User Manual

PathHunter® Aflibercept Bioassay HS Kit

For Measuring Aflibercept-Mediated Inhibition of VEGF Receptor Dimerization

For Bioassay Kits:

93-0996Y1-00153: 2-Plate Kit

93-0996Y1-00154: 10-Plate Kit

Document Number 70-424 Revision 1

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Table of Contents

Important: Please read this entire user manual before proceeding with the assay.

Overview 1

Assay Principle 1

Materials Provided 2

Storage Conditions 2

Additional Materials Required 3

Protocol Schematic 4

Detailed Protocol 5

- Day 1: Bioassay Cell Preparation 5
- Day 1: Control Agonist Preparation 6
- Day 1: Aflibercept or Biosimilar Preparation 7
- Day 2: Detection 10

Typical Results 11

Troubleshooting Guide 12

Document Revision History 13

Limited Use License Agreement 14

Contact Information 14

Table of Figures

Figure 1. Assay Principle 1

Figure 2. Agonist Serial Dilutions. 6

Figure 3. Aflibercept Dilution Series 8

Figure 4. Representative Assay Plate Map 9

Figure 5. Typical Results 11

For additional information or Technical Support, see contact information at the bottom of this page.

Overview

PathHunter Aflibercept Bioassay HS Kit delivers a robust, highly sensitive, and easy-to-use functional cell-based assay to determine Aflibercept drug potency and detect neutralizing antibodies. This bioassay HS kit has been optimized further to deliver reproducible results with lower variability. The bioassay kit contains all the materials needed for a complete assay, including cryopreserved cells, detection reagents, cell plating reagent, positive control agonist, and assay plates. The ready-to-use cryopreserved cells have been manufactured to ensure assay reproducibility and faster implementation from characterization to lot release. This bioassay has been optimized and qualified with the marketed biologic drug, Eylea® which is not provided in the bioassay kit.

Eylea® is a registered trademark of Regeneron Pharmaceuticals, Inc.

Assay Principle

The PathHunter Aflibercept bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, the ProLink™ (PK), and the Enzyme Acceptor (EA). Independently, these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

This bioassay is an application of the Eurofins DiscoverX dimerization assay platform. The assay is designed to detect VEGF-A-induced functional homodimerization of the kinase insert domain (KDR), also known as the VEGFR2 receptor. Activation of the KDR receptor through VEGF-A leads to receptor dimerization, which is an essential event in the receptor's signaling cascade. The bioassay cells have been engineered to co-express KDR fused to PK, and another KDR construct fused to EA. Receptor dimerization forces the two enzyme fragments to complement, resulting in the formation of a functional β-gal enzyme. The enzyme hydrolyzes a substrate to generate a chemiluminescent signal. Aflibercept binds to and traps the VEGF-A ligand, preventing it from activating the VEGFR2 receptors. This results in a dose-dependent reduction of the signal.

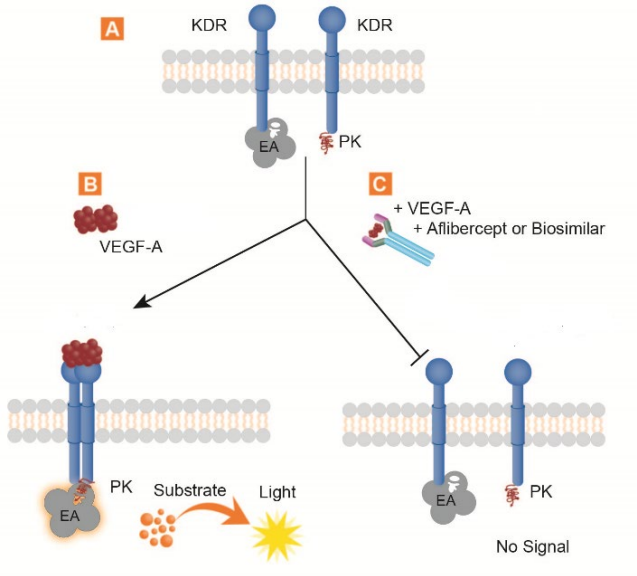


Figure 1. Assay Principle: Interaction of the ligand with the receptor causes receptor dimerization, forcing the complementation of the two β-gal fragments which forms an active enzyme capable of hydrolyzing a substrate. On the other hand, blocking the ligand with aflibercept or a similar ligand-trap prevents receptor dimerization, resulting in a loss of signal.

Materials Provided

List of Components	93-0996Y1-00153	93-0996Y1-00154
PathHunter HEK 293 KDR/KDR Bioassay Cells (1.2×10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 0 Reagent (100 mL per bottle)	1	3
Recombinant Human $VEGF_{165}$ (10 µg per vial)	1	1
AssayComplete Protein Dilution Buffer (50 mL per bottle)	1	2
PathHunter Bioassay Detection Kit Detection Reagent 1 (Bottle) Detection Reagent 2 (Bottle)	 1 x 3 mL 1 x 12 mL	 1 x 15 mL 1x 60 mL
96-Well White, Flat-Bottom, Poly-D-Lysine-Coated, Sterile Plates with Lid	2	10

Storage Conditions

PathHunter HEK 293 KDR/KDR Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of bioassay cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

- Short-term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Use tongs to remove cryovials from liquid nitrogen storage, and place the vials immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen that may be present inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

AssayComplete™ Cell Plating 0 Reagent (CP0)

Upon receipt, store at -20°C. Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 20 mL of reagent per aliquot can be dispensed and frozen down.

Recombinant Human $VEGF_{165}$

Upon receipt, store at -20°C until ready to use (up to the date listed in the kit's Certificate of Analysis). Centrifuge the vial before opening to maximize recovery. Once prepared, the excess stock solution can be

stored as suitable aliquots (e.g. 30 µL) at -20°C until needed. Do not freeze/thaw more than twice.

AssayComplete™ Protein Dilution Buffer

Upon receipt, store at -20°C. Once thawed, the Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on the stock sample concentrations, and should be adjusted accordingly

PathHunter Bioassay Detection Kit

Upon receipt, store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For long-term storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

For the ten-plate kit: If all the plates will not be used at the same time, we recommend making five aliquots for each of the two detection reagents. Each aliquot will be adequate for two assay plates. Make five aliquots of 3 mL each for Detection Reagent 1, and five aliquots of 12 mL each for Detection Reagent 2. Sufficient reagent volumes are provided in the kit to make these aliquots.

96-Well White, Flat-Bottom, Poly-D-Lysine Coated Sterile Plates with Lid

Upon receipt, store at room temperature.

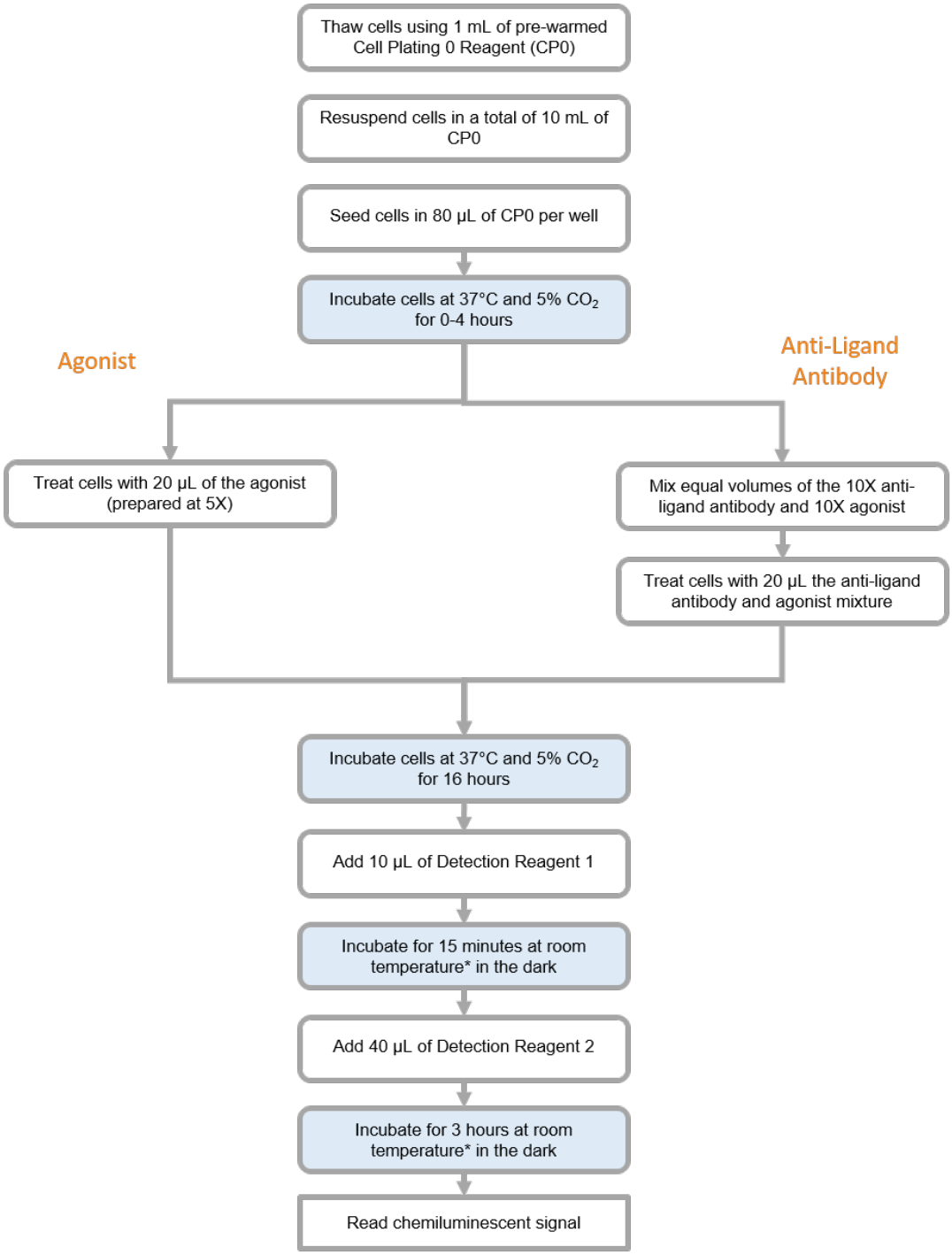
Additional Materials Required

The following equipment and additional materials are required to perform these assays:

Material	Ordering Information
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Multimode or luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micropipettes and pipette tips (10 µL-1000 µL)	
Polypropylene tubes (50 mL and 15 mL)	
Microcentrifuge tubes (1.5 mL)	

Protocol Schematic

Quick-start Procedure: In a 96-well plate, perform the following steps.



*Room temperature refers to a range of 23-25°C.

Detailed Protocol

This user manual provides a protocol for determining potency in a 96-well format. To run this bioassay in a 384-well format, or to optimize the assay for the detection of neutralizing antibodies, contact Technical Support at DRX_SupportUS@eurofinsUS.com.

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating cryopreserved PathHunter HEK 293 KDR/KDR bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the materials required are set up in the tissue culture hood. These include:
   a. One sterile 25 mL reagent reservoir
   b. One sterile 15 mL conical tube
   c. A micropipette (P1000) set to dispense 1 mL
   d. A multichannel pipette and tips set to dispense 80 µL
   e. A bottle of AssayComplete™ Cell Plating 0 Reagent (CP0), pre-warmed in a 37°C water bath for 15 minutes
   f. A 96-Well White, Flat-Bottom, Poly-D-Lysine (PDL)-coated, Sterile Plate with Lid (provided with the kit)
   g. A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, to be used as the master dilution plate
2. Dispense 10 mL of CP0 into the 15 mL conical tube.
3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.

DO NOT use a heated water bath to thaw the vial. Hold the cryovials by the cap; **DO NOT** touch the sides or bottom of the vial to avoid thawing of the cell pellet. Wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood.

4. Add 1 mL of pre-warmed CP0 from the 15 mL conical tube into the cryovial to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet the cell suspension up and down, several times, to uniformly resuspend the cells.
5. Transfer the cell suspension to the conical tube containing the remaining 9 mL of CP0. Remove any remaining suspension from the cryovial to ensure maximum recovery of all the cells.
6. Replace the cap on the conical tube and gently invert it several times to ensure that the cells are properly resuspended in CP0, without creating any froth in the suspension. Immediately pour the suspension into the sterile 25 mL reagent reservoir.
7. Transfer 80 µL of the cell suspension to each well of the 96-well PDL-coated assay plate using a multichannel pipette. Replace the lid and leave the plate at room temperature for 15 minutes, to allow the cells to settle uniformly in the well and minimize potential edge effects.
8. Incubate the assay plate at 37°C and 5% CO_2 while preparing sample dilutions.

   Note: An optimal assay window is observed when samples are added to cells within one hour of plating.

Day 1: Control Agonist Preparation

The following protocol is designed for testing purified biologics. The PathHunter assays can also be run in the presence of high levels of serum or plasma without significantly impacting assay performance. In some assays, standard curves of the control can be prepared in neat serum or plasma, and added directly to cells without further dilution. For best results, the optimized minimum required dilution of crude samples should be empirically determined.

Recombinant $VEGF_{165}$ will serve as a positive control (agonist) in the assay. Preparation of a 1:3 serial dilution of $VEGF_{165}$ is described in this protocol. The volumes listed below are designed for running samples from one dose-response curve in duplicate on the assay plate (Refer to Figure 4. Representative Assay Plate Map)

1. The agonist is prepared at 5X the desired final concentration as it will be diluted five-fold by adding 20 µL to 80 µL of media present in the assay plate.

    1.1. Add 200 µL of the AssayComplete™ Protein Dilution Buffer (PDB) to Wells A2 to A12 of the master dilution plate.

    1.2. Add 100 µL of PDB to the $VEGF_{165}$ vial containing 10 µg of the lyophilized powder to make a 100 µg/mL stock solution.

    1.3. Add 190 µL of PDB to Well A1 of the master dilution plate. Add 10 µL of the 100 µg/mL $VEGF_{165}$ stock prepared in Step 1.2 to Well A1. Mix thoroughly by pipetting up and down several times. This results in a 5 µg/mL solution (5X the final dose of 1 µg/mL).

    1.4. Using a clean tip, transfer 100 µL from Well A11 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 100 µL from Well A2 into Well A3. Mix by pipetting up and down several times. Repeat this process until Well A11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to Well A12 as this will serve as a negative control.

    Note: If only running the control agonist ($VEGF_{165}$), the dose curve may be added to the cells within 0-1 hour after plating.

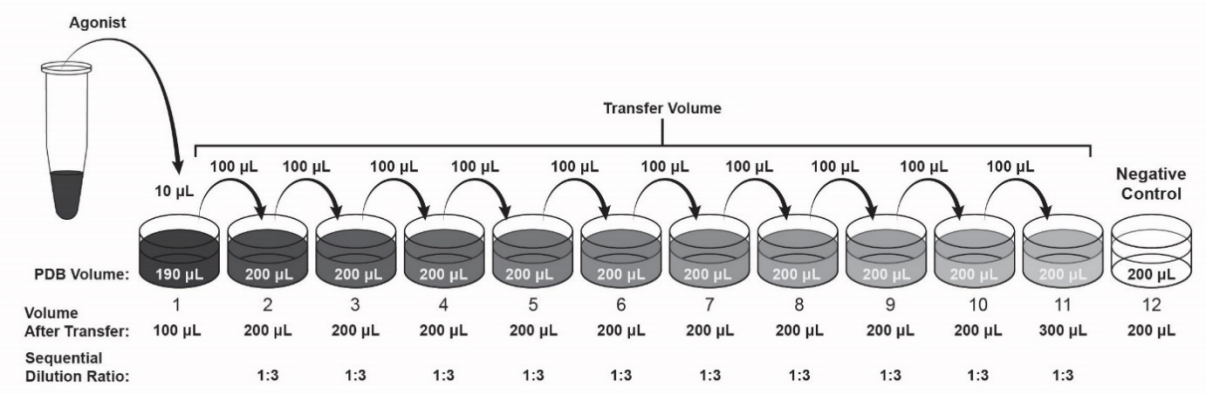


Figure 2. Agonist Serial Dilutions: Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

Day 1: Aflibercept or Biosimilar Preparation

The following protocol is used for determining the potency of aflibercept or other biosimilars. It is not recommended to pre-incubate the aflibercept and $VEGF_{165}$ mixture at room temperature prior to addition to the assay plate. Prolonged incubation at room temperature may lead to steep HillSlopes, which may negatively impact potency estimation.

1. **Prepare the agonist challenge.**

   The optimal stimulation concentration of the Eurofins DiscoverX $VEGF_{165}$ was determined to be approximately 20 ng/mL. If $VEGF_{165}$ from a different vendor is used, the EC_{80} should be determined empirically prior to running samples. Prepare the agonist challenge at 10X the desired final concentration. For enough agonist challenge for a single biosimilar curve run in triplicate, dilute 2 µL of the 100 µg/mL stock with 998 µL of PDB in an Eppendorf tube.

2. **Prepare the aflibercept reference curve.**

   Aflibercept is prepared at 10X the desired final concentration. The suggested final concentration of the top dose for aflibercept is 333 ng/mL. The dilution series are made in the master dilution plate (96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate).

   2.1. Prepare a 10X stock concentration of aflibercept at 3.33 µg/mL and transfer 100 µL to Well B1 of the master dilution plate.

   2.2. Prepare the aflibercept dilution series using the volumes recommended in the table below. Each column in the table pertains to a specific aspect of preparing the dilution series:

   - **Aflibercept Solution Transfer Volume** refers to the volume being transferred from a given well into the adjacent destination well.
   - **Transfer from Well No.** lists the well from which the dilution volume is collected and transferred to the adjacent destination well
   - **Diluent Volume** refers to the volume of PDB that should be added to each destination well. Add the indicated volumes of PDB into the corresponding destination wells prior to making dilutions of the aflibercept solution.
   - **Destination Well No.** refers to the well in which the dilution is being prepared.

Destination Well No.	Aflibercept Solution Transfer Volume (µL)	Transfer from Well No.	Dilution Factor	Diluent Volume (µL)	Resulting Aflibercept Concentration (10X), µg/mL	Final Concentration in Assay (1X), ng/mL
B1	Volume from stock	NA	0	0	3.33	333
B2	30	B1	8	210	0.42	42
B3	150	B2	1.5	75	0.28	28
B4	150	B3	1.5	75	0.19	19
B5	150	B4	1.5	75	0.12	12
B6	150	B5	1.5	75	0.08	8
B7	150	B6	1.5	75	0.05	5
B8	150	B7	1.5	75	0.036	3.6
B9	100	B8	3	200	0.012	1.2
B10	100	B9	3	200	0.004	0.4
B11	100	B10	3	200	0.001	0.1
B12	0	NA	0	200	0	0

2.3. Continue to transfer the recommended volumes listed in the “Aflibercept Solution Transfer Volume” column to its corresponding well numbers listed in the “Destination Well No.” column. No antibody is transferred to Well B12 as this will serve as a negative control.

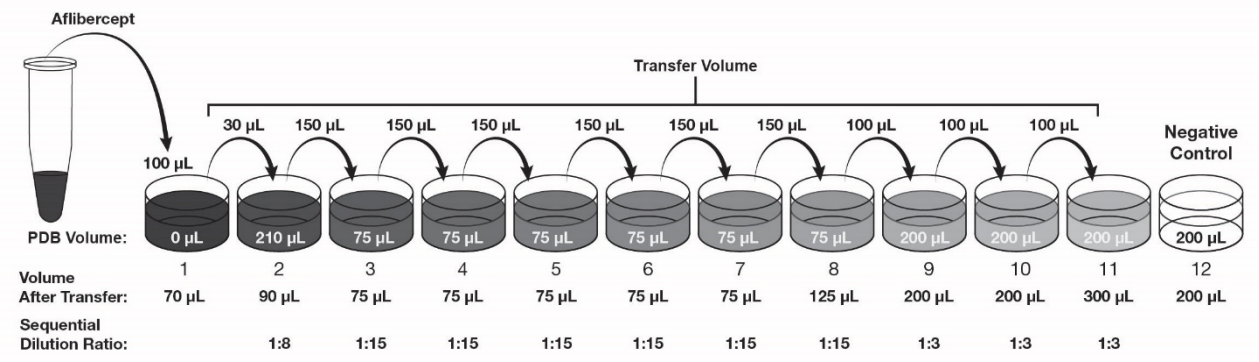


Figure 3. Aflibercept Dilution Series: Make eleven serial dilutions, using the dilution ratios indicated in the figure.

3. Prepare additional test samples in a new row of the master dilution plate (one row per sample) using the same serial dilutions as indicated for aflibercept.
4. Transfer 40 µL from the aflibercept curve prepared in Step 2 (from Row B of the master dilution plate) to the same row on a fresh ligand-neutralizing plate (e.g. Row B of a V-Bottom Polypropylene 96-Well Dilution Plate, Eurofins DiscoverX, Cat. No. 92-0011 or similar).
5. Transfer 40 µL from the additional test samples on the master dilution plate to the same row on the ligand-neutralizing plate.
6. Add 40 µL of the $VEGF_{165}$ agonist challenge prepared in Step 1, to Wells 1-11 of each row of the ligand-neutralizing plate containing antibody serial dilutions. 40 µL of PDB can be added to the negative control wells (Column 12) to maintain equal volumes in all wells.
7. Mix the contents of the wells in the ligand-neutralizing plate thoroughly by pipetting up and down several times with a clean tip for each well, or with a multi-channel pipette.

   Note: It is not recommended to pre-incubate the aflibercept and $VEGF_{165}$ mixture at room temperature prior to addition of samples to the assay plate. Prolonged incubation at room temperature may lead to steep HillSlopes, which may negatively impact potency estimation.
8. Remove the assay plate from the incubator and bring it into the tissue culture hood.
9. If using the agonist control in the assay plate, add 20 µL from the agonist reference curve prepared in Step 1 of the Control Agonist Preparation section on the master dilution plate, to the appropriate wells of the assay plate (e.g. Rows A and H).
10. From each row of the ligand-neutralizing plate, transfer 20 µL of the pre-mixed aflibercept and $VEGF_{165}$ dilution series mixture to the appropriate wells of the assay plate. Refer to Figure 4. Representative Assay Plate Map for suggested sample layout.

11. Return the assay plate to the incubator and incubate at 37°C and 5% CO_2 overnight (16-18 hours).

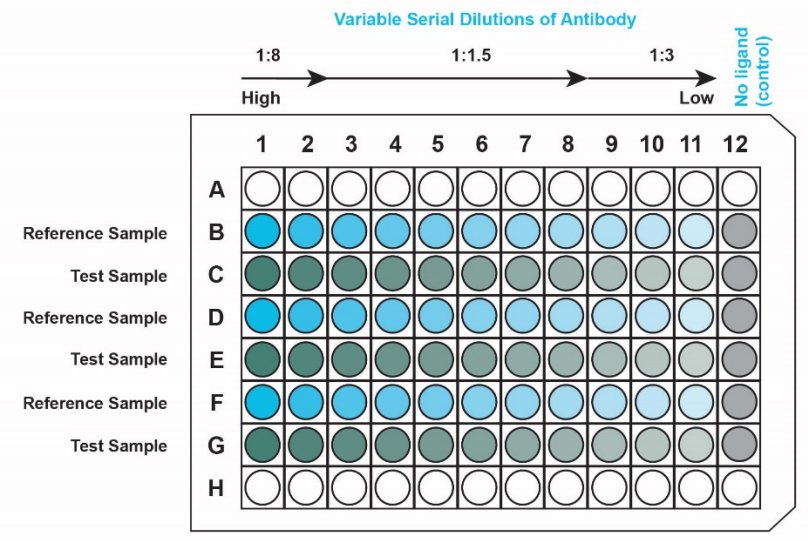


Figure 4. Representative Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test and reference sample tested using a variable dilution scheme. Optional: The reference agonist dose curve can be added to Rows A and H.

Day 2: Detection

The following steps for adding detection reagents are applicable when testing purified biologics. For crude biologic samples, gently removing the liquid from all wells of the assay plate and replacing it with 100 µL of the Cell Plating Reagent before the addition of detection reagents, can result in a higher signal. Additional Cell Plating Reagent will be required for this method.

1. Add 10 µL of the Detection Reagent 1 to each well of the assay plate.
2. Incubate the plate at room temperature (23-25°C) for 15 minutes in the dark.

   Room temperature refers to a range of 23-25°C

   Detection kit reagents are light sensitive, hence incubation in the dark is necessary.
3. Add 40 µL of the Detection Reagent 2 to each well of the assay plate.
4. Incubate the plate at room temperature for 3 hours in the dark.
5. Read the sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

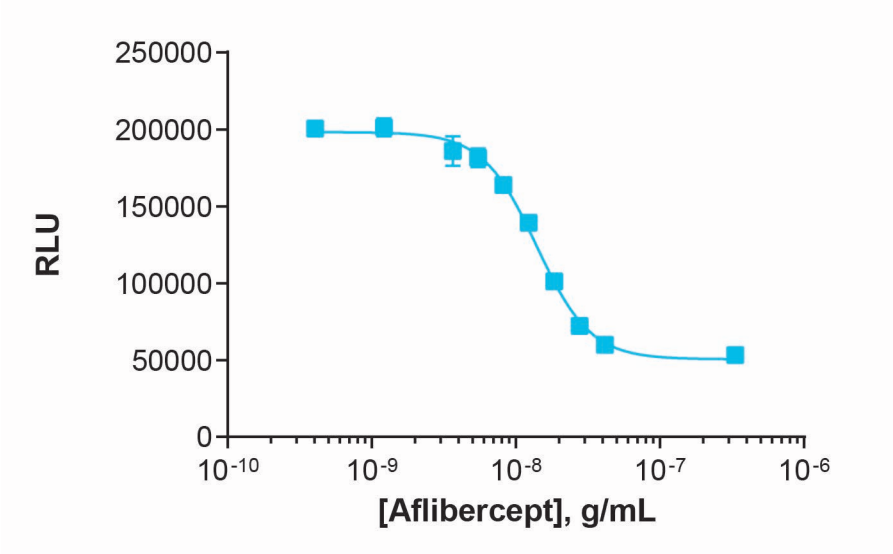
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel.

Typical Results

The following graph is an example of a typical dose-response curve for the Aflibercept Bioassay generated using the protocol outlined in this manual. The data shows potent, dose-dependent inhibition of KDR homodimerization.

The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.

A.



B.

IC_{50} (ng/mL)	14
S/B	3.9

Figure 5. Typical Results: Representative **A**, dose-response curve and **B**, the IC_{50} and assay window for inhibition of KDR homodimerization when aflibercept successfully blocks $VEGF_{165}$-mediated dimerization.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to the thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or improper ligand incubation time	Refer to the Certificate of Analysis for recommended ligand and assay conditions.
	Incorrect preparation of the ligand (agonist or antagonist)	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of the ligand.
	Sub-optimal time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased or no response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using a microscope.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should ideally be prepared just prior to use.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 second/well.
Experimental S/B does not match the value noted in the Certificate of Analysis provided	Incorrect incubation temperature	Confirm assay conditions.
		Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Incorrect preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper ligand handling or storage	Ensure that the ligands are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Certificate of Analysis.
	Problems with dynamic range or dilutions	Changing tips during dilution can help in avoiding carryover.
High variability between replicates	Instrument calibration	Ensure that the dispensing equipment is properly calibrated, and proper pipetting technique is used.

For questions on using this product, please contact Technical Support at 1.866.448.4864 or DRX_SupportUS@eurofinsUS.com

Document Revision History

Revision Number	Date Released	Revision Details
0	April 2020	• New document
1	December 2020	• Updated protocol with dilution series table on page 7

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