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# White Paper: Accelerating Biosimilar & Biobetter Drug Development: Ready-to-Use, Cell-Based Assays for Potency and Lot Release Testing

With the drug industry's expanding emphasis on biologics, the need for robust cell-based assays has grown at all stages of development. Requirements for efficacy, quality, and potency testing often demand a complex set of bioassays and/or cell-based assays for new therapeutics or biosimilars. Developers of the latter have found this need for cell-based assays to be particularly challenging.

Commercially available, ready-to-use cell-based assays provide a robust functional response from specific therapeutic targets. They can significantly shorten assay development time while facilitating adoption and greater reproducibility across multiple global sites. Examples discussed in this white paper include cell-based assays for bevacizumab (Avastin), insulin, glucagon-like peptide 1 (GLP1), growth hormone (GH), and anti-tumor necrosis factor alpha (anti-TNFα) antibodies. These case studies highlight how commercial tools can reduce assay development time, enabling a biologic developer to move quickly with the goal of getting to market or submission before their competitors.

## Bioassays for Potency and Lot Release Testing

Biologics are complex protein structures that are usually made by and purified from live cells. Such manufacturing processes are highly complicated and can be difficult to control. Lack of control can lead to physicochemical variations that affect drug function. Additionally, biologics are often extremely sensitive to physical conditions (temperature, shear forces, chemical phase, and light) and enzymatic action. These products typically require complex bioassays for batch release and stability assessment rather than (or in addition to) simple chemical tests for identity and purity. Good measurement of biological activity is essential before the drug substance can be released or packaged.

Manufacturers often implement a panel of quality control tests for drug-substance and drug-product release. Such assays vary in output, complexity, and time requirements. Depending on the specific drug, assays can range from enzymatic tests (e.g., enzyme-linked immunosorbent assays, ELISAs) to cell-based assays (CBAs) and even animal testing. To this end, the industry needs rapid, robust, and reproducible cell-based assays that can expedite the process of lot release and stability testing.

**Biosimilar & biobetter drug developers need to move quickly to get to market before their competitors. Commercial cell-based assays facilitate this by reducing assay development time for critical comparability and QC lot release assays.**

## Why Cell-Based Assays?

CBAs that test for drug potency offer a number of advantages over animal models or highly complicated *in vitro* assays. Compared to animal testing for potency bioassays, CBAs typically:

- Reduce testing costs by >90%
- Achieve quicker implementation
- Reduce hands-on time with cryopreserved ready-to-assay cells
- Reduce assay variation with clonal cell lines and validated cell banks
- Eliminate ethical questions on animal testing

For all these and many more reasons, CBAs are becoming the method of choice for potency assay development. However, a question often emerges concerning which CBA or bioassay should be used to test for potency of a given drug.

## Choosing the Ideal Cell-Based Assay

An ideal potency bioassay should mimic a given therapeutic molecule's mechanism of action (MOA) while producing highly precise, accurate, and reproducible data in a quality-controlled environment compliant with good laboratory practice (GLP) and/or good manufacturing practice (GMP) (based on the ICH Q2A and Q2B guidelines). Traditionally, bioassay developers have adapted existing phenotypic readouts for their drug targets, often reflecting cellular events that occur far downstream of the target: cell death, reporter gene expression, cell viability, cytokine release, and cell proliferation. Most assays require significant retooling or extensive development before they can be implemented for batch release/validation testing, adding significant time to method development and optimization. In addition, "home-brewed" phenotypic assays have a number of distinct disadvantages ranging from low specificity, long assay time (up to seven days in some cases), high complexity, and variability.

**Eurofins DiscoverX assays provide a biologically relevant readout that is proximal to the target — in contrast to the distal readouts of phenotypic and reporter-gene assays — providing a highly specific signal for each drug tested.**

Commercially available cell-based potency assays offer a number of advantages over the above-mentioned systems, including reduced development and validation times, easily adopted methods, simplified global transfer processes, reduced overall costs, and global support from their providers. To address the need for such products, companies such as Eurofins DiscoverX have developed a broad array of CBAs that have been implemented around the world by numerous companies for potency and lot release testing.

Eurofins DiscoverX has developed more than 750 CBAs relying on the native biology of target receptors to quantitatively measure biologic drug potency using industry-validated PathHunter® enzyme-fragment complementation (EFC) technology (Figure 1). The flexibility of this platform enables interrogation of a diverse array of therapeutic targets (Figure 2), including biosimilar targets important in diabetes management (GLP1, exendin-4, and insulin) and inflammation (anti-TNFα and ustekinumab). These assays provide a readout that is proximal to the target — in contrast to the distal readouts of phenotypic and reporter-gene assays — providing a highly specific signal for each drug tested. The elegance of this technology is emphasized by its implementation of a simple, homogeneous protocol for each available assay, allowing for rapid assay readout in 24–48 hours.

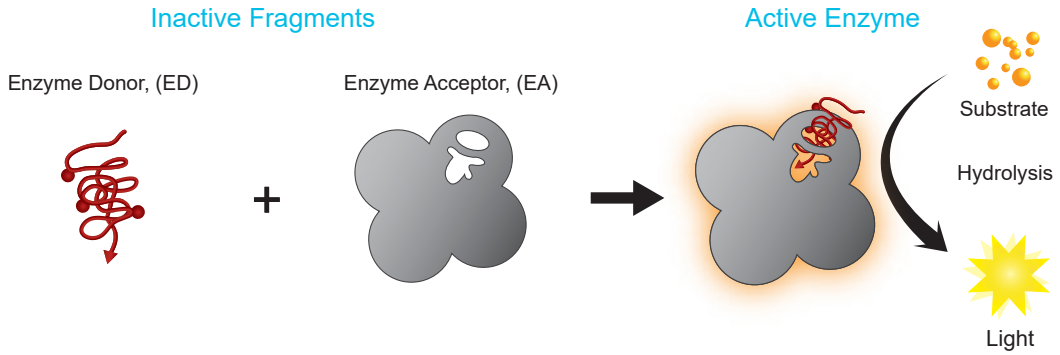


Figure 1. Eurofins DiscoverX's proprietary PathHunter enzyme fragment complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme split into two inactive components, the enzyme donor peptide (ED) and an enzyme acceptor (EA). When brought together in close proximity, ED complements with EA to form active β-gal. The active enzyme then catalyzes the substrate to generate chemiluminescent light, providing a highly amplified signal to make for a high-sensitivity assay.

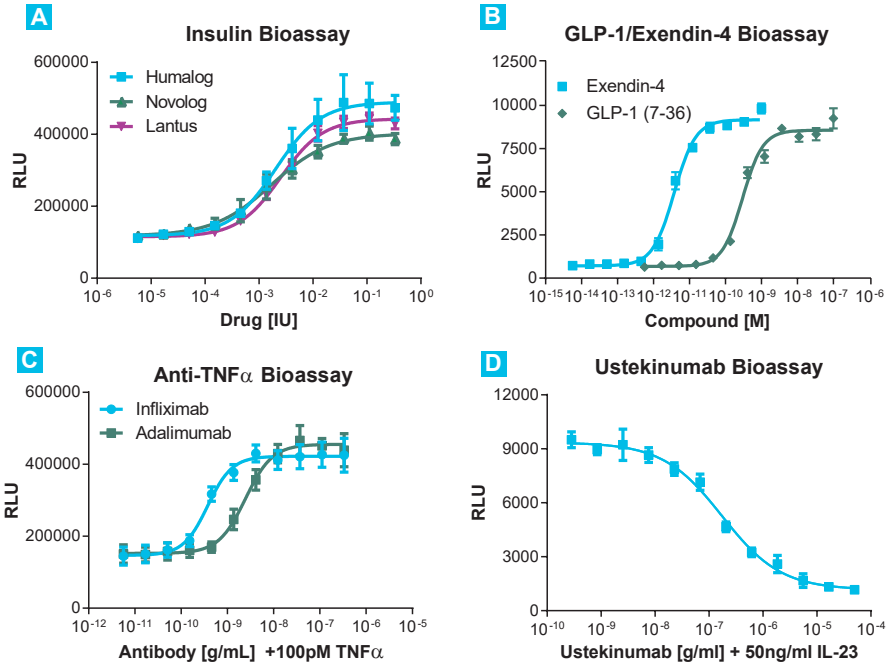


Figure 2. Examples of eight bioassays developed as both potency and neutralizing antibody (NAb) assays for biosimilars; (A) response to three commercial insulin therapeutics (Humalog, Novolog, and Lantus) in a cell-based functional assay that measures activation of human insulin receptor; (B) a GLP1 and Exendin-4 bioassay was developed to accelerate development of biosimilars to metabolic drugs using a cyclic AMP readout in live cells. (C) A bioassay developed for anti-TNFα drugs enables users to benchmark drugs such as adalimumab, infliximab, etanercept, golimumab, and certolizumab against their respective biosimilar molecules. (D) A simple assay determines potency of the anti-inflammatory antibody ustekinumab.

## Industry Trends for Potency Assays

In addition, the industry is moving away from continuous culturing of cells to increase the efficiency of potency assay development. Although immortalized cell lines have been used for potency assays, continuous culturing requires thawing and culturing of a new vial of cells before testing each batch of drugs, which increases the cycle time for routine quality control (QC) testing. Continuous culture assays can significantly increase the per sample cost, reduce efficiency, increase variability, and add up to two hours per day of culture and documentation time in a quality environment. Finally, cultured cells can change their performance over time through cellular drift, which limits their use to only a few validated passages before a new vial needs to be thawed.

**The industry is rapidly moving toward the use of cryopreserved ready-to-assay cells, where the analyst thaws cells and performs the assay without culturing the cells, saving several weeks of cell culture time per assay.**

To overcome those challenges, the industry is rapidly moving toward the use of ready-to-assay, cryopreserved cells. The analyst can thaw cryopreserved cells directly onto an assay plate and perform an assay within 0–48 hours. This approach completely eliminates the need for end users to maintain cells in continuous culture, to harvest flasks, or to count cells before running an assay. Despite the fact that these cells are not cultured before an assay, they are optimized to ensure predictable performance immediately after thawing. This approach has been pioneered by both Genentech and Amgen's potency assay development teams for developing bioassays in QC and lot release testing (1, 2).

## Optimized Ready-to-Assay Cells From Eurofins DiscoverX

With deep experience in developing and optimizing cryopreserved ready-to-assay cells from continuous culture cell lines, Eurofins DiscoverX has demonstrated broad-scale application of the approach. A frequently asked question is whether cryopreserved cells perform comparably to continuously cultured cells. Figure 3A compares data obtained using both assay formats, with $EC_{50}$ derived from continuous-culture cells on the y axis and cryopreserved ready-to-assay cells on the x axis. For an overwhelming majority of targets, the performance of the two formats match closely and provide equivalent responses, as determined by an excellent $R^2$ of 0.814.

Figures 3B and 3C compare raw data generated through using continuously cultured cells and cryopreserved ready-to-assay cells in a VEGFR2 assay. Here, continuous culture cells were lifted, counted, and plated along with the ligand (VEGF121), then incubated for 16 hours (Figure 3B). Cells from the same single-cell–derived clone were frozen at an early passage and then used in a ready-to-assay format, in which they were thawed and plated directly with ligand onto an assay plate, followed by a 16-hour incubation (Figure 3C). Both assays produced comparable results in terms of signal-to-background ratio and $EC_{50}$ for VEGF121.

With comparable functional output, the advantages of cryopreserved ready-to-assay cells become increasingly evident as noted by both Genentech and Amgen (1, 2).

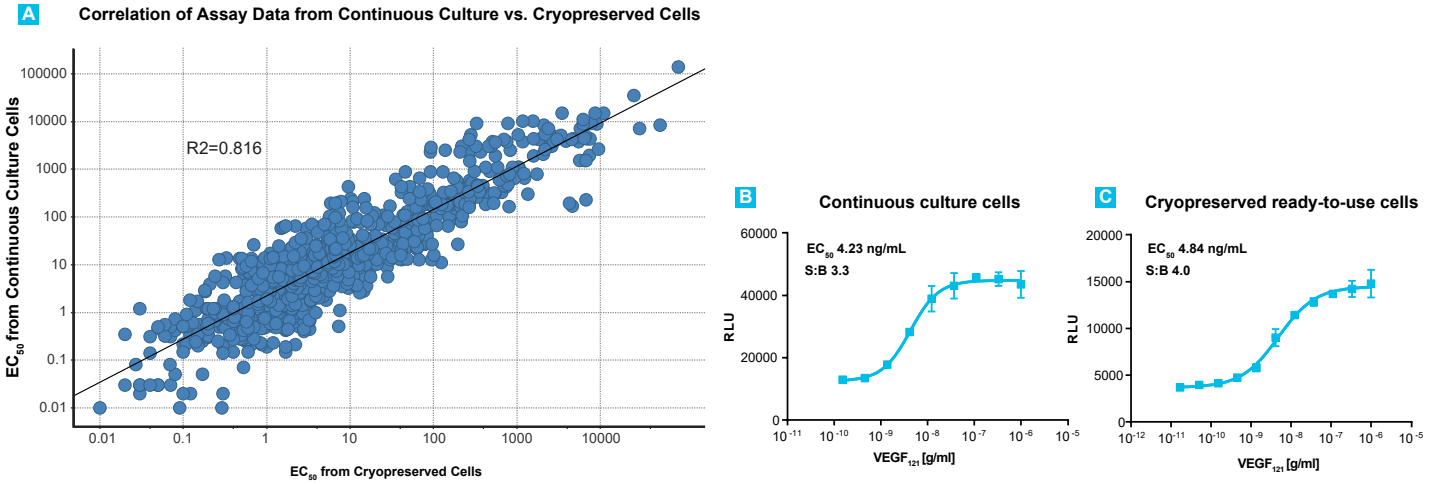


Figure 3. Comparing results for assays run with cells in continuous culture and cryopreserved ready-to-assay cells; (A) correlation of calculated $EC_{50}$ from 842 cell-based assays using cells in continuous culture (y axis) and cryopreserved ready-to-assay format (x axis); a line of best fit includes an $R^2$ value. To compare VEGFR2 assay conducted on cells in continuous culture (B) and cryopreserved ready-to-assay cells (C), cultured cells were lifted and plated with ligand for 16 h, then incubated with detection reagent; cryopreserved ready-to-assay cells were directly plated with ligand for 16 h before addition of detection reagent.

Use of such cells significantly increases:

- Efficiency (fewer personnel needed for cell maintenance and media preparation)
- Data consistency and assay reproducibility (all cells in a given bank are frozen at the same passage number)
- Operational flexibility (assays can be performed as and when needed)
- Cost savings (personnel time, culture media, and reagents)
- Ease of assay development and method transfer to global sites (handling of cells during continuous culture can account for many transfer problems)

Ultimately, these benefits improve overall assay success and significantly reduce timelines for assay development and implementation. However, using a bank of frozen, ready-to-use cells requires proper qualification of those critical reagents to ensure long-term success. A recent paper by industry scientists and consultants offers guidelines for the proper creation, storage, and use of cryopreserved cell banks in quality environments (3).

Eurofins DiscoverX has strictly adhered to the guidelines prescribed in the industry paper, such as:

- The use of a two-tiered cell banking with a master cell bank (MCB) and a working cell bank (WCB)
- Controlled freezing of the cell banks in optimized freezing media
- Storage of the cell banks in the vapor phase of LN2 freezers
- Rigorous testing of cell banks for post-thaw cell viability, mycoplasma, and bacterial sterility
- Functional performance testing with three randomly selected vials

Each lot of cryopreserved cells goes through robust QC checks, the results of which are provided in a detailed certificate of analysis including full traceability to the original cell bank.

## Case Study: Bevacizumab Bioassay

The power of PathHunter® EFC technology for potency assay development is illustrated by a bioassay developed for the angiogenesis inhibitor, bevacizumab. Its mechanism of action is to inhibit the growth of blood vessels in tumors by binding to vascular endothelial growth factor A (VEGF-A), preventing that protein from activating VEGFR2, the primary VEGF receptor found on endothelial cells. VEGF-A–induced activation of VEGFR2 promotes proliferation of endothelial cells, so proliferation of primary human umbilical vein endothelial cells (HUVECs) has been a historical endpoint used in bevacizumab characterization (4, 5).

Figure 4A shows an example of bevacizumab inhibiting VEGF-stimulated proliferation of HUVEC cells with an MTT dye-based readout. That assay is time-consuming (three to four days of assay time) and uses primary HUVECs, which can be highly variable in their performance for passage numbers, lots, and vendors because of donor heterogeneity. Additionally, culturing such cells is significantly challenging, with a risk of high variability and many failed runs. Furthermore, using cell proliferation as the assay endpoint reduces specificity because a number of factors can affect the proliferation of the cells, including other growth factors or media contaminants.

Alternatively, the PathHunter Bevacizumab assay targets an early event in the VEGFR2 signaling. This assay quantifies VEGF-A–induced homodimerization of the VEGFR2 receptor, which is an early step in the receptor's activation cascade. This activation event is blocked by the presence of anti-VEGF-A antibodies such as bevacizumab. As Figure 4B shows, VEGF-A stimulates VEGFR2 dimerization at an $EC_{50}$ consistent with the reported $ED_{50}$ for VEGF-induced proliferation in HUVECs of 1–6 ng/mL (4, 5). In addition, dose-dependent inhibition of dimerization by bevacizumab produces an $IC_{50}$ of ~39 ng/mL, which is comparable to the published $ED_{50}$ of 50 ng/mL for bevacizumab in a HUVEC proliferation assay (6).

The PathHunter method for testing bevacizumab potency has several advantages over HUVEC assays.

1. The PathHunter assay takes significantly less time (results obtained <24 hours rather than >96 hours), which reduces cycle time and increases assay throughput.

2. The simple add-and-read protocol of the PathHunter assays uses ready-to-assay cryopreserved cells, bringing with them all the advantages discussed earlier.

3. The PathHunter assay is robust with high accuracy and precision and a larger assay window than the HUVEC proliferation assay (compare Figures 4A and Figure 4B).

4. The PathHunter assay has since been qualified and validated, based on the USP <1033> Biological Assay Validation guidelines, for QC lot release of biosimilar drugs at partner CROs (data available on request).

5. Additionally, good matrix tolerance (Figure 4C) enables the use of the PathHunter assay in testing clinical samples for neutralizing anti-drug antibodies.

To assess the suitability of the assay for potency testing, we performed an assay qualification exercise over three days. Relative to a reference sample of 100% potency, we tested samples of varying potency ranging from 150% to 50%, according to guidelines from the International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (7, 8). As Table 1 shows, the assay was found to be very accurate (95.9%) and precise (4.1%) over the three day exercise, with a relative standard deviation (RSD) <6%. To demonstrate linearity of sample measurements, we plotted "measured" against "expected" relative potencies (Figure 5A) and calculated the slope and $R^2$ values, arriving at a respectable $R^2$ value of 0.985.

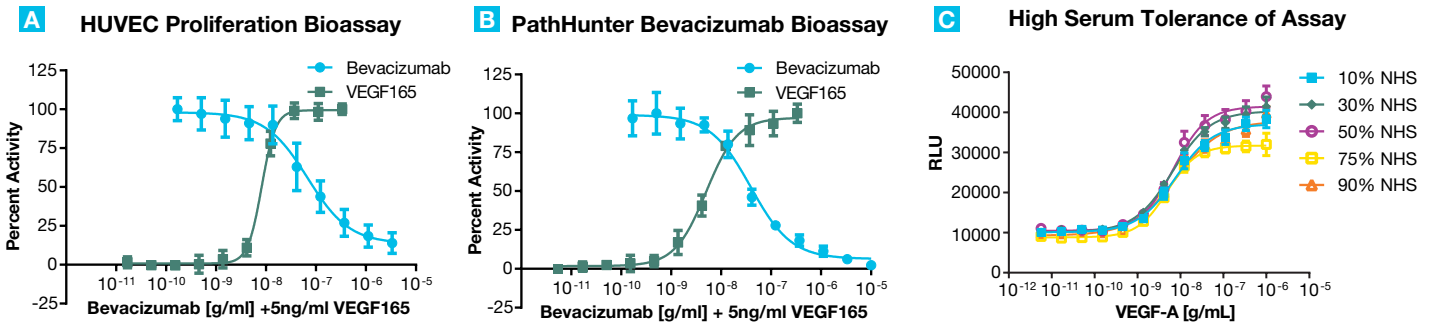


Figure 4. VEGF-A is known to cause homodimerization of VEGFR2 (KDR) as the first step in the activation cascade of these receptors. Anti-VEGF-A antibodies such as Bevacizumab prevent that dimer formation, leading to inhibition of VEGF-A–dependent signaling. (A) Testing of VEGF-A and bevacizumab in the HUVEC proliferation assay provides a functional readout of VEGF-A inhibition. (B) VEGFR2 homodimer bioassay treated with VEGF-A and bevacizumab; the latter displays a robust and precise response to both agents, with EC/$IC_{50}$ results comparable to those obtained with the HUVEC proliferation assay. (C) The VEGFR2 assay is also highly tolerant of matrix (pooled normal human serum) concentrations as high as 90%.

## 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% and Precision = 4.1%**

Table 1. Parallelism and relative potency of a reference standard can be measured with PathHunter assays (see Figure 5). The VEGFR2 dimerization assay was tested with four potency conditions from 50% to 150% and compared with a reference standard (100%).

Finally, we created and tested three independent lots of ready-to-use PathHunter® VEGFR2 dimerization cells to show the reproducibility of their response to the ligand VEGF-165, an active splice variant of VEGF-A. As Figure 5B shows, intra-lot and inter-lot variability met our acceptance criteria, showing <15% CV in $EC_{50}$ between lots and 9.9–11% CV within lots (data not shown). Based on our results, PathHunter VEGFR2 dimerization cells performed well for potency and stability assays, as well as potentially for neutralizing antibody assays, with a simple, robust, and reproducible ready-to-assay protocol.

Table 2 lists additional examples of PathHunter bioassays that are suitable for potency applications. These include bioassays for biosimilar drugs such as growth hormone, insulin, and granulocyte colony-stimulating factor (G-CSF), among others. All represent robust assays as demonstrated by their repeatability, precision, accuracy, and linearity values.

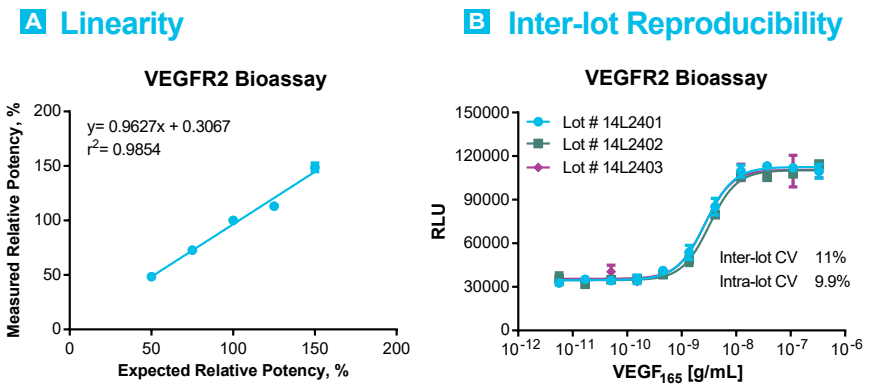


Figure 5. Parallelism and relative potency of a reference standard (A) Measured relative potencies are plotted against expected relative potencies. The assay performed with high degrees of accuracy, precision, and linearity. (B) Variation in $EC_{50}$ of VEGF165 was determined from three independently manufactured VEGFR2 bioassay lots. Excellent reproducibility is observed both within individual lots and among lots, with CVs <11%.

## Qualified Bioassay Examples

| Assay | Drug | Indication | Repeatability (%CV) | Accuracy (%) | Precision (% RSD) | Linearity (r2) |
|---|---|---|---|---|---|---|
| KDR/KDR | Bevacizumab | Cancer | 7.3 | 95.9 | 4.1 | 0.99 |
| GHR | hGH (somatotropin) | Growth Hormone Deficiency (GHD) | 10.1 | 95.3 | 3.1 | 0.99 |
| G-CSF3R | Filgrastim (G-CSF) | Neutropenia | 16.7 | 97.7 | 16.5 | 0.96 |
| INSR | Insulin | Diabetes | 15.5 | 91.7 | n.d. | 0.97 |
| GLP1R | Liraglutide | Diabetes | 8.4 | 98.3 | 6.3 | 0.99 |
| EpoR/EpoR | Epoetin Alfa | Anemia | 10.6 | 92.9 | 6.4 | 0.96 |
| EGFR/ErbB2 | Panitumumab | Cancer | 4.4 | 95.9 | 4.1 | 0.99 |
| IL1R/IL1RAP | Anakinra | Rheumatoid arthritis | 3.9 | 97.7 | 4.6 | 0.97 |
| ErbB2/ErbB3 | Pertuzumab | Cancer | 7.2 | 96.8 | 4.8 | 0.99 |

Table 2. Examples of Eurofins DiscoverX assays qualified for potency and lot release use; performance metrics are listed for nine different therapeutic targets that are high profile targets for biosimilar development. All nine assays met standard acceptance criteria for repeatability, accuracy, precision, and linearity.

## The Need for Speed

With increased global emphasis on development of innovator biologics and biosimilars alike, and with multiple developers targeting the same molecules, there is a clear race to be the first to submission for any molecule. This is driving the need for specific bioassays to accelerate and shorten their development timelines. Availability of commercial ready-to-assay cryopreserved cells in a qualified kit will enable developers to skip difficult and time-consuming method development and move directly to qualification and assay validation for potency and neutralizing antibody assays. For biosimilar developers, the target specificity and robust assay performance demonstrated by the examples shown are also important factors in establishing similarity with respect to quality and efficacy between their follow-on molecules and the innovator products they are meant to replace.

**Commercially available and qualified ready-to-assay kits will enable biosimilar & biobetter developers to skip difficult and time-consuming method development, and move directly to assay validation, supporting their race to be the first to submission for their molecule.**

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