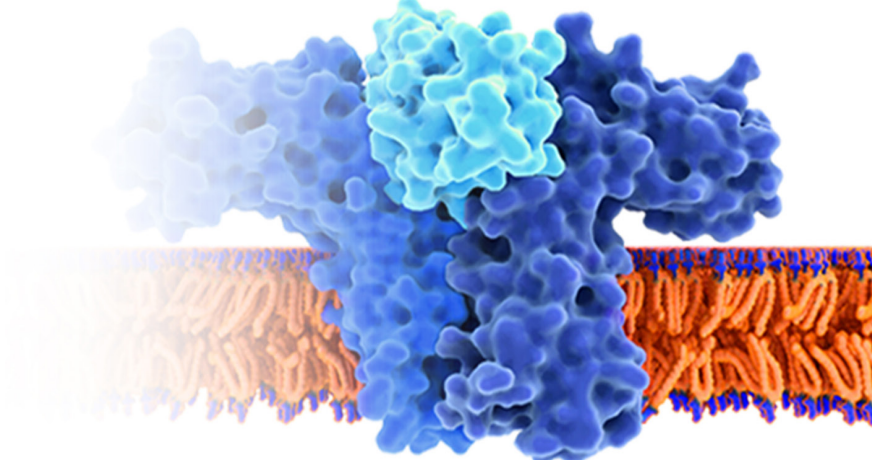


APPLICATION NOTE

Functional, Cell-based Assays For Cytokines Signaling and Relative Potency Testing in Lot-release Applications

INTRODUCTION

Cytokines play a crucial role in immune responses and have become important drug targets for treating inflammatory and autoimmune diseases. Over the past decade, numerous cytokine-based clinical and preclinical trials have emerged, resulting in several approved drugs such as Humira® (targets TNFα), Dupixent® (targets IL-4 and IL-13), Stelara® (targets IL-12 and IL-23), Tremfya® (targets IL-23), and Actemra® (targets IL-6). Advances in understanding of the diverse cytokine signaling pathways and receptor combinations have improved knowledge of cytokine functions. However, developing therapeutics targeting complex cytokine pathways remains challenging, necessitating specific tools. Eurofins DiscoverX® offers a range of cell-based assays designed for implementation from discovery to development and into the clinical release of cytokine therapeutics targeting several conditions, including inflammatory and autoimmune diseases.

CYTOKINE RECEPTOR DIMERIZATION AND SIGNALING REPORTER ASSAYS

Eurofins DiscoverX offers one of the largest portfolios of cell-based assays for the cytokine family of targets including interleukin receptors and chemokines that use the patented enzyme fragment complementation (EFC) technology. These assays are available in two different assay formats:

(1) Receptor Dimerization Assays – Dimerization assays allow for the interrogation of interleukin binding that results in receptor subunit homo- or hetero-dimerization (dimerization format).

(2) Signaling Reporter Assays – Reporter assays measure the activation of a transcriptional reporter downstream of an interleukin binding to its receptor (reporter format).

KEY RESOURCES

- Cytokine and interleukin receptor assays
- Signaling pathway reporter assays
- Ready-to-use, cell-based bioassays

The former offers unparalleled specificity by measuring ligand-mediated dimerization of the specific interleukin receptor subunits. In contrast, the latter offers large assay windows due to signaling pathway amplification and the downstream readout. Both assay formats are available as cell lines or ready-to-use assays. Results with these homogeneous, robust assays demonstrate that they are fit-for-purpose during drug development from early discovery to relative potency testing in quality control (QC) lot release applications.

Cytokine-induced signaling via dimerization and reporter assays based on the application of EFC technology are shown in Figure 1.

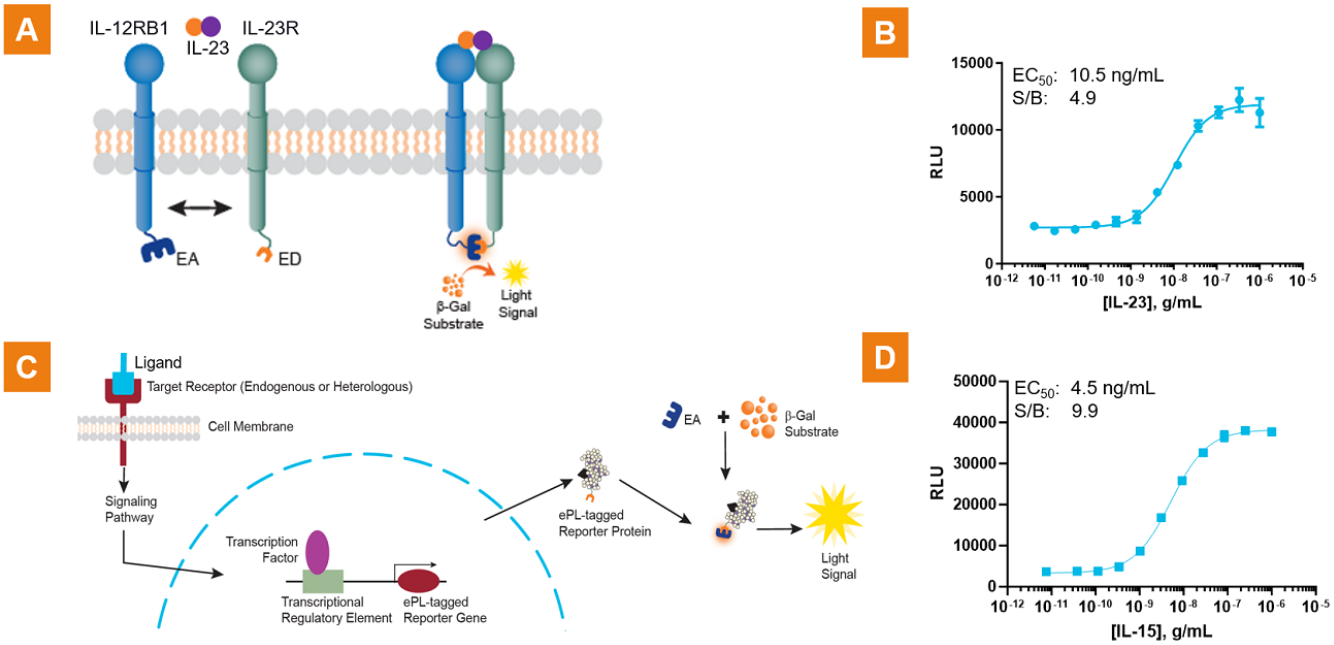


Figure 1. IL-23 induced receptor dimerization assay and IL-15 signaling reporter assay. A. and B. demonstrates the quantitation of receptor dimerization and gain of signal response using a PathHunter® IL-23 dimerization cell-based assay. IL-23 receptor subunits are tagged with EFC β-galactosidase (β-gal) fragments EA (enzyme acceptor) and ED (enzyme donor). Upon agonist treatment, the two subunits dimerize, resulting in the complementation of the two β-gal fragments to create a functional β-gal enzyme, whose activity is measured with a luminometer. A quantitative gain-of-signal sigmoidal response as a measure of IL-23 receptor dimerization is observed. C. and D. show cytokine activation measured by quantifying a distal receptor signaling event (transcriptional activation) using an EFC assay format, wherein cytokine binding to the receptor initiates a signaling cascade resulting in ED-tagged reporter protein production and increased enzyme activity with the ED fragment addition upon cell lysis (C.). A quantitative gain-of-signal sigmoidal response as a measure of IL-15 signaling is observed (D.).

Both dimerization and reporter assays can be used for measuring signaling pathways. Dimerization assays are more specific in their cytokine activation, while reporter assays often show larger assay windows and good sensitivity. An example of dimerization assay specificity is observed when different cytokines (IL-1β, IL-33, TNFα, etc.) are tested; only IL-1β results in IL-1R1/IL-1RAP dimerization (Figure 2. A). In contrast, when an NF-kB signaling reporter assay is used to measure cytokine activation signaling, a number of cytokines (e.g., TNFα, CD40L) in addition to IL-1β activate NF-kB signaling (Figure 2. B.). However, the signal/background (S/B) window observed for IL-1β is much larger with the NF-kB signaling reporter assay (S/B = 58.3, EC_{50} = 1.4 ng/mL) compared to that observed with the IL-1/IL-1RA dimerization assay (S/B = 5.7, EC_{50} = 0.83 ng/mL).

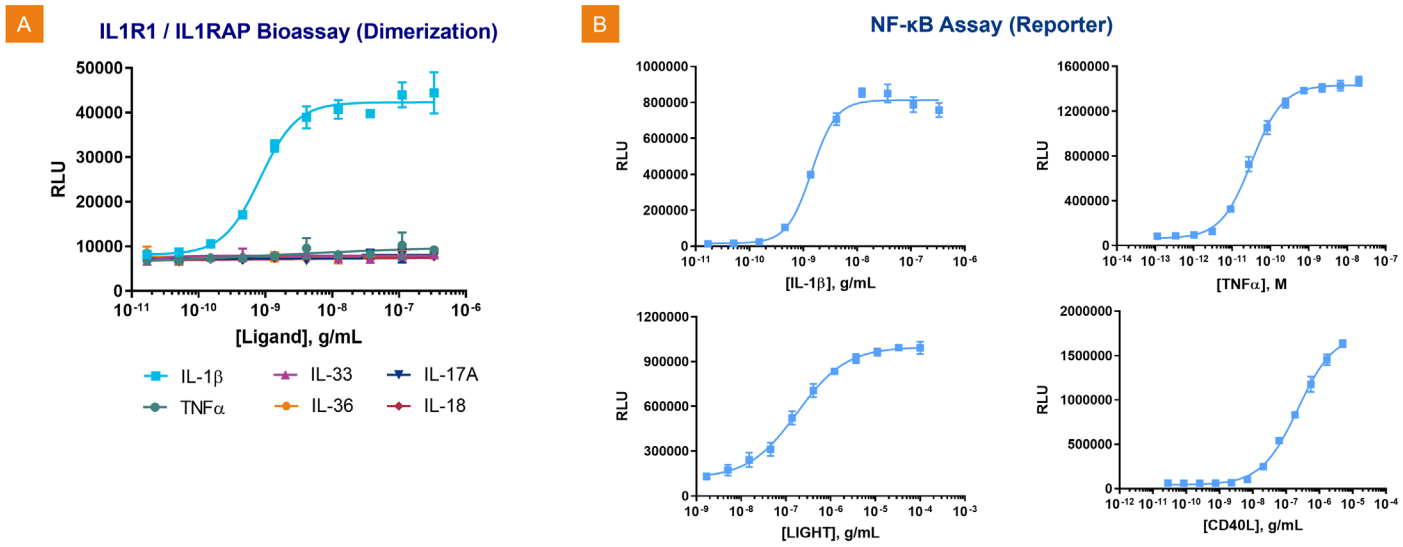


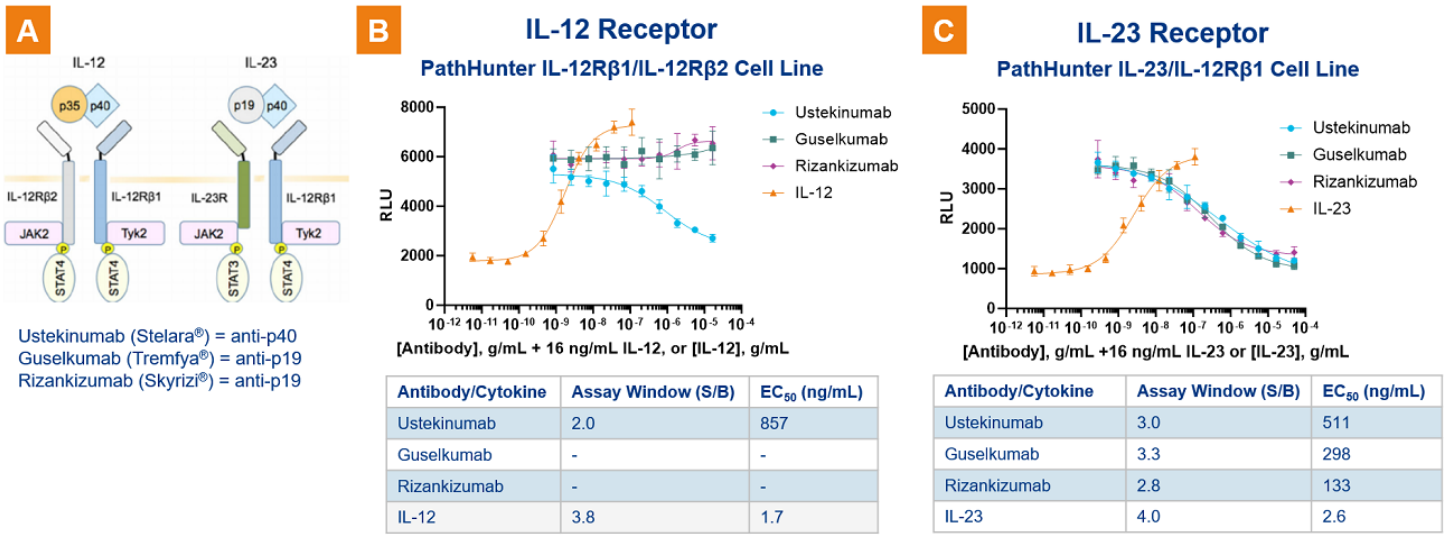
Figure 2. Receptor dimerization vs. signaling reporter assay. A. Multiple cytokines tested for IL-1R1/IL-1RAP dimerization, in which only IL-1β caused dimerization, and B. demonstrates the activation of a reporter gene (NF-kB) in a signaling reporter assay by different ligands including IL-1β.

SPECIFICITY OF RECEPTOR DIMERIZATION ASSAYS

Greater specificity of receptor dimerization assays is further demonstrated with two members of the IL-12 family, IL-12 and IL-23 cytokines, both validated therapeutic targets for Crohn's disease, plaque psoriasis, and psoriatic arthritis. IL-12 and IL-23 are heterodimers containing the common cytokine subunit (p40) that binds to the IL-12Rβ1 receptor subunit. Their unique cytokine subunits (p35 and p19 for IL-12 and IL-23, respectively) and receptor partners (IL-12Rβ2 and IL-23R for IL-12 and IL-23, respectively) are responsible for specific signaling pathway activation. When IL-12, IL-23, and a homodimer of the p40 subunit are used in the assays (see Figures 3. B.), only IL-12 activates IL-12Rβ1/IL-12Rβ2 dimerization and not IL-23R/IL-12Rβ1 dimerization. Similarly, only IL-23 activates IL-12Rβ1/IL-23R (see Figure 3. C).

The p40 subunit monomer of IL-12 or IL-23 results in equally poor activation in both assays as expected (data not shown).

When using a therapeutic antibody targeting the common p40 subunit found in IL-12 and IL-23 (e.g., Ustekinumab marketed as Stelara), the antibody inhibits both IL-12R and IL-23R dimerization (see the blue curves in Figures 3. B. and C.). However, antibodies targeting the p19 subunit specific for IL-23 (e.g., guselkumab (Tremfya) and riazankizumab (Skyrizi®)) result in an antagonistic IL-23 signaling in the IL-23R dimerization assay, but have no effect on IL-12 activation of IL-12 receptor dimerization. The data with these therapeutic antibodies demonstrate how the assays can be used to compare the activity and potency of different drugs.



Antibody/Cytokine	Assay Window (S/B)	EC_{50} (ng/mL)
Ustekinumab	2.0	857
Guselkumab	-	-
Rizankizumab	-	-
IL-12	3.8	1.7

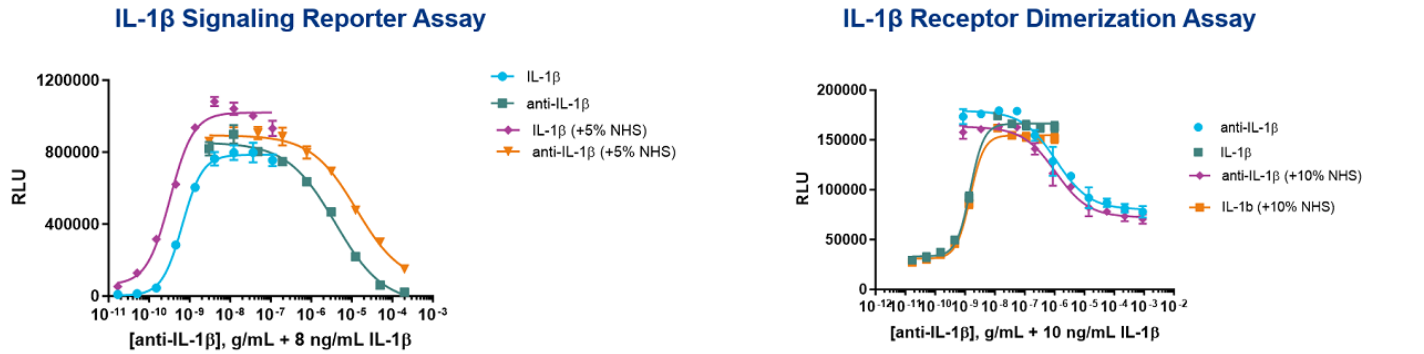
Antibody/Cytokine	Assay Window (S/B)	EC_{50} (ng/mL)
Ustekinumab	3.0	511
Guselkumab	3.3	298
Rizankizumab	2.8	133
IL-23	4.0	2.6

Figure 3. Therapeutics targeting the p40 subunit of IL-23 and IL-12 or the p19 subunit of IL-23. The effects of antibodies (anti-p40 and anti-p19) on IL-12 and IL-23 receptor dimerization are shown wherein ustekinumab targeting the common p40 subunit inhibits dimerization of IL-12Rβ1 to either IL-12Rβ2 or IL-23R. Anti-p19 antibodies, however, only inhibit IL-23 signaling in the IL-23 receptor dimerization assay and have no effect in the IL-12 receptor dimerization assay.

SENSITIVITY OF SIGNALING REPORTER ASSAYS

An NF-kB reporter assay was used to compare IL-1β agonist and antagonist responses and sensitivities to the IL-1RA/IL-1RAP dimerization assay. IL-1β activation (agonist) and its inhibition (in antagonist mode with anti-IL-1β antibody) was measured with or without 5% normal human serum (NHS). The S/B ratios obtained with the reporter assay format were larger in both agonist and antagonist mode, but this reporter assay sensitivity was attenuated in antagonist mode, particularly in the presence of 5% NHS (Figure 4. A.). When using the dimerization assay for IL-1β signaling, there was no change in the assay window or the IC_{50} observed with anti-IL-1β drug in the presence of 10% NHS (Figure 4. B.).

This example demonstrates that the signaling reporter assay format results in a greater response to IL-1β activation than the dimerization assay, even in the presence of NHS. However, the reporter assay exhibits a susceptibility to changes in S/B or potency in the presence of human serum since components in the serum may affect other signaling pathways in the cell or other steps in the IL-1β pathway downstream of dimerization, and thereby impact NF-kB mediated transcription.



Sample	PBS		+ 5% NHS	
	S/B	EC/ IC_{50}, ng/mL	S/B	EC/ IC_{50}, ng/mL
IL-1β (agonist mode)	**50.1**	0.66	**19.5**	0.33
Anti-IL-1β + 8 ng/mL IL-1β	**40.3**	4,445	**5.7**	13,020

Sample	PBS		+ 10% NHS	
	S/B	EC/ IC_{50}, ng/mL	S/B	EC/ IC_{50}, ng/mL
IL-1β (agonist mode)	**6.0**	1.49	**5.5**	1.49
Anti-IL-1β + 10 ng/mL IL-1β	**2.2**	1,104	**2.2**	1,082

Figure 4. IL-1βR signaling reporter and receptor dimerization assays. The signaling reporter assay used to measure IL-1β signaling shows attenuated assay windows and increased IC_{50} in the presence of NHS, while dimerization assay results are similar in the presence or absence of NHS.

As shown in this example, the receptor dimerization assay produces robust agonist and antagonist responses in the presence of 10% NHS required for quantifying anti-drug neutralizing antibodies (NAb) in human serum samples. Hence, dimerization assays are better suited for NAb screening as agonist and antagonist responses exhibit little change when human serum is present.

APPLICATION OF CYTOKINE ASSAYS FOR RELATIVE POTENCY, CHARACTERIZATION, AND LOT RELEASE

The cytokine assays developed by Eurofins DiscoverX are available for use in the ready-to-use bioassay format and have been qualified for their ability to meet the criteria needed for assay qualification of candidate therapeutics. These assays reflect the drug's mechanism of action (MOA), can measure changes in drug potency, and are optimized to give robust and reproducible results. When used in potency testing, the relative potencies of the drug can be measured in response to cytokine activation or dimerization. For instance, relative potency analysis of Actemra (tocilizumab) using IL-6 bioassay cells demonstrates IL-6 induced IL-6R/IL-6ST dimerization (Figure 5).

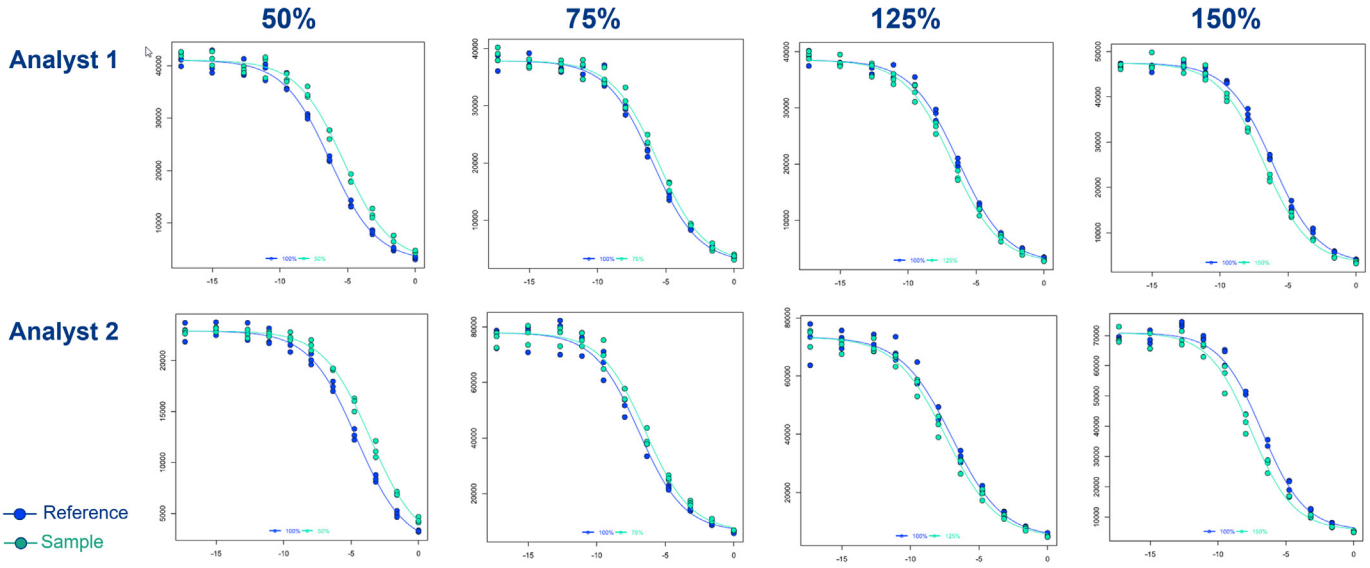


Figure 5. Relative potency assessment for the tocilizumab (IL-6) qualified bioassay. A wide range of potencies was demonstrated using 4 nominal concentrations of the drug (tocilizumab) in an IL-6 bioassay to measure IL-6-induced dimerization conducted by two different analysts.

Further, qualification of Actemra using the tocilizumab bioassay shows a high degree of accuracy and exceptional linearity, validating its suitability in measuring relative potency with high confidence.

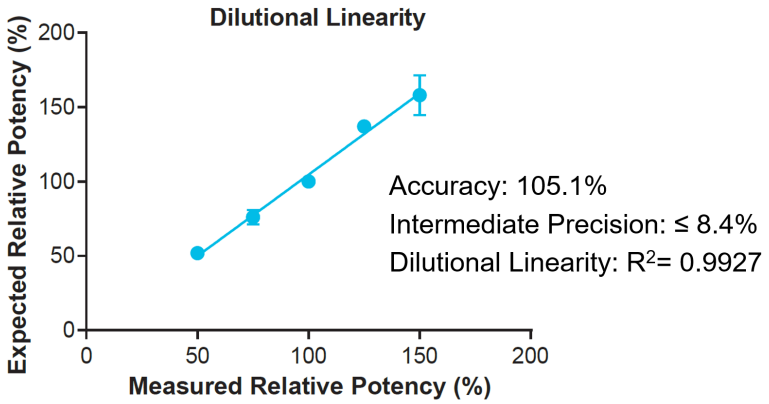


Figure 6. Tocilizumab bioassay qualification results using Actemra. Actemra, an IL-6R antagonist, is qualified using the tocilizumab bioassay displaying a high degree of relative potency associated with a high level of dilutional linearity and accuracy.

SUMMARY

Eurofins DiscoverX's cytokine assays are available in multiple formats to measure receptor activation and dimerization as well as downstream signaling. PathHunter receptor dimerization assays show greater specificity over reporter assays and are suitable for NAb testing due to their insensitivity in the presence of human serum. PathHunter reporter assays also enable the measurement of cytokine signaling changes, often providing larger assay windows and greater sensitivity for screening.

The PathHunter cytokine assays include target-specific, assay-ready formats addressing both short-term and cell-line evaluation needs to anchor early discovery phase applications. The RTU bioassay kit format is carefully crafted for QC lot-release testing programs, specific for the target being studied, and qualified using marketed drugs. Overall, Eurofins DiscoverX's qualified bioassays (including that of cytokines) have primarily supported the qualification and validation of hundreds of therapeutic drugs, and effortless and routine transfer to other sites.

PathHunter cytokine receptor assays aid in cytokine drug discovery and development:

- Offer MOA-based dimerization, signaling reporter, and other assay types covering over 80% of interleukins
- Available in stable cell lines, including working cell banks, as well as ready-to-use eXpress™ assay and bioassay kit formats with many bioassays qualified with a reference ligand or therapeutic
- Used from discovery through development and into commercial release and stability QC lot testing

Visit discoverx.com/target-class/cytokine-receptor/ to access functional cell-based assays for over 80% of human interleukins, including IL-1, IL-2, IL-6, IL-12, IL-7, IL-15, TNFα, GM-CSF, and more.

RESOURCES

- Cytokine and interleukin receptor assays
- BLOG: Advancing therapeutics targeting cytokines
- Ready-to-use, cell-based bioassays for comparability and QC lot release testing
- Functional cell-based assays to support COVID-19 drug discovery through QC lot release programs
- Signaling pathway reporter assays for understanding therapeutic MOAs
- EFC platform for interrogating biomolecular reactions

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