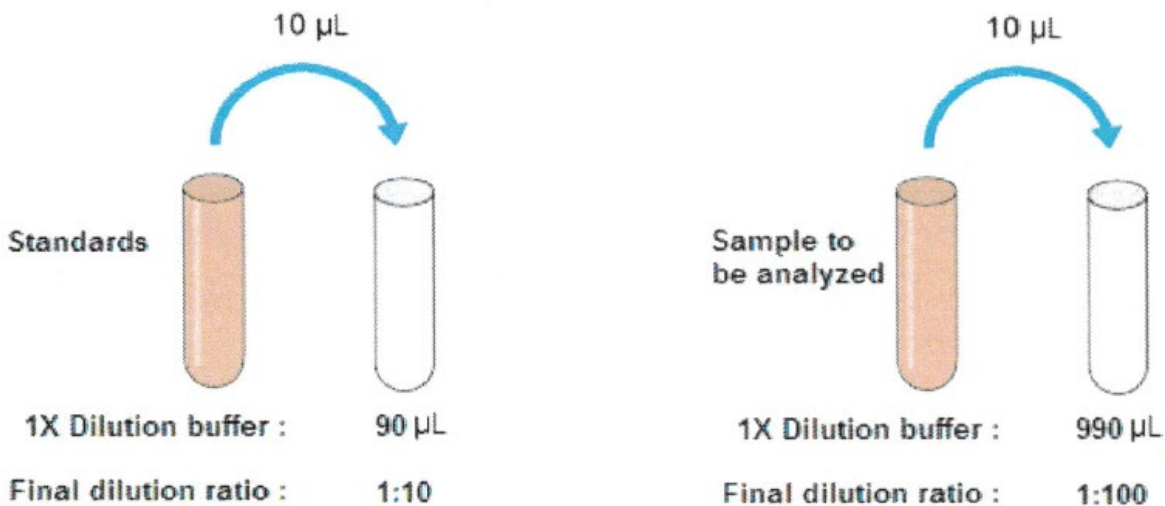


## Dilutions of Standards and Samples

The dilutions depicted below are examples of how to obtain final dilutions for standards and samples. 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  $\mu\text{L}$  of standard added to 90  $\mu\text{L}$  of 1X dilution buffer, giving the total volume of 100  $\mu\text{L}$  and a dilution of 1:10.
2. 10  $\mu\text{L}$  of sample added to 990  $\mu\text{L}$  of 1X dilution buffer, giving the total volume of 1000  $\mu\text{L}$  and a dilution of 1:100.
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.

### Standard/Sample Dilution



### TEST PROCEDURE

- 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.
- All Standards should be run with each series of unknown samples.
- Standards should be subject to the same manipulations and incubation times as the samples being tested.
- All steps of the test should be completed without interruption.
- Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.
- 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.



## ASSAY PROCEDURE

1. Pipette **100 µL** of **Assay Buffer** into each of the wells to be used
2. Pipette **75 µL** of each **1:10 Diluted Standard, and 1:100 Diluted Samples** into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided.

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 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 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 by tapping several times. **Incubate the plate on a bench top for 30 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 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** (Reference at OD620 nm is optional) within **15 min** after pipetting the Stop Solution.

## 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.

## CALCULATION OF RESULTS

A standard curve should be constructed 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/>.

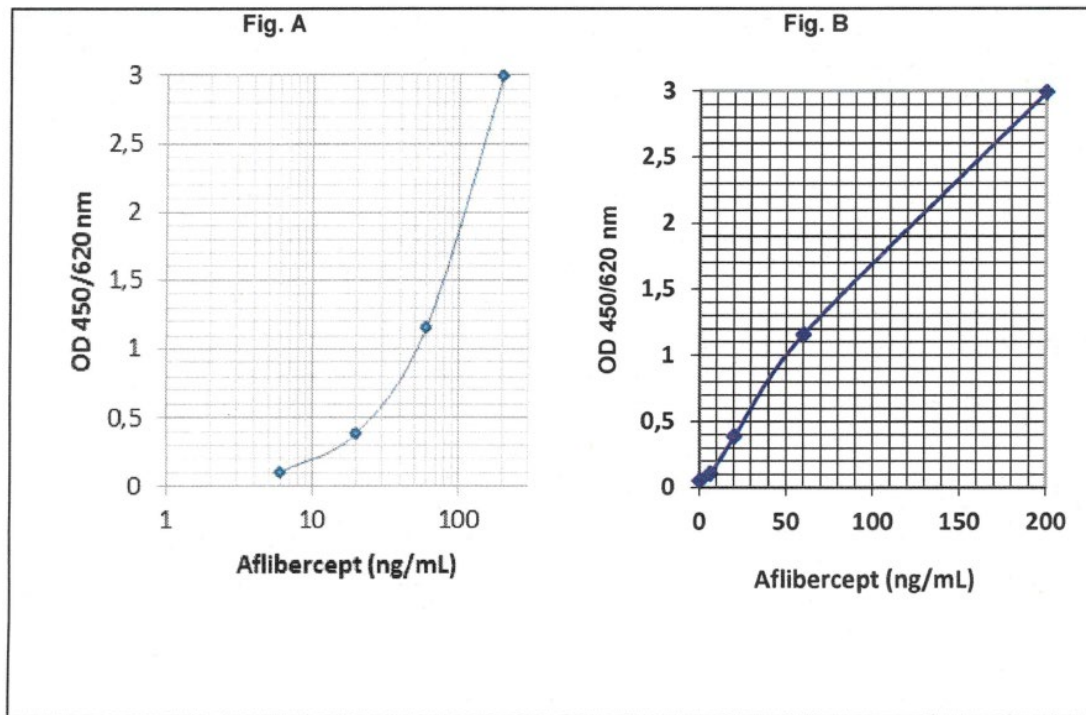
The concentration of the samples can be read from this standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 100). If any diluted sample is reading greater than the highest standard, it should be further diluted (in such a case, a 1:500 final dilution of the sample is proposed) appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of 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!)

1:10 Diluted Standard	A	B	C	D	E
Concentration (ng/mL)	200	60	20	6	0
Mean OD <sub>450/620 nm</sub>	2.989	1.155	0.387	0.103	0.048

### Typical Graph



### ASSAY CHARACTERISTICS

#### 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 OD<sub>450/620 nm</sub> values less than the mean OD of standard D. No cross reaction was observed with the other therapeutic antibodies tested (Tocilizumab, Cetuximab, Etanercept, Infliximab, Rituximab and Trastuzumab) at



concentrations up to 2 mg/mL. However, Bevacizumab resulted in full cross- reaction as expected. Ranibizumab, another anti-VEGF therapeutic antibody consisted of Fab fragment only, produced a mean OD less than the mean OD of standard D.

**Sensitivity:**

The lowest detectable level that can be clearly distinguished from the zero standard is 5 ng/mL (zero standard +2SD read from the curve) under the above- described conditions. Analytical sensitivity is 5 ng/mL, and corresponding to the detection limit (limit of quantification) of 0.5 µg/mL for undiluted clinical samples because the serum or plasma samples are instructed to be diluted at 1:100 before starting the assay

**Precision:**

Intra-assay CV: <10%.

Inter-assay CV: <10%.

**Recovery:**

Recovery rate was found to be >95% with native serum and plasma samples when spiked with exogenous Aflibercept.

**Automation**

The Aflibercept ELISA is suitable also for being used by an automated ELISA processor.

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