Measuring Receptor Dimerization To Create Functional Cell-Based Assays for ~85% of the Interleukin Receptor Family

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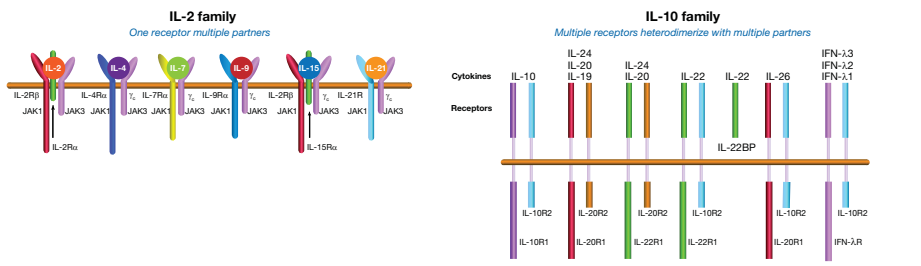
Abstract

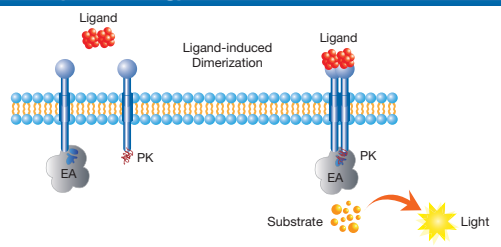
Ligand-induced receptor dimerization is an early functional step in receptor activation, representing the most proximal, functional read-out for receptor activation. It is well understood that the family of Interleukin receptors will dimerize with the other members of its family leading to a complicated signaling cascade that is critically involved in a variety of auto-immune, inflammatory and oncogenic diseases. Surprisingly, existing cellular assays have been unable to faithfully monitor these interactions in a proximal manner to the receptor. Here we present a novel application of the Enzyme Fragment Complementation system to monitor receptor-receptor interactions at the surface of intact live cells, applicable to diverse receptor types such as Interleukin receptors, BMP receptors, receptor tyrosine kinases and cytokine receptors, with a specific focus on the interleukin family of receptors. The high specificity, simplicity of the assay protocol, large signal to noise ratio, serum tolerance and reproducibility of these assays has enabled their use in cell-based screening, functional characterization, QC lot release assays and neutralizing antibody studies. Examples discussed here include assays for the IL-1, IL-2, IL-6, IL-10 receptor families, with assays developed or in development for ~85% of the Interleukins and their receptors.

Structural Diversity in Interleukin Receptor Families



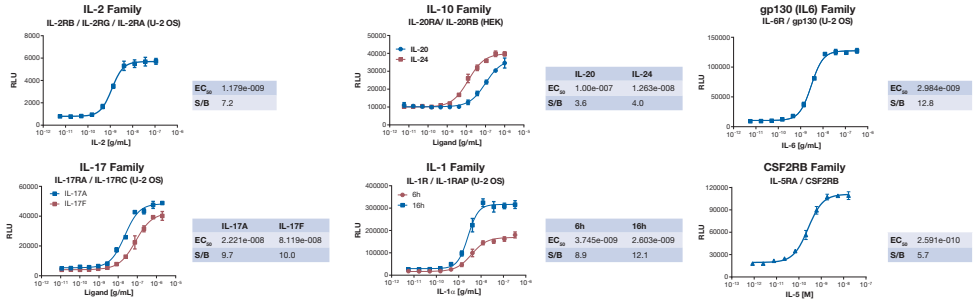
Structural diversity in interleukin receptors. Receptors for interleukins are arranged in families. Each receptor is composed of at least two subunits. **A.** All IL-2 family receptors share a subunit, known as the common γc subunit, while also encoding a high affinity receptor subunit that recognizes and binds its specific ligand with high affinity. Both receptor subunits are essential for transducing signals to downstream pathways. Two receptors (for IL-2 and IL-15) have evolved to require an additional receptor subunit (high specificity receptor α) for functionality (Figure adapted from Liao et al., 2011. Curr Opin Immun 23(5): 598-604) . **B.** IL-10 family receptors involve heterodimerization of multiple receptor subunits to generate responses to different ligands (Figure adapted from Zdanov A., 2010. Cytokine Growth Factor Rev. 21(5): 325-30).

PathHunter® Dimerization Assay Technology



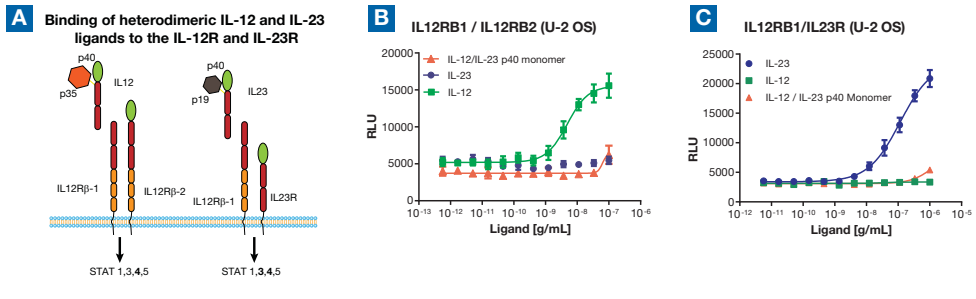
PathHunter dimerization assay technology has been utilized to construct assays to interrogate an early event in the interleukin pathway signaling cascade. In brief, one receptor in the dimer pair is tagged with the PK fragment of the β-gal reporter, while the second subunit is fused to the EA component of β-gal. When the ligand engages the high affinity receptor, it causes it to functionally dimerize with its heterodimer partner, bringing the fragments of the β-gal reporter into close proximity which forces them to reconstitute enzymatic function, which can be detected by the addition of enzyme substrate.

Highly Specific Assays for Interleukin Receptors Across Multiple Families



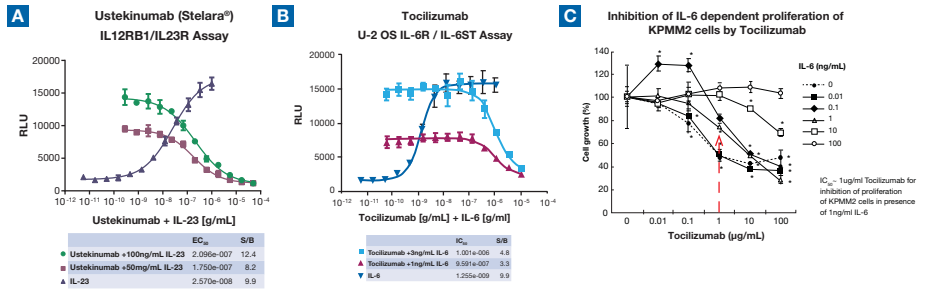
Representative examples of assays for interleukin receptors from 6 different families of interleukins / receptors. Each plot shows a dose response for the relevant ligand(s) in a given assay from the indicated family. Data plotted are mean RLU and standard deviation from at least triplicate wells for each dose. These assays are characterized by robust assay windows and low CVs.

Dimerization of inflammation targets IL-23R and IL-12R is highly specific



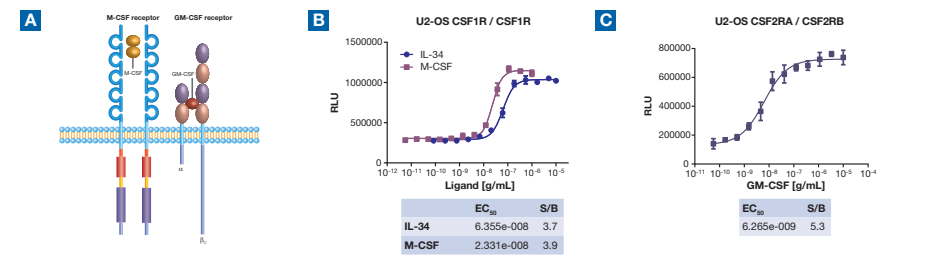
Dimerization of IL-23R and IL-12R is highly specific. **A.** IL23R and IL12R share a common subunit (IL12RB1). Each receptor binds a heterodimeric ligand, which share the p40 subunit. Both receptors have been linked to Crohn's disease by GWAS studies. **B.** Dimerization of IL-12R (IL12RB1/IL12RB2) is stimulated by IL-12, but not by IL-23 or p40 monomer. **C.** Dimerization of IL-23R (IL23R/ IL12RB1) is stimulated by IL-23 but not by IL-12 or p40 monomer.

Simple, Functional Cell-based Assays for Ustekinumab & Tocilizumab



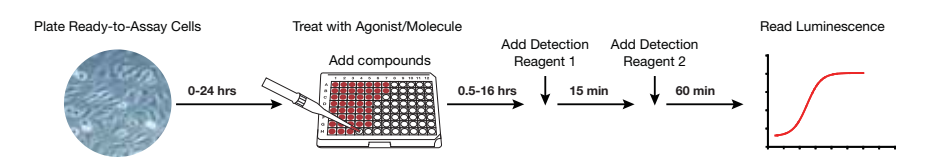
A. To treat inflammatory diseases induced by IL-23 or IL-12, antibodies targeting the p40 subunit have been developed as therapeutics, for example Ustekinumab or Stelara® (Stelara is a registered trademark of Janssen/Centacor). Response of IL23R assay to the therapeutic antibody Ustekinumab is shown above. The assay has been tested with two different concentrations of IL-23 agonist, at EC_{80} and EC_{50} of the agnoist response. **B.** Cell-based assay for detecting stimulation and inhibition of IL-6 signaling through the membrane-bound IL-6R. Assay responds to IL-6 with an EC_{50} of 1.26ng/ml, while tocilizumab (a therapeutic antibody targeting IL-6R) inhibits IL-6-induced dimerization. **C.** Inhibition of IL-6-dependent proliferation of KPMM2 cells with Tocilizumab. IC_{50} of 1ug/ml for Tocilizumab obtained with a stimulation dose of 1ng/ml (red arrow in the figure above) is consistent with the IC_{50} value for tocilizumab in the IL6R dimerization assay (as in figure B) run with the same stimulation condition. *Figure adopted from Mihara et al., (2005) Int. Immunopharmacology 5: 1731-40.*

Robust cell-based assays for IL-34, M-CSF & GM-CSF



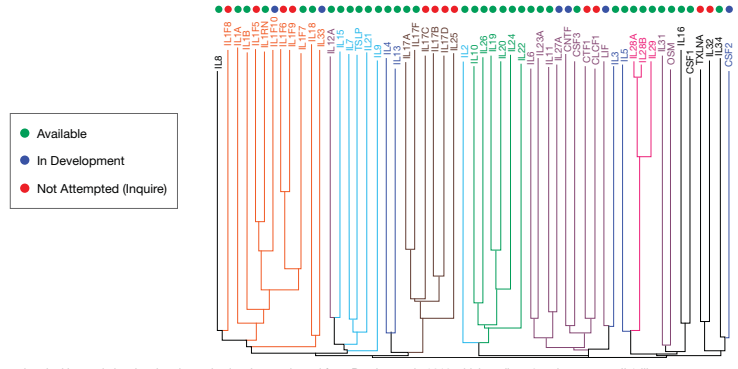
Robust cell-based assays for IL-34, M-CSF & GM-CSF. **A.** Schematic representation of the receptors for macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage CSF (GM-CSF). The M-CSF receptor (CSF1R) is a homodimeric receptor tyrosine kinase; the GM-CSF receptor (CSF2R) is a heterodimeric receptor. **B.** The CSF1R homodimerization assay measures a robust response to IL-34 and M-CSF. **C.** The CSF2R heterodimer assay measures a robust response to GM-CSF.

A Simple Homogenous Protocol With Rapid Results



PathHunter bioassay kits use a simple homogenous protocol with rapid results. 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. Cells manufactured in bioassay kits are meant for single use in ready-to-assay vials.

Phylogram of human interleukins & available assays



This phylogram of human interleukins and closely related proteins has been adapted from Brocker et al., 2010 which outlines 8 major groups – IL1-like (orange), γ-chain utilizing (light blue), IL4-like (dark blue), IL6/12-like (purple)), IL10-like (green), IL28-like (pink) and IL17-like (brown), and the non-classified cytokines (black). The blue, green and red dots in front of each name highlights our progress with the development of the assay – green means that we have that assay available, blue means that it is in development and red dots indicate that we have not yet attempted construction of that assay.

[Image adopted from Brocker *et al*., 2010. Evolutionary divergence and functions of the human interleukin (IL) gene family. *Human Genomics*. 5(1): 30-55]

Summary & Conclusions

- Robust cell-based assays for over 85% of human interleukins and related cytokines
- Assays are highly specific and based on native biology of receptors
  - Proximal readout– monitors early functional activation of interleukin pathway
  - Response specific to receptor/ligand (interleukin) pair
  - Applicable to monitoring activation or inhibition of receptor with large molecules
- Simple assay protocol
  - Simple, homogenous, one-step add & read protocol
- Scalable for screening or high throughput analysis of samples
  - Optimized for 96-well and 384-well applications
- Excellent reproducibility and precision

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