

# Simple, Reproducible & Robust Bioassays In Cryopreserved Ready-To-Assay Format

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## Abstract

Cell-based bioassays often pose a hurdle during a rapidly moving biologics development program. High standards for assay accuracy, precision, reproducibility and robustness are additionally put to the test by the use of continuous culture cells that can add to assay variability and increase the cost and complexity of each assay.

Advent of new technology platforms can enable rapid development of cell-based bioassays that are simple, accurate, precise and robust. Here, we discuss the development and application of diverse PathHunter® cell-based assays that cover distinct cellular mechanisms either distal from or proximal to the receptor. These quantitative assays are highly specific, scalable, robust and utilize a homogenous mix-and-read protocol, which facilitates rapid and reproducible detection of drug potency. In order to increase the reproducibility and reduce the complexity of the assay by eliminating cell culture, these assays are developed as cryopreserved ready-to-assay cells that can be plated directly from the frozen state onto the assay plate. The cell preparation, bioassay protocol and reagents have been optimized to provide superior bioassay performance with high reproducibility (<10%RSD). Examples discussed here include assays for Bevacizumab, human growth hormone, GLP1 receptor agonists and erythropoietin.

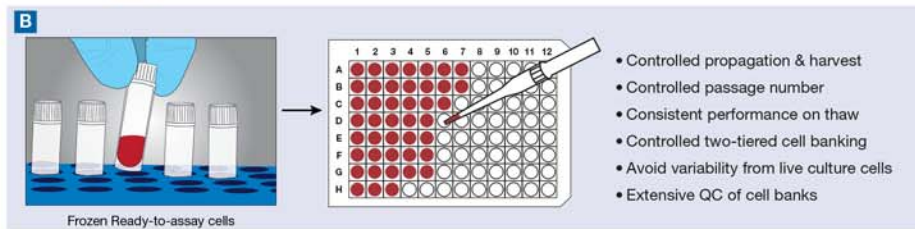
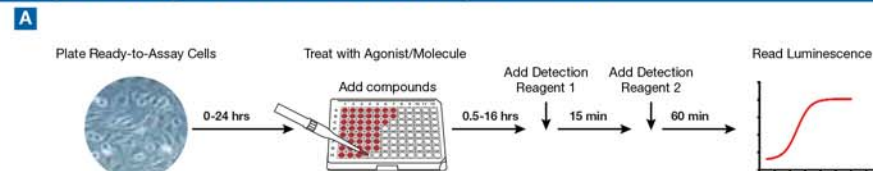
## PathHunter Enzyme Fragment Complementation

Inactive Fragments      Active Enzyme



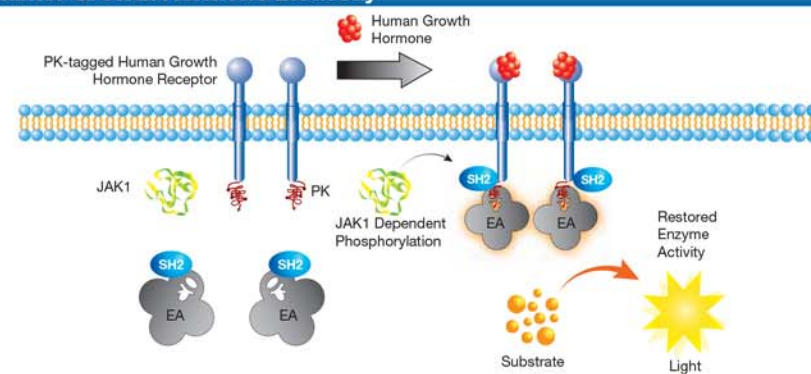
DiscoverX's proprietary PathHunter Enzyme Fragment Complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme, split into two inactive components, the enzyme donor peptide (ED) and enzyme acceptor (EA). When brought together in close proximity, ED complements with EA forming active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

## 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 0-24 hours at 37°C. The agonist/test molecule is added to the plate and incubated for 0.5-16 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.

## PathHunter Growth Hormone Bioassay

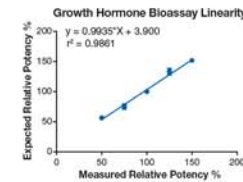


PathHunter Growth Hormone bioassay cells are engineered to co-express a ProLink™ (PK) tagged human growth hormone receptor (GHR), an untagged JAK1 kinase, and an Enzyme Acceptor (EA) tagged SH2 domain protein. Activation of the GHR by hGH induces phosphorylation of the receptor by JAK1 and this leads to SH2-EA recruitment, forcing complementation of the two β-galactosidase enzyme fragments (EA and PK). The resulting functional enzyme hydrolyzes substrate to generate a chemiluminescent signal.

## High Accuracy and Precision for PathHunter Growth Hormone Bioassay

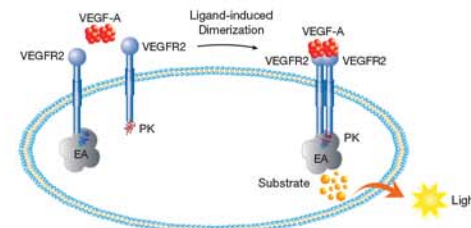
Assay Accuracy & Precision						
Day	Expected Potency, %	Measured Potency, %	Mean Potency, %	SD	Recovery, %	RSD, %
Day 1	150	152.0	151.7	1.53	98.9	1.01
Day 2	125	133.0	133.0	5.29	94.0	3.98
Day 3	75	79.8	75.2	4.10	99.7	5.46
Day 1	50	57.3	56.4	1.01	88.7	1.8
Day 2	50	57.3	56.4	1.01	88.7	1.8
Day 3	50	56.6				

Accuracy= 95.3% Precision= 3.06%



The Growth Hormone bioassay was tested with four test samples of human growth hormone, from 50% to 150% expected potency, compared to the reference (100%). The measured relative potencies were plotted against the expected potency with a high degree of accuracy (95.3%) and precision (3.06%).

## PathHunter Bevacizumab Bioassay

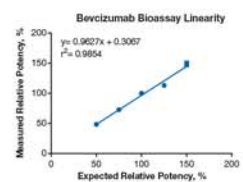


Using EFC, we have generated a simple cell-based assay for VEGF-A and the VEGFR2 receptor. The assay measures VEGFR2 receptor homodimerization by VEGF-A as the first step in its activation cascade that eventually leads to angiogenesis. The VEGFR2 receptor is tagged with both the ProLink (PK) and the Enzyme Acceptor (EA). Upon ligand induced activation, receptor dimerization occurs which forces the two β-gal components to complement. This creates an active enzyme that hydrolyzes substrate generating a chemiluminescent signal.

## Excellent Accuracy and Precision for PathHunter Bevacizumab Bioassay

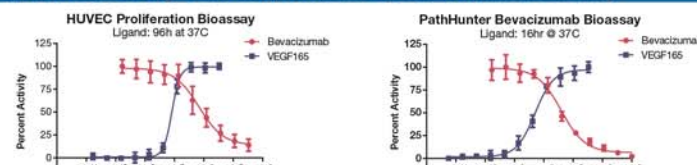
Assay Accuracy & Precision						
Day	Expected Potency, %	Measured Potency, %	Mean Potency, %	SD	Recovery, %	RSD, %
Day 1	150	151.0	148.7	4.04	99.1	5.12
Day 2	125	144.0	143.0	3.46	90.4	3.07
Day 3	75	109.0	113.0	3.46	90.4	3.07
Day 1	50	74.4	72.7	2.47	97.0	3.12
Day 2	50	73.9	72.7	2.47	97.0	3.12
Day 3	50	69.9				
Day 1	50	48.8	48.5	2.42	96.9	4.99
Day 2	50	45.9				
Day 3	50	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), Ranibizumab and Afibercept bind to VEGF-A and 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 VEGFR2 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.

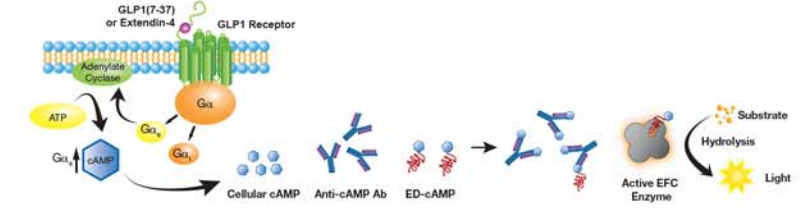
## HUVEC Proliferation Assay vs. PathHunter Bevacizumab Bioassay



	HUVEC Proliferation Assay	PathHunter Bevacizumab Bioassay
EC <sub>50</sub> Bevacizumab	8.18 ng/mL	5 ng/mL
EC <sub>50</sub> VEGF165	67.88 ng/mL	38.96 ng/mL
S/B Ratio	2.5 fold	>4.7 fold
Assay run time	96 hours	16 hours
Specificity	Low	High
Assay Output	MTT Readout	Chemiluminescence
Cell type	Primary cells with donor variability	Clonal, frozen ready-to-assay cells
Cell Culture	Required	No cell culture necessary

The HUVEC proliferation assay is another method to test drug potency for the anti-VEGF drugs. VEGF-A (VEGF165 is an active splice variant of VEGF-A) will cause HUVEC cells to proliferate and Bevacizumab will inhibit this by inhibiting VEGF-A interaction with VEGFR2. Here we have tested the PathHunter Bevacizumab bioassay with VEGF-A and bevacizumab (top right), demonstrating the robust and precise response to both agents, and EC<sub>50</sub>'s comparable to those obtained in the HUVEC proliferation assay (top left). The table below compares the proliferation assay and the PathHunter assay, demonstrating that the latter is quicker, more robust, highly specific and generates the better data without the need for any cell culture.

## cAMP Hunter Bioassay for GLP1R Agonists

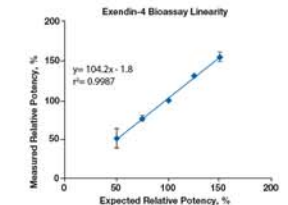


cAMP Hunter Bioassay for GLP1 receptor agonists contains cells that overexpress naturally Gs coupled wildtype GLP1 receptor. The assay is designed to detect increases in intracellular cAMP levels in response to agonist stimulation of the GLP1 receptor, through GLP1(7-37) or Exendin-4. The cellular cAMP levels are detected using the cAMP detection kit, where cAMP from the cells and ED-labeled cAMP (ED-cAMP) compete for anti-cAMP antibody (Ab) binding sites. Antibody-bound ED-cAMP will not be able to complement with EA, but unbound ED-cAMP is free to complement EA to form active enzyme, which subsequently produces a luminescent signal. The amount of signal produced is directly proportional to the amount of cAMP in the cells.

## High accuracy with Exendin-4 in the GLP1R Bioassay

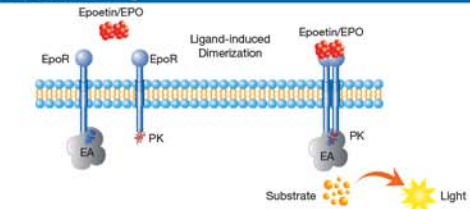
Assay Accuracy & Precision						
Day	Expected Potency, %	Measured Potency, %	Mean Potency, %	SD	Recovery, %	RSD, %
Day 1	150	159.0	154.5	6.36	103	4.12
Day 2	125	131.0	130.0	5.29	104	1.09
Day 1	100	100.0	100.0	0.0	100	0.00
Day 2	75	74.0	75.5	3.53	102	4.62
Day 1	50	42.0	51.0	12.72	102	24.96
Day 2	50	60.0				

Accuracy= 102% Precision= 6.96%



The bioassay for GLP1 receptor agonists was tested with four test samples of Exendin-4, from 50% to 150% expected potency, compared to the reference (100%). Each assay was run in triplicate on each day. The measured relative potencies were plotted against the expected potency with a high degree of accuracy (102%) and precision (6.96%).

## PathHunter Epoetin Alfa Bioassay

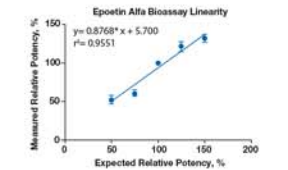


The PathHunter Epoetin Alfa Bioassay is used to detect ligand induced dimerization of two subunits of a receptor-dimer pair. This assay relies on the native biology of the erythropoietin receptor (EpoR), where erythropoietin induces receptor homodimerization as the first step in the pathway activation cascade. The cells have been engineered to coexpress EpoR fused to PK, and another EpoR construct fused to EA. Binding of Epoetin alfa or darbepoetin alfa to EpoR induces it to homodimerize, forcing complementation of the two enzyme fragments, PK and EA. This results in the formation of a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.

## Potency, Linearity, Precision & Accuracy of the Epoetin Alfa Bioassay

Assay Accuracy & Precision						
Day	Expected Potency, %	Measured Potency, %	Mean Potency, %	SD	Recovery, %	RSD, %
Day 1	150	128.0	132.0	5.2	88.0	3.94
Day 2	125	135.0	130.0	5.29	97.1	5.04
Day 3	125	128.0	121.3	6.1	97.1	5.04
Day 1	75	57.0	61.0	3.6	81.3	5.83
Day 2	75	62.2				
Day 3	50	43.8	52.6	5.6	105.1	10.61
Day 1	50	54.9				
Day 2	50	46.2				
Day 3	50	56.6				

Accuracy= 92.9% Precision= 6.36%



The Epoetin alfa bioassay was tested with four test samples of erythropoietin, from 50% to 150% expected potency, compared to the reference (100%). Each assay was run in triplicate on each of the days. The measured relative potencies were plotted against the expected potency with a high degree of accuracy (92.9%) and precision (6.36%).

## Summary & Conclusions

- Cell-based assays with functional response based on drug mechanism of action & native biology
- Excellent potency and linearity with high accuracy and precision of the bioassay
- Simple protocol with rapid results on any benchtop luminometer
- Highly reproducible and robust
- Used for both Nab and potency bioassays
- 750+ cell-based assays & 30+ bioassays for biosimilars available
- Developed as cryopreserved ready-to-assay cells

Go to [www.discoverx.com/biologics](http://www.discoverx.com/biologics) to see additional validation data and a full list of targets.