Robust and Simple Potency and NAb Bioassays for Avastin® Biosimilar Development

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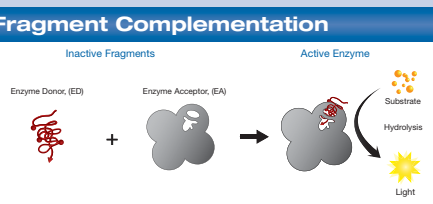
Abstract

One of the major bottlenecks in the development of biosimilars is the need for good bioassays to create potency, stability and neutralizing antibody (NAb) assays. Ideal bioassays need to reflect the clinical mechanism of action (MOA) of the originator drug and should be simple, precise, reproducible and robust. In addition, serum/matrix tolerance and sensitivity are extremely important factors in the development of quantitative NAb assays. Avastin (bevacizumab), a 149 kDa humanized IgG1 monoclonal antibody that selectively binds and inhibits vascular endothelial growth factor A (VEGF-A), is a particularly challenging product for biosimilar assay development, as relevant functional readouts can be time-consuming and require handling of finicky HUVEC cells.

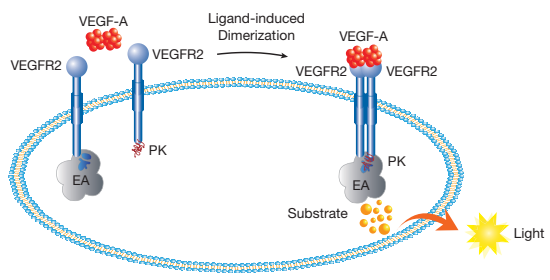
Here, we discuss the development and application of PathHunter® cell-based assays that rely on the native biology of the VEGF receptor (VEGFR), thereby reflecting the clinical MOA of the originator drug. Specifically, the Avastin assay quantifies VEGFR2 homodimerization upon binding of VEGF-A, which is blocked by addition of Avastin or appropriate biosimilar molecules.
This assay is highly specific, robust and utilizes a homogenous mix-and-read protocol, facilitating rapid and reproducible quantitation of drug potency. The technology also enables accurate and sensitive detection of neutralizing antibodies even in high concentrations of human serum through a simple chemiluminescent output. The assays are developed in a convenient ready-to-use format that minimizes assay variability that may result from cell culture. The cell preparation, bioassay protocol and reagents have been optimized to provide superior bioassay performance with high reproducibility (<7% RSD).

PathHunter Enzyme Fragment Complementation



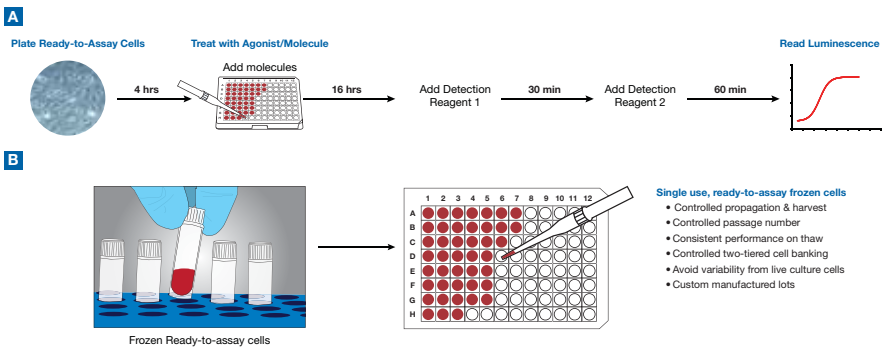
DiscoveRx’s proprietary PathHunter Enzyme Fragment Complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme, split into two inactive components, the enzyme donor peptide (ED) and enzyme acceptor (EA). When brought together in close proximity, ED complements with EA forming active β-gal. The active enzyme catalyzes the substrate generating chemiluminescent light, providing a highly amplified signal and thus an assay of high sensitivity.

Assay Principle for Avastin Bioassay



Using EFC, assays have been created to measure receptor heterodimerization, homodimerization or co-receptor recruitment. The two target receptors are tagged with ProLink™ (PK) or Enzyme Acceptor (EA). Upon ligand-induced activation, the receptors naturally dimerize forcing the two β-gal components to complement and create an active enzyme. Active β-gal generates a chemiluminescent signal in the presence of substrate, signaling the formation of receptor dimers.

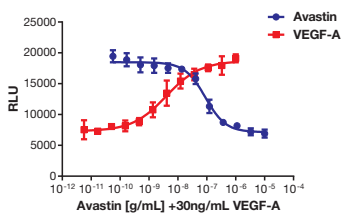
A Simple Homogenous Protocol With Rapid Results



PathHunter bioassay kits use a simple homogenous protocol with rapid results. (**A**) Ready-to-assay cells from DiscoveRx are plated on a 96-well plate and incubated for 4 hours at 37°C. The agonist/test molecule is added to the plate and incubated for 16 hours. The detection reagents are added sequentially in two addition steps and the chemiluminescent signal can be detected on any plate-reading luminometer. (**B**) Cells manufactured in bioassay kits are meant for single use in ready-to-assay vials. The frozen cells are plated directly onto plates to run the assay and this format has several advantages as outlined above.

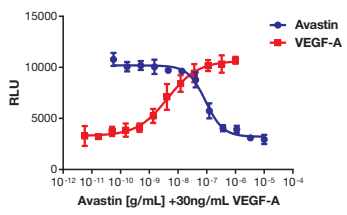
Avastin Inhibits VEGF-A Dependent VEGFR2 Dimerization with Stable Signal for 24 hrs.

Avastin Bioassay Signal at 1 Hour



	Avastin	VEGF-A
HillSlope	-1.226	0.7391
EC_{50}	8.872e-008	3.972e-009
S/B	2.6	2.6

Avastin Bioassay Signal at 24 Hours



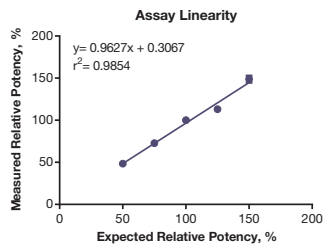
	Avastin	VEGF-A
HillSlope	-1.414	0.8171
EC_{50}	9.108e-008	4.345e-009
S/B	3.2	3.2

PathHunter bioassays and the signal from these assays are robust and stable. After the addition of detection reagent 2, the assay was read at 1 hour and 24 hours. As observed, there was no noticeable change in IC_{50} of Avastin or EC_{50} of ligand in the assay with increased incubation time, while assay window improves by 20% with 24hr incubation, indicating that the assay signal is extremely stable over this period of time.

Assay Reproducibility, Accuracy, Precision & Linearity

Assay Accuracy & Precision

	Expected Potency, %	Measured Potency, %	Mean Potency, %	SD	Recovery, %	RSD, %
Day 1	150	151.0	148.7	4.04	99.1	5.12
Day 2		144.0				
Day 3		151.0				
Day 1	125	109.0	113.0	3.46	90.4	3.07
Day 2		115.0				
Day 3		115.0				
Day 1	75	74.4	72.7	2.47	97.0	3.12
Day 2		73.9				
Day 3		69.9				
Day 1	50	48.8	48.5	2.42	96.9	4.99
Day 2		45.9				
Day 3		50.7				

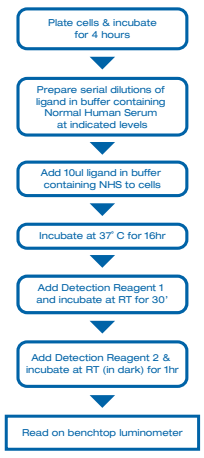


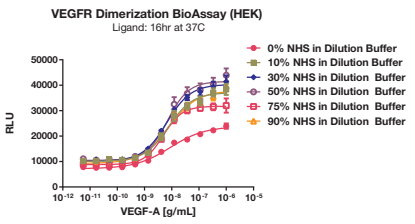
Accuracy= 95.9% **Precision= 4.1%**

VEGF-A is known to cause homodimerization of VEGFR2 (KDR), as the first step in the activation cascade of these receptors. Anti-VEGF-A antibodies such as Avastin (Bevacizumab) and Aflibercept prevent this dimer formation, leading to inhibition of VEGF-A dependent signaling. Here we have tested the VEGFR2 homodimer assay with VEGF-A, demonstrating a robust response and a high level of reproducibility with multiple runs.

The VEGFR dimerization assay was tested with four test samples, from 50% to 150%, compared to a reference standard (100%). The measured relative potencies were plotted against the expected relative potencies with a very high degree of accuracy and precision.

Analysis of Minimal Required Dilution with Normal Human Serum

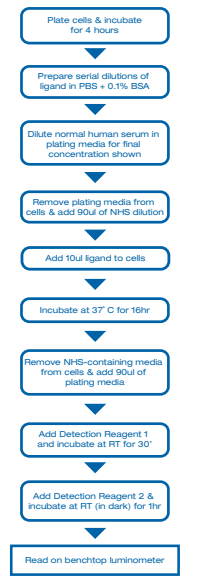


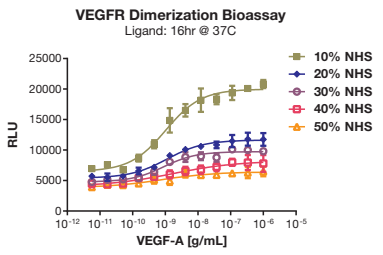


	HillSlope	EC_{50}	S/B
0% NHS	0.6821	8.747e-009	3.3
10% NHS	0.9854	7.540e-009	3.7
30% NHS	0.9842	6.514e-009	4.0
50% NHS	1.124	6.898e-009	3.9
75% NHS	1.263	4.883e-009	3.6
90% NHS	0.8393	7.811e-009	4.1

Effect of sample matrix on VEGFR dimerization assay was evaluated by addition of small volumes of neat normal human serum (NHS) using the protocol on the left. As shown on the right, addition of concentrated serum samples has a negligible effect on EC_{50} even at 90% NHS. Furthermore, the effect on assay window also differed negligibly between 10% and 90% serum, suggesting that the minimal required dilution is 0.9. Note: 100% serum was not tested in this experiment.

Tolerance of VEGFR Dimerization Assay to Human Serum





	HillSlope	EC_{50}	S/B
10% NHS	0.7989	1.024e-009	3.1
20% HS	0.7747	1.124e-009	2.2
30% NHS	0.8698	8.895e-010	2.0
40% NHS	0.4322	1.655e-009	2.1
50% NHS	0.5410	7.698e-010	1.7

S/B for 0% NHS = 3.7

Tolerance of the VEGFR dimerization to varying final concentrations of normal human serum was evaluated using the protocol on the left. As shown on the right, the assay is tolerant of a up to a final assay volume of 10% human serum, although typically much lower volumes of patient samples could be utilized. More than 10% serum in this assay may cause a decrease in SB ratios.

Summary & Conclusions

- An assay for Avastin has been developed with a simple chemiluminescent readout
- Avastin assay relies on VEGFR2 homodimer formation through VEGF-A
- PathHunter Avastin bioassay provides a target-specific response and can tolerate up to 10% human serum
- Avastin assays are robust and highly reproducible with a simple, homogenous protocol
- To minimize assay variability, the Avastin bioassays are prepared in ready-to-assay kits with all required assay components, including frozen, single use cell vials
  - Cells in ready-to-assay format minimize assay variability stemming from the use of continuous culture cells
  - Each kit contains enough material for 1000 data points (10 x 96-well plates)
  - Customized lots of up to 1000 vials are prepared for each project
- Similar assays are available for >700 targets, including >30 Biosimilar targets

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