Development of Simple, Robust Bioassays for Biosimilar & Biobetter Development

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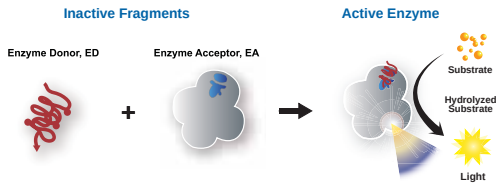
Abstract

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 biopharmaceutical drug and should be simple, precise, reproducible and robust. To further enable NAb assays, there is an added need for serum/matrix tolerance and sensitivity.

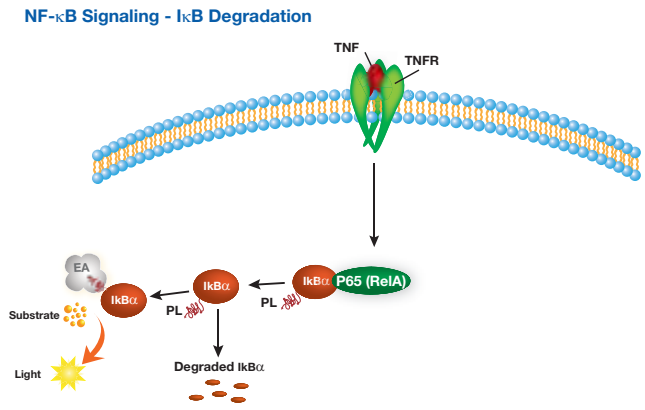
Here, we discuss the development and application of diverse PathHunter® cell-based assays that cover distinct cellular mechanisms either downstream of or at the level of receptor engagement by large molecules. Importantly many of these assays rely on expression of native receptors thereby reflecting the clinical MOA of the biopharmaceutical drug. These assays are highly specific, quantitative, scalable, robust and utilize a homogenous mix-and-read protocol, which facilitates rapid and reproducible detection of drug potency. The technology is also amenable to 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 often occurring due to 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



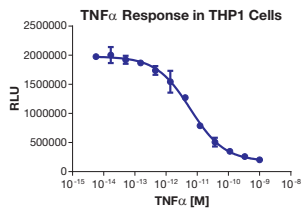
DiscoveRx's proprietary PathHunter Enzyme Fragment Complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme, split into two inactive components, the enzyme donor peptide (ED) and enzyme acceptor (EA). When brought together in close proximity, ED complements with EA forming active 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 Anti-TNFα Bioassay

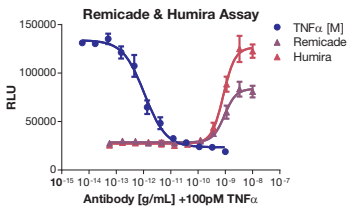


Assay Principle. TNFα binds to and stimulates the TNF receptor, triggering a downstream signaling cascade that activates the NFκB pathway. This leads to the degradation of the IκB protein. Here, the PL-tagged IκB protein is expressed in THP-1 cells. Stimulation of cells with TNFα results in dose-dependent degradation of IκB by the proteasome. The activation of the receptor can be detected via IκB degradation in a very sensitive manner by the addition of detection reagents containing EA and the substrate, resulting in chemiluminescent light that is proportional to the amount of PL-tagged IκB present in the cells. Therefore, a higher activation of TNFR will result in lower assay signal.

Robust Bioassay Response with Anti-TNF drugs - Infliximab and Adalimumab



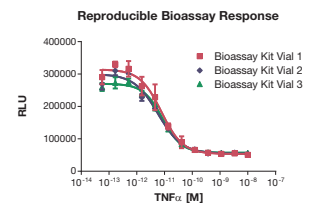
HillSlope	-0.7918
EC_{50}	6.001e-012
S/B	9.7



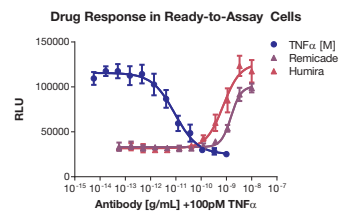
	TNFα [M]	Remicade [g/mL]	Humira [g/mL]
HillSlope	-1.099	2.025	2.024
EC_{50}	1.085e-012	9.257e-010	8.470e-010
S/B	5.6	3.2	4.8

Continuous culture cells for the Anti-TNF assay. Cells were cultured and plated overnight before the assay was conducted. These assays demonstrate a high reproducibility (<15% intra-plate CV) in response to TNFα (left), and when treated with the innovator molecules Infliximab (Remicade®) and Adalimumab (Humira®) (right).

Highly Reproducible Ready-to-Use Bioassay Kits for Anti-TNF Drugs



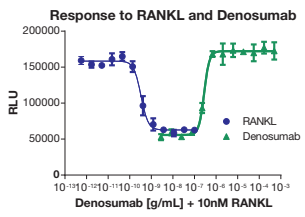
	Vial 1	Vial 2	Vial 3
HillSlope	-1.128	-1.006	-1.253
EC_{50}	7.688e-012	5.825e-012	8.370e-012
S/B	5.8	5.8	4.7



	TNFα [M]	Remicade [g/mL]	Humira [g/mL]
HillSlope	-0.9576	2.213	1.288
EC_{50}	9.090e-012	1.536e-009	7.467e-010
S/B	4.7	3.1	3.6

Frozen Ready-to-Assay Cells Provide Highly Reproducible Data. Anti-TNFα bioassay from frozen ready-to-use cells, shows excellent sensitivity and reproducibility in response to TNFα (left), and when treated with innovator molecules Remicade and Humira (right). As noted above, frozen cells were plated directly onto the assay plate post thaw, incubated overnight to allow them to attach and assayed the next day with TNFα and the inhibitor molecules.

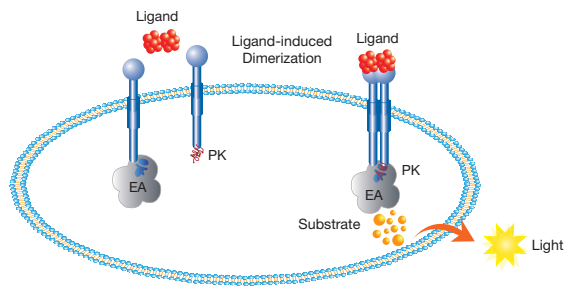
Robust Bioassay For Anti-RANKL Antibody - Denosumab



	RANKL	Denosumab
HillSlope	-2.628	3.565
EC_{50}	3.329e-010	2.816e-007
S/B	2.5	3.3

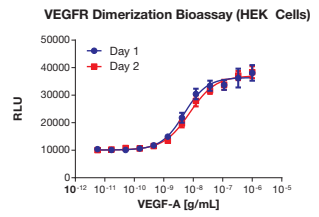
Assay Principle & Robust Response to Denosumab (Anti-RANKL) – RANKL activates TNFR superfamily member, RANK, which leads to the activation on the NFκB response. By expressing PL-tagged IkB in U2OS cells that express RANK on their surface, we can monitor receptor activation, in a similar manner to TNFR activation. 24 hours after plating the engineered cells, Denosumab was mixed with 10nM RANK ligand (RANKL) and added to the cells for 1hr. Following the addition of PathHunter detection reagent we observe a robust response to both agonist and inhibitor drug.

Assay Principle for Avastin & IL17 Receptor Assays

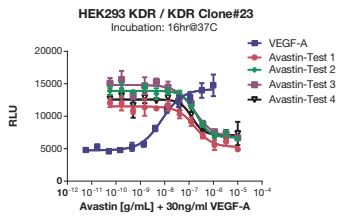


Using EFC, assays have been created to measure receptor heterodimerization, homodimerization or co-receptor recruitment. The two target receptors are tagged with ProLink™ (PK) or Enzyme Acceptor (EA). Upon ligand-induced activation, the receptors naturally dimerize forcing the two β-gal components to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

Bioassay for Avastin and Other Anti-VEGF-A Drugs



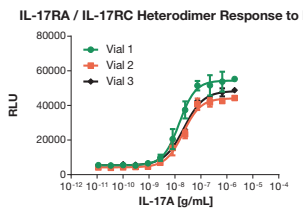
	Day 1	Day 2
HillSlope	1.117	0.9854
EC_{50}	4.884e-009	7.540e-009
S/B	3.8	3.8

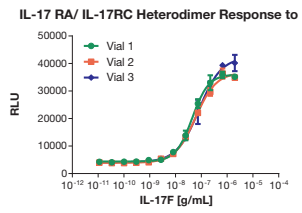


	Avastin-Test 1	Avastin-Test 2	Avastin-Test 3	Avastin-Test 4	VEGF-A
HillSlope	-1.227	-1.597	-1.131	-1.815	1.009
EC_{50}	1.725e-007	1.965e-007	1.692e-007	1.315e-007	7.657e-009
S/B	2.5	2.1	2.1	1.7	3.1

Avastin Assay Provides Highly Reproducible Data. 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 prevent this dimer formation, leading to inhibition of VEGF-A dependent signaling. Here we have tested the VEGFR2 homodimer assay with VEGF-A and Avastin, demonstrating a robust response and a high level of reproducibility with multiple runs. This assay provides a much simpler method for testing Avastin potency and NAb assay development than the available HUVEC cell proliferation assay.

Bioassay for Anti-IL-17A and Anti-IL-17F Antibodies



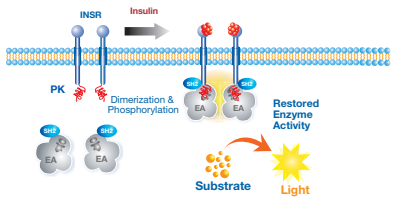


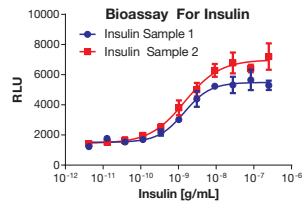
	Vial#01	Vial#02	Vial#3
Best-fit values			
Bottom	5417	4245	5397
Top	54406	44141	48382
$LogEC_{50}$	-7.827	-7.679	-7.653
HillSlope	1.393	1.293	1.151
EC_{50}	1.489e-008	2.092e-008	2.221e-008
S/B	10.5	11.7	9.7

	Vial#01	Vial#02	Vial#3
Best-fit values			
Bottom	4398	3900	4110
Top	36021	36775	42297
$LogEC_{50}$	-7.350	-7.221	-7.090
HillSlope	1.370	1.084	1.008
EC_{50}	4.462e-008	6.010e-008	8.119e-008
S/B	7.5	8.0	10.0

Highly Reproducible IL17RA/IL17RC Assay to Develop Anti-IL-17 Drugs. IL-17A or IL-17F will cause heterodimerization of IL-17RA and IL-17RC receptors, as the first step in their activation cascade. Anti-IL-17 antibodies will prevent the formation of this heterodimer, leading to inhibition of IL-17 receptor signaling. Here, we have developed and tested the IL-17A and IL-17F in this assay, demonstrating a robust response and a highly reproducible response. This assay provides a much simpler method for testing IL-17 potency and NAb assay development than the available assays.

Simple, Robust Assay for Insulin Biosimilar Development

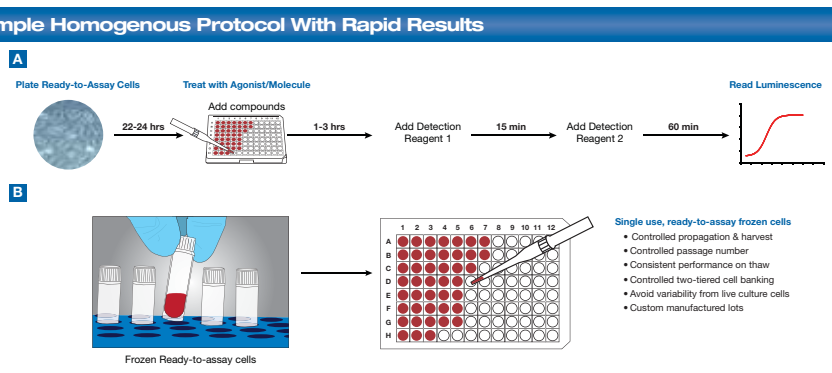




	Sample 1	Sample 2
HillSlope	1.254	0.9321
EC_{50}	1.431e-009	1.480e-009
S/B	3.6	4.8

Bioassay development for Insulin Biosimilars and Biobetters – (Left) Insulin binds and activates the Insulin receptor, leading to conformation change and trans-phosphorylation. This results in the recruitment of an SH2-domain protein to the receptor, as part of the natural signaling cascade. By expressing a PK-tagged Insulin receptor and an EA-tagged SH2-domain protein, we can monitor target-proximal receptor activation with a simple chemiluminescent readout. (Right) Two different sample of Insulin provide an appropriate response in the Insulin bioassay.

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 or 384-well plate and incubated for about 24 hours at 37°C. The agonist/test molecule is added to the plate and incubated for 1-3 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.

Summary & Conclusions

- Potency and NAb assays available for > 30 biosimilar targets.
- To minimize assay variability, the 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 datapoints (10 x 96-well plates)
  - Customized lots of up to 1000 vials are prepared for each project

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