Advancing Therapeutics Targeting Inflammatory Cytokines with Functional Cell-based Assays

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Abstract

Cytokines have emerged as major drug targets for inflammatory and autoimmune diseases owing to their involvement in the function of both adaptive and innate immune systems. A significant number of cytokine-based clinical and preclinical development trials have surfaced in the last decade with many approved drugs targeting inflammatory cytokines such as Humira® (TNFα), Dupixent® (IL-4/IL-13), Stelara® (IL-12/IL-23), and Actemra® (IL-6), while many others are under development.

Eurofins DiscoverX® offers one of the largest portfolios of cell-based assays for Interleukins and cytokines that uses the industry-validated enzyme fragment complementation (EFC) technology. Two different assay formats are available to interrogate either (1) interleukin binding that results in receptor subunit hetero-dimerization (dimerization format), or (2) activation of a transcriptional reporter downstream of interleukin binding to its receptor (reporter format). The former offers unparalleled specificity by measuring ligand-mediated dimerization of the specific interleukin receptor subunits, while the latter offers large assay windows due to its distal nature and pathway amplification. Both assay formats are available as cell lines or ready-to-use assay kits. These homogeneous and robust assays are fit-for-purpose for implementing from discovery to QC lot-release.

In this poster, we present case studies on the use of cell-based assays that monitor both mechanisms of actions (MOAs) – receptor dimerization and signaling reporter activation (e.g. transcriptional reporters) targeting key inflammatory cytokines including IL-12, IL-23, IL-15, IL-17, and IL-4/IL-13 for a variety of applications, including rank ordering of antibodies, evaluating antibody specificity, as well as detection of anti-drug neutralizing antibodies.

Multiple MOAs for Monitoring Cytokine Signaling

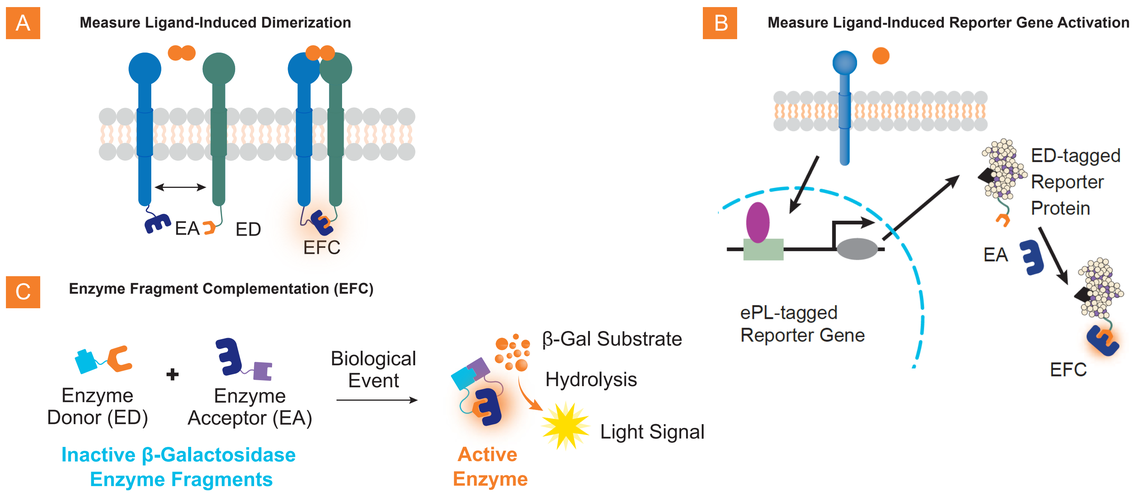
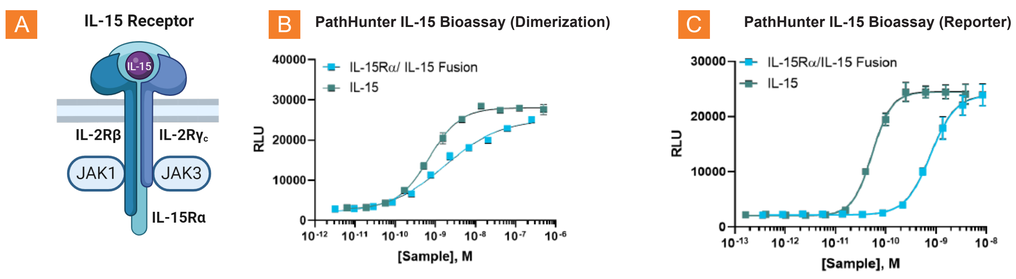


Figure 1. PathHunter® assay to monitor two MOAs for interleukin/cytokine signaling. Interleukin or cytokine activation of membrane receptors are detected by either receptor subunit dimerization occurring immediately after ligand-receptor binding or downstream in the signaling pathway via activation of a transcriptional reporter gene. Both MOAs employ the proprietary Eurofins DiscoverX EFC technology that is based on a split β-galactosidase (β-gal) enzyme system. **A.** For the dimerization assay, each partner receptor subunit is fused to either the complementary β-gal. enzyme acceptor (EA) or enzyme donor (ED) fragments. Upon ligand-receptor engagement, receptor heterodimerization and enzyme complementation occurs resulting in increased enzyme activity. **B.** For the signaling reporter assay, cytokine-receptor engagement activates a signaling cascade that subsequently drives expression of a reporter protein tagged with the small enhanced ProLabel (ePL) ED fragment. The EA fragment is added prior to EFC. **C.** EFC overview. Enzyme activity in both assays is measured with addition of detection reagent containing luminescent enzyme substrate and detecting the complementation of the ED and EA fragments.

Fit-for-purpose Solutions for IL-15 (target) in Two Assay Formats



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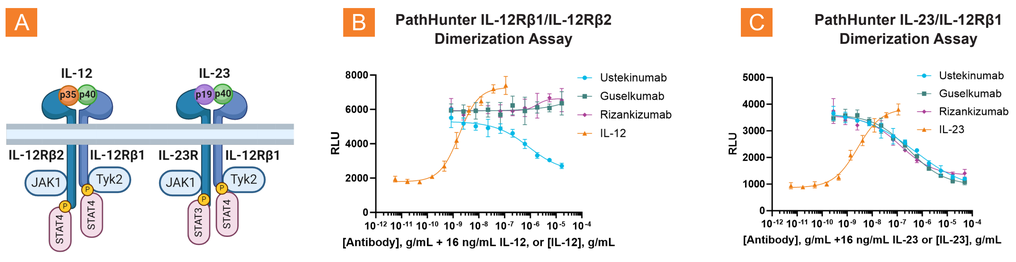
Assay Format	Dimerization Cell Line	Reporter Cell Line
Cell Background	U2OS	REH
Receptor Subunits: IL-2RB, IL-2RG, IL-15RA	Engineered (Truncated)	Engineered (Full Length)
Assay Window (S/B): IL-15 / IL-15Rα-IL-15 Fusion Protein	8.8 / 8.7	14.7
EC_{50} (nM): IL-15 / IL-15Rα-IL-15 Fusion Protein	0.69 / 1.7	0.054 / 0.78
Incubation Time (hours)	18-20	16-18

Figure 2. PathHunter cell-based assays for IL-15. **A.** The IL-15 receptor is a heterotrimeric receptor comprised of the signaling subunits IL-2Rβ (IL-2RB) and γc (IL-2RG) that are shared with the IL-2 receptor, and a high affinity IL-15Rα (IL-15RA) subunit. *In vivo*, IL-15 is usually presented in trans by the IL-15Rα chain to the IL-2Rβ/IL-2Rγc complex displayed on the surface of T cells and natural killer (NK) cells. However, recombinant IL-15 does not require presentation by IL-15Ra to activate the IL-2Rβ/IL-2Rγc complex. Binding of IL-15 leads to activation of signaling through JAK/STAT pathway, primarily through STAT5. **B.** PathHunter bioassays for IL-15 receptor heterodimerization in U2OS bioassay cells or (**C.**) detection of IL-15 signaling pathway activation through a STAT5 transcriptional reporter gene in REH bioassay cells. Both assay formats utilize engineered receptors (Extracellular and transmembrane domains only for the dimerization format, and full-length receptors for the reporter assay). A slightly larger assay window and lower EC_{50} are observed with the PathHunter IL-15 Reporter Bioassay as compared to the PathHunter IL-15 Dimerization Bioassay. Both assay formats are suitable for assessing potency of soluble IL-15 and IL-15Rα-IL-15 fusion proteins.

Specificity of Receptor Dimerization Assays using Therapeutics Targeting Cytokines



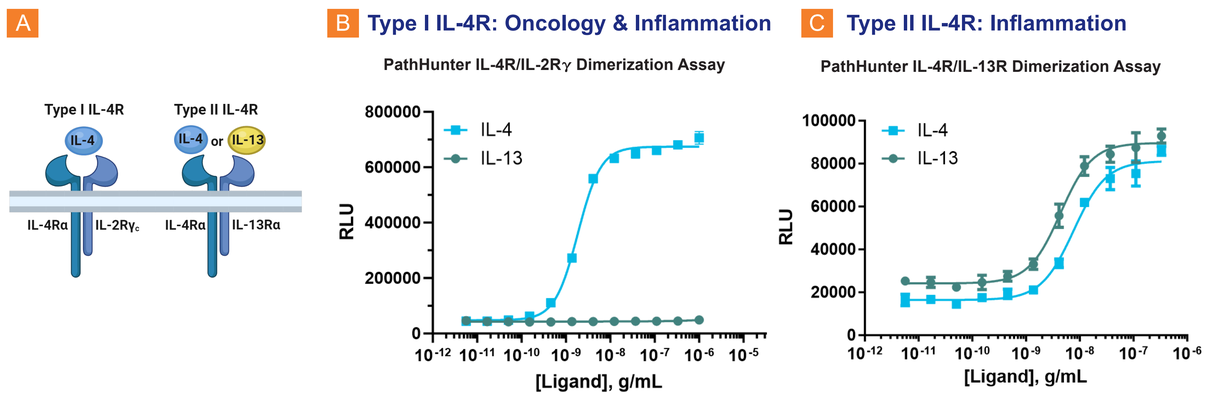
Ustekinumab (Stelara®) = anti-p40
Guselkumab (Tremfya®) = anti-p19
Rizankizumab (Skyrizi®) = anti-p19

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. PathHunter receptor dimerization assay testing with therapeutics. **A.** IL-12 and IL-23 signaling involves common cytokine (p40) and receptor (IL-12Rβ1) subunits. Their unique cytokine subunits (p35 and p19) and receptor partners (IL-12Rβ2 and IL-23R) are responsible for specific signaling pathway activation. **B.** and **C.** 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 both IL-12Rβ2 and 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. The data with these therapeutic antibodies demonstrate how the assays can be used to compare the activity and potency of different drugs.

IL-4 Receptor-specific Signaling using Dimerization and Reporter Assays



Cytokine	Assay Window (S/B)	EC_{50} (ng/mL)
IL-4	16	1.9
IL-13	-	-

Cytokine	Assay Window (S/B)	EC_{50} (ng/mL)
IL-4	5.2	1.9
IL-13	3.8	4.4

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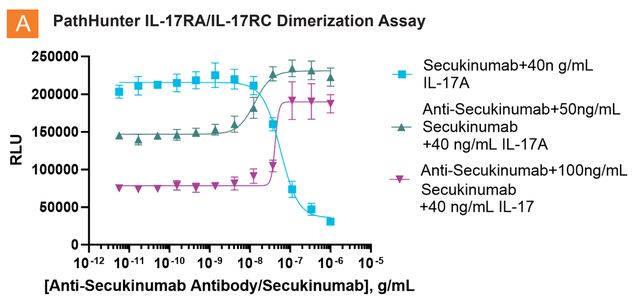
Type I + Type II IL-4R: Oncology & Inflammation
PathHunter IL-4R/IL-13R Signaling Reporter Assay
RLU
IL-4
IL-13
[IL-4/IL-13], g/mL

Cytokine	Assay Window (S/B)	EC_{50} (ng/mL)
IL-4	6.3	10.0
IL-13	7.7	61.4

Figure 4. IL-4 receptor dimerization and signaling reporter assays to characterize receptor signaling of IL-4/IL-13. **A.** IL-4 initiates signaling through type I or type II receptor complexes. The type I receptor consists of IL-4Rα and γc (IL-2Rγ) subunits and is activated only by IL-4 in immune cells, while the type II receptor consists of IL-4Rα and IL-13Rα subunits and can be activated by either IL-4 or IL-13 in epithelial cells. **B.** and **C.** PathHunter IL-4R/IL-2RG dimerization cell line tested with IL-4 and IL-13 shows only IL-4 activated dimerization, while both IL-4 and IL-13 activated dimerization were achieved using the PathHunter IL-4R/IL-13R dimerization cell line. The two dimerization assays monitor effects on the different types of receptor complexes, which in turn would enable characterization of therapeutics effects on specific cell types. **D.** The PathHunter IL-4/IL-13 signaling reporter assay was used to quantify IL-4 and IL-13 signaling. The IL-4 activation measured with the reporter assay is a combination of IL-4 activated signaling from type I + II receptors, since IL-2R γ is expressed in the cell line. The IL-4/IL-13 signaling reporter assay cannot discriminate between IL-4 activation from type I vs. type II IL-4R receptor complexes.

Quantitation of Neutralizing Antibodies (NAb) to Secukinumab



B

Antibodies/Cytokine	Assay Window (S/B)	EC / IC / NC_{50} (ng/mL)
IL-17A	9.5	8.4
Secukinumab +40ng/mL IL-17A	6.6	57
Anti-Secukinumab +50ng/mL Secukinumab +40ng/mL IL-17A	1.5	13
Anti-Secukinumab +100ng/mL Secukinumab +40ng/mL IL-17A	2.5	42

FDA guidelines specify a minimum sensitivity of 1 µg/mL for detection of nAbs in human serum

Figure 5. PathHunter IL-17R dimerization assay to detect neutralizing Secukinumab antibodies. IL-17 receptor dimerization assay results demonstrating robust agonist and antagonist activity were performed with samples prepared in the presence of 10% normal human serum (NHS). Serum tolerance is required for quantifying anti-drug NAbs in human serum samples. **A.** Increasing concentrations of an anti-secukinumab (anti-IL17A) antibody blocks the inhibition of IL-17A signaling by secukinumab. **B.** The measured effective neutralizing concentration (NC50) is in the 10-100ng/mL range meeting the FDA guideline of a minimum sensitivity of 1 µg/mL. The anti-secukinumab antibody exhibits potent neutralizing activity of secukinamb IL-17A inhibition and demonstrates assay suitability for NAb screening of patient serum.

Multitude of Assays for Cytokine/Interleukin Targets

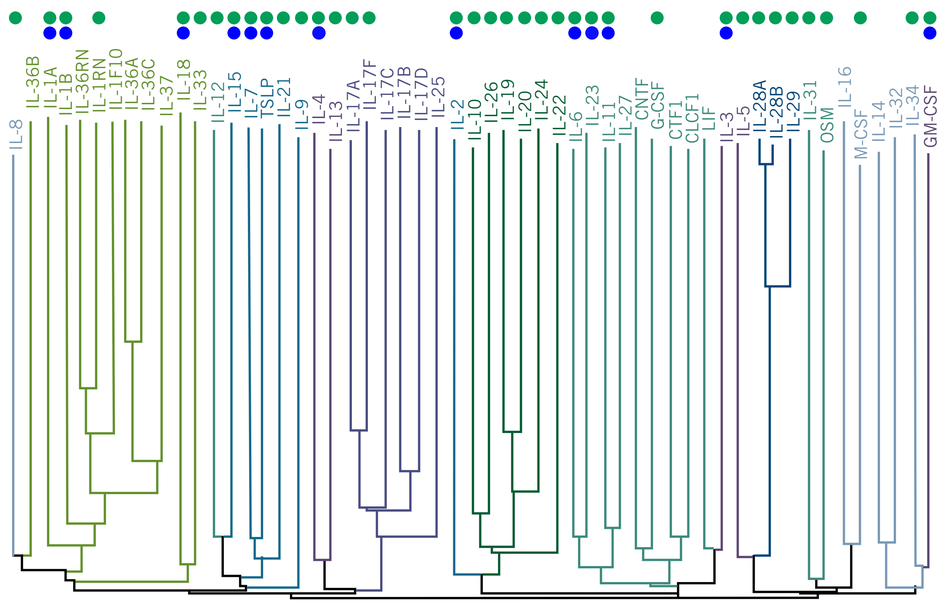


Figure 6. Phylogram of human interleukins and closely related proteins outline 8 major groups. Interleukin groups include IL-1-like, IL-4-like, IL-10-like, IL-28-like, IL-17-like, and the non-classified cytokines. **Green dots** indicate existing assays developed at Eurofins DiscoverX (largely in the dimerization portfolio). **Blue dots** indicate new reporter format assays available or in development. Additional assays can be developed under our custom development capabilities program.

Summary

- PathHunter cytokine and interleukin assay platforms offer MOA-based dimerization and signaling reporter assays covering over 80% of interleukins, including a variety of interleukins targeting inflammation.
- PathHunter dimerization MOA, offered as cell line and bioassays, are sensitive and highly specific assays that are minimally impacted by the presence of human serum, making them suitable for both relative potency and neutralizing antibody detection applications
- PathHunter reporter assays provide large assay windows and excellent sensitivity, making them excellent choices for screening and relative potency assays

Learn more about Eurofins DiscoverX cytokine receptor assays at **discoverx.com/cytokine** or contact us at **discoverx.com/contact-us** for custom assay solutions.

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