Comprehensive and Fit-for-Purpose Solutions to Enable Drug Development for Cytokine Targets

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Abstract

Interleukins are known for their involvement in functioning of both the adaptive and innate immune systems. Interleukins direct immune cells to divide and differentiate and are classified as either pro-inflammatory or anti-inflammatory. Interleukins and their receptors are well-validated targets for therapeutic intervention in immuno-oncology and for a variety of disorders, such as psoriatic arthritis, Crohn's disease, and many other indications. Examples of interleukins that are current targets for therapeutic intervention with marketed drugs include, IL-2, IL-6, IL-4/IL-13, IL-17, and IL-23.

Eurofins DiscoverX® offers one of the largest portfolios of cell-based assays for Interleukins and cytokines which uses the patented EFC technology. These assays are available as two different assay formats to interrogate either (1) interleukin binding that results in receptor subunit hetero-dimerization (dimerization format), or (2) activation of a transcriptional reporter downstream of an 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 the distal nature of pathway amplification. Both assay formats are available as cell lines or ready-to-use assays. These homogeneous and robust assays are fit-for-purpose for implementing from discovery to QC lot release.

In this poster, we present representative data for a variety of interleukins and cytokines (such as IL-2, IL-15, GM-CSF, IL-6 and TSLP) highlighting their performance in both the dimerization and transcriptional reporter assay formats.

Multiple MOAs for Monitoring Cytokine Signaling

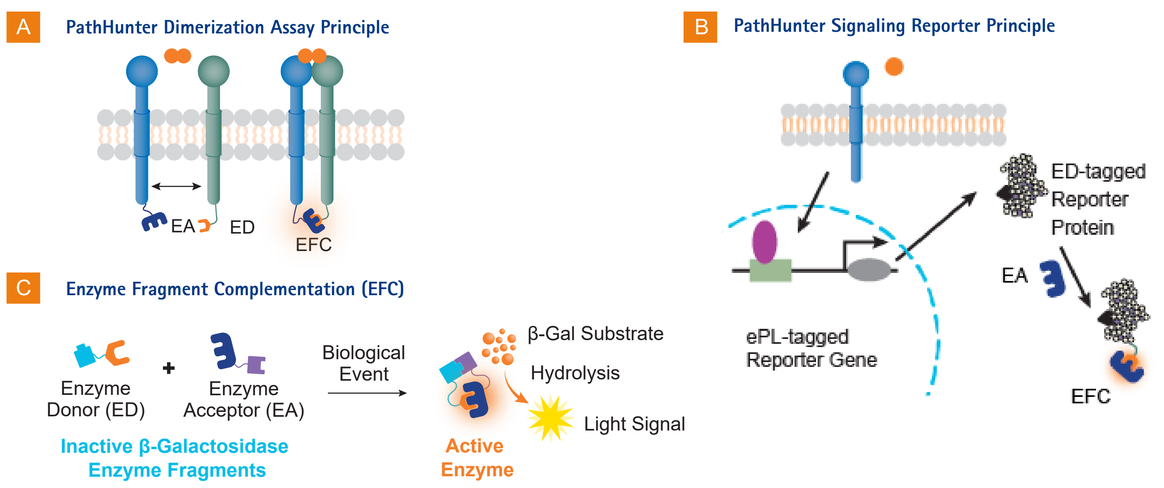
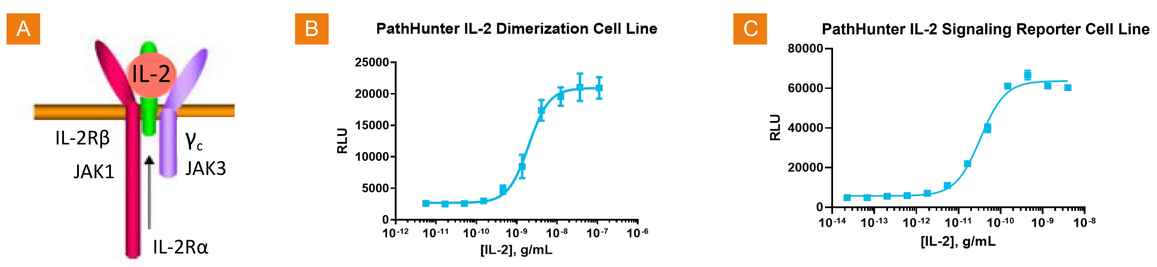


Figure 1. PathHunter® dimerization and transcriptional reporter assay formats to monitor interleukin/cytokine signaling. Interleukin or cytokine activation of membrane receptors are detected by either receptor subunit dimerization occurring immediately after ligand-receptor binding or downstream in the signaling pathway activation of a transcriptional reporter gene. Both mechanism of actions (MOAs) employ the proprietary Eurofins DiscoverX Enzyme Fragment Complementation (EFC) technology that is based on a split β-galactosidase (β-gal) enzyme. A. For the dimerization assay, each partner receptor subunit is fused to either the complementary β-gal enzyme acceptor (EA) or enzyme donor (ED) fragment. Upon ligand-receptor engagement, receptor hetero-dimerization and EFC occurs resulting in increased enzyme activity. B. For the signaling reporter assay, cytokine-receptor engagement activates the signaling pathway that subsequently drives expression of a reporter protein tagged with the small enhanced ProLabel® (ePL), the β-gal 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.

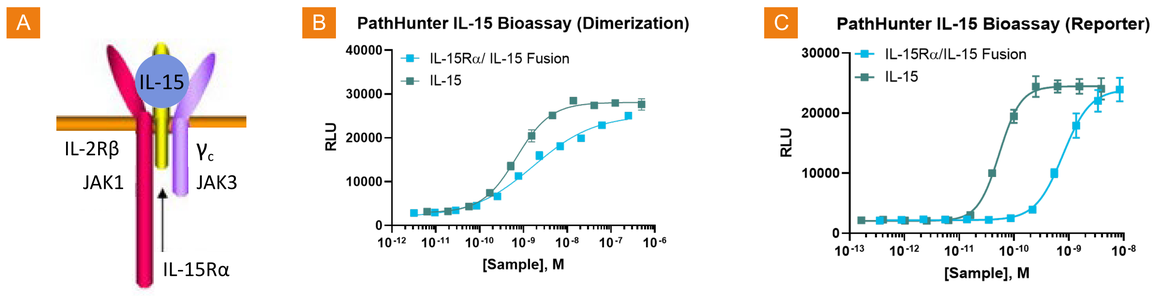
IL-2 Receptor Cell-Based Assays to Measure Dimerization and Downstream Transcription



D	Assay Format	Dimerization Cell Line	Reporter Cell Line
	Cell Background	U2OS	REH
	Receptor Subunits: IL2RA, IL2RB, IL2RG	Engineered (Truncated)	Engineered (Full Length)
	Assay Window (S/B)	9.6	13.9
	EC_{50} (ng/mL)	2	0.33
	Incubation Time (hours)	6	16

Figure 2. PathHunter cell-based assays for IL-2. A. The IL-2 receptor is a heterotrimeric receptor comprised of the three receptor subunits: the ligand-specific IL-2Rα subunit (encoded by IL2RA), the signaling subunits IL-2Rβ (encoded by IL2RB), and γ_c (encoded by IL2RG). IL-2 initially binds to IL-2Rα, then IL-2Rβ is recruited, and finally γ_c, leading to activation of signaling through JAK/STAT pathway, primarily through STAT5. All three subunits are required for high affinity binding, although IL-2 can bind to the IL2RB/IL2RG heterodimer with lower affinity. B. The dimerization assay for IL-2 receptor hetero-dimerization in U2OS cells. C. Detection of signaling pathway activation through a STAT5 transcriptional reporter gene in REH cells. Both assay formats utilized engineered receptors (extracellular domain through transmembrane domain only for the dimerization format, and full length receptors for the reporter assay). A slightly larger assay window (S/B = signal-to-background) and lower EC50 was observed with the IL-2 signaling reporter cell line as compared to the dimerization cell line. However, a longer IL-2 incubation time with cells was required for the reporter assay. D. Comparison of assay formats with corresponding assay windows, EC_{50}, and incubation times.
Note: The dimerization assay is available as a ready-to-use PathHunter IL-2R bioassay kit to accelerate your development program from discovery to post-market QC lot-release testing.

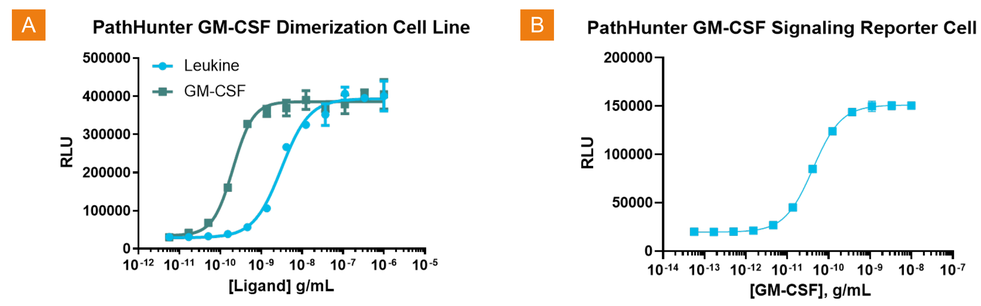
IL-15 Receptor Cell-Based Assays to Measure Dimerization and Downstream Transcription



D	Assay Format	Dimerization Bioassay	Reporter Bioassay
	Cell Background	U2OS	REH
	Receptor Subunits: IL2RB, IL2RG	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 3. PathHunter cell-based assays for IL-15. A. The IL-15 receptor is a heteromeric receptor comprised of the signaling subunits IL-2Rβ (IL2RB) and γ_c (IL2RG) that are shared with the IL-2 receptor, and a high affinity IL-15Rα subunit. *In vivo*, IL-15 is presented in trans by the IL-15Rα chain to the IL-2/15Rβγ_c complex displayed on the surface of T-cells and natural killer (NK) cells. However, recombinant IL-15 does not require presentation by the IL-15Rα to activate the IL-2/15Rβγ_c complex. Binding of IL-15 leads to activation of signaling through JAK/STAT pathway, primarily through STAT5. B. The bioassay for IL-15 receptor hetero-dimerization in U2OS cells. C. Detection of signaling pathway activation through a STAT5 transcriptional reporter gene in REH bioassay cells. Both assay formats utilized engineered receptors (extracellular domain through transmembrane domain only for the dimerization format, and full length receptors for the reporter assay). D. A slightly larger assay window and lower EC_{50} is observed with the IL-15 reporter bioassay as compared to the IL-15 dimerization bioassay. Both assay formats are suitable for assessing potency of soluble IL-15 and IL-15Rα/IL-15 fusion proteins, and assay-ready formats for both are in development.

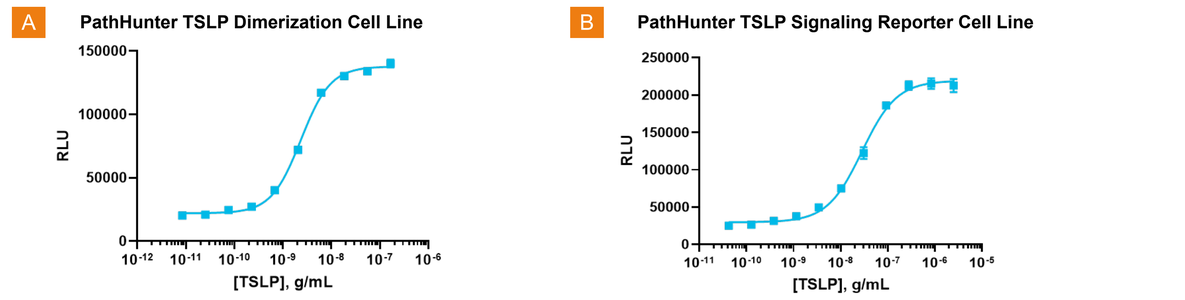
Cell-Based Assays for GM-CSF Measure Dimerization or Transcription



C	Assay Format	Dimerization Cell Line	Reporter Cell Line
	Cell Background	U2OS	U937
	Receptor Subunits: CSF2RA, CSF2RB	Engineered (Truncated)	Endogenous
	Assay Window (S/B)	13.3	7.6
	GM-CSF EC_{50} (pg/mL)	203.6	42
	Incubation Time (hours)	16	16

Figure 4. PathHunter cell lines for GM-CSF. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic regulator of inflammation in the response to pathogens, in autoimmune disease, and in cancer, making it an attractive target for therapeutic manipulation. GM-CSF binds to a heterodimeric receptor comprised of the common βc receptor subunit (CSF2RB) that is shared with the related receptors for IL-3 and IL-5, and a cytokine-specific α chain (CSF2RA) that is expressed at low levels on target cells. A. The cell line for GM-CSF receptor dimerization, engineered in U2OS cells, measures ligand-induced hetero-dimerization of CSF2RA and CSF2RB subunits that have been engineered as fusion proteins with complementary EFC tags. B. The GM-CSF signaling reporter cell line was engineered to express a STAT5 transcriptional reporter in U937 cells that endogenously expresses the GM-CSF receptor. While the assay window for GM-CSF is slightly larger in the dimer format, the reporter assay in U937 cells shows ~5-fold greater sensitivity to GM-CSF, with an EC_{50} of 42 pg/mL. C. Comparison of assay formats with corresponding assay windows, EC_{50}, and incubation times.
Note: The dimerization assay is available as a ready-to-use PathHunter GM-CSF bioassay kit and qualified with Sargramostim (Leukine®, Partner Therapeutics), a recombinant GM-CSF (representative dose curve for Leukine shown in A.).

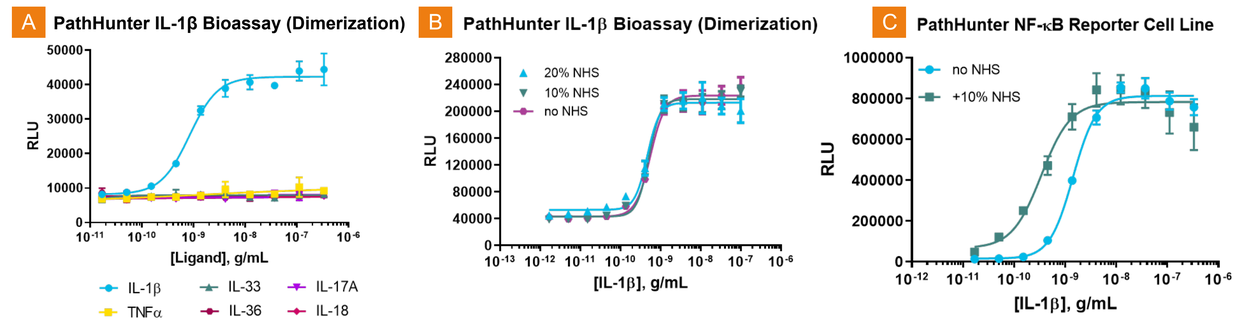
Cell-Based Assays for TSLP Measure Dimerization or Transcription



C	Assay Format	Dimerization Cell Line	Reporter Cell Line
	Cell Background	DLD1	REH
	Receptor Subunits: IL-7RA and CRLF2	Engineered (Truncated)	Endogenous (IL-7RA) and Engineered (Full Length, CRLF2)
	Assay Window (S/B)	7.0	8.5
	EC_{50} (ng/mL)	2.8	28.4
	Incubation Time (hours)	16	16

Figure 5. PathHunter cell-based assays for TSLP (Thymic Stromal Lymphopoietin). TSLP binds to a heterodimeric receptor comprised of the ligand-specific TSLP receptor chain (TSLPR; CRLF2) and the IL-7 receptor-α. Activation of the receptor polarizes dendritic cells to induce type 2 inflammation and directly expand and/or activate Th2 cells, group 2 innate lymphoid cells, basophils, and other immune cells. As such, TSLP is considered a master regulator of type 2 immune responses at the barrier surfaces of skin and the respiratory/gastrointestinal tract, and modulation of the TSLP-TSLPR axis is currently being investigated as a therapeutic modality for treatment of allergic diseases such as atopic dermatitis and asthma. A. The TSLP dimerization assay was engineered in DLD1 to measure TSLP-induced hetero-dimerization of the IL-7R and CRLF2 receptor chains as EFC fusion proteins. B. The TSLP signaling reporter cell line was engineered to heterologously express CRLF2 and a STAT5 transcriptional reporter in REH cells that endogenously express IL-7R. While the assay window in response to TSLP is slightly larger in the reporter assay, EC_{50} was 10-fold lower in the dimerization assay.

Specificity of Assays for IL-1β and Sensitivity to Human Serum



Assay Format	No NHS	+ 10% NHS	+20% NHS
Assay Window (S/B)	6.3	6.6	4.3
EC_{50} (ng/mL)	0.58	0.52	0.46
Assay Format:	No NHS	+ 10% NHS	+20% NHS

Assay Format	No NHS	+ 10% NHS
Assay Window (S/B)	31.7	17.2
EC_{50} (ng/mL)	1.4	0.34

Figure 6. Specificity of PathHunter dimerization assays and sensitivity to normal human serum (NHS). A. The IL-1 dimerization bioassay measures dimerization of the IL-1 receptor subunits (IL-1RA and IL-1RAP) in response to several ligands. Specificity is demonstrated by a dose dependent increase in assay signal in response to IL-1β, but no response to the 5 other related interleukins or TNFα. B. Sensitivity of the IL-1β bioassay to NHS. A dose curve of IL-1β was prepared in vehicles containing 0%, 10%, or 20% NHS. Both concentrations of NHS had minimal impact on assay window and EC_{50} in the IL-1β dimerization bioassay. C. By contrast, a transcriptional reporter assay for IL-1β, using the NF-kB reporter, demonstrated a nearly 2-fold drop in assay window in the presence of only 10% NHS, and a roughly 4-fold change in EC_{50}. These data suggest that the IL-1β dimerization bioassay is more suitable for neutralizing antibody (NAb) detection than the reporter assay format.

Phylogenic Analysis of Human Interleukins and Available Assays

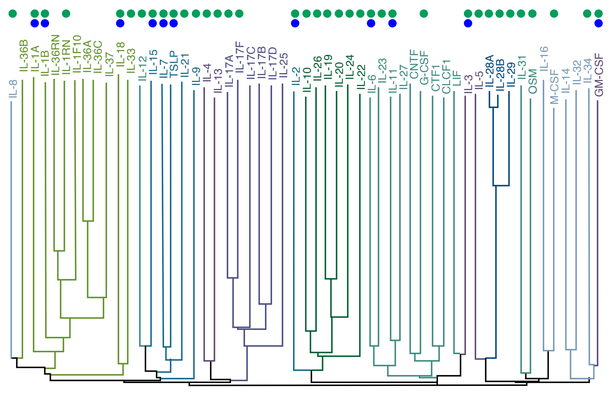


Figure 7. Phylogenic analysis of human interleukins and closely related proteins across 8 major groups. Interleukin groups include IL-1-like, IL-4-like, IL-10-like, IL-28-like, IL-17-like, and the non-classified cytokines. Green dots indicate existing assays developed at Eurofins DiscoverX (largely in the dimerization portfolio). Blue dots indicate new reporter assay formats available or in development. Additional assays may be developed via our custom development capabilities program.

Summary

- PathHunter cytokine and interleukin assays offer MOA-based dimerization and signaling reporter assays covering over 80% of interleukin targets
- PathHunter dimerization MOA (offered as cell lines 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 assay formats typically provide large assay windows and excellent sensitivity to ligand, making them excellent choices for screening and relative potency assays

Learn more about the Eurofins DiscoverX assays for cytokines and interleukins at discoverx.com