New PathHunter Reporter Assays: Analysis of PD-1 Checkpoint Receptor and NFAT Signaling Pathways

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Abstract

Reporter genes are a well-established method used to develop cell-based assays for testing drugs that inhibit targets involved in specific signaling pathways. Here, we introduce new PathHunter® Signaling Pathway Reporter Assays that utilize the industry-validated Enzyme Fragment Complementation (EFC) technology to detect reporter gene activity. Reporter cells with endogenous or heterologously introduced target receptors are used to readout signaling pathway activation, resulting in transcriptional activation of a reporter gene encoding a protein tagged with a small enzyme fragment. Reporter gene activity is measured by the addition of lysis buffer, luminescent enzyme substrate and the complementary larger enzyme fragment in an easy-to-use format. For example, an NF-κB reporter assay was developed and validated with CD40L to measure endogenous CD40 activation in U2OS cells.

Another assay developed is the PathHunter Jurkat NFAT Pathway Reporter cell line, which measures T-cell activation but has been modified further to build an assay for testing therapeutic compounds inhibiting the inhibitory PD-1 checkpoint receptor effects on T-cell activation. The PathHunter Jurkat PD-1 Pathway Reporter Assay provides an alternative means of investigating the PD-1 signaling pathway that is complementary to our PathHunter Jurkat PD-1 Signaling Assay, which reads out effects on more proximal events in the PD-1 pathway independent of T-cell receptor activation.

PathHunter Signaling Pathway Reporter Assays

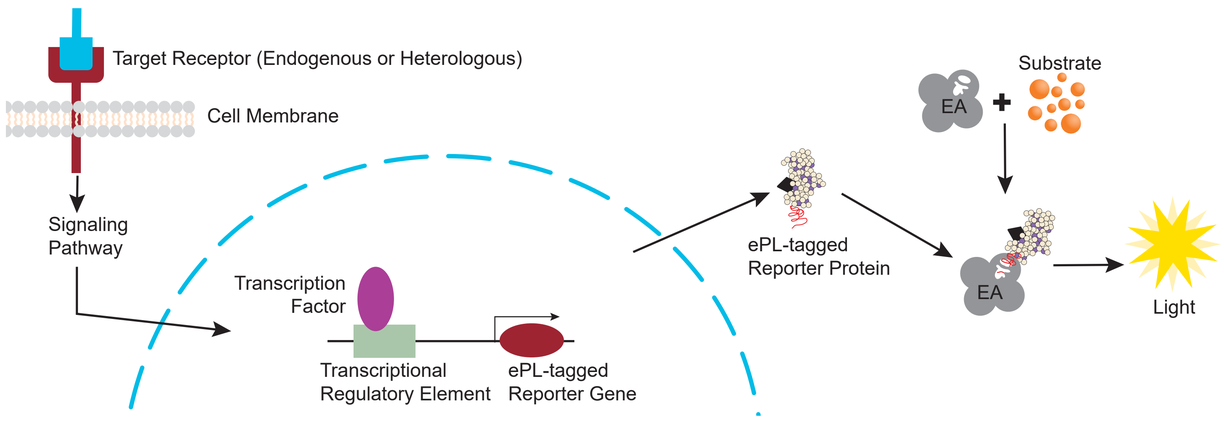

Figure 1. The PathHunter signaling pathway reporter assay detects target pathway signaling through the activation of endogenous receptors or receptors introduced into cells with the reporter gene construct. Ligand-mediated stimulation of these receptors initiates pathway signaling and subsequent activation of transcription factors, which bind to a regulatory transcriptional element controlling reporter gene expression. In this assay, the activated signaling pathway drives the expression of the reporter protein tagged with the small enhanced ProLabel (ePL) β-galactosidase enzyme donor fragment. Reporter activity is measured by lysing reporter pathway cells with a detection reagent containing the complementary enzyme acceptor (EA) fragment and luminescent enzyme substrate. The enzyme activity is then detected as a result of EFC.

Cell-Based PathHunter Signaling Pathway Reporter Assays Using Endogenous or Heterologously-Expressed Target Receptors

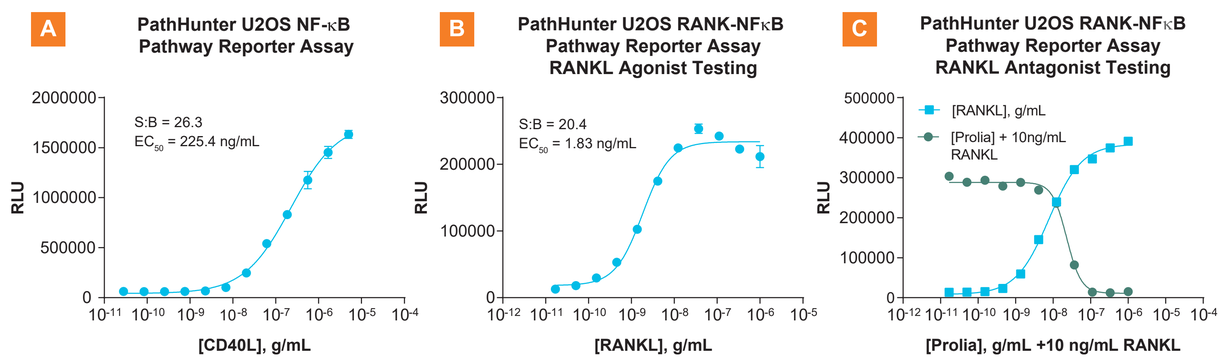

Figure 2. PathHunter signaling reporter assays for endogenous or heterologously-expressed target receptors. A. The PathHunter U2OS NF-κB Pathway Reporter Assay detects CD40L activation of endogenous CD40 receptors. B. The PathHunter U2OS RANK-NFκB Pathway Reporter Assay was created by RANK gene transfer into PathHunter U2OS NFκB Pathway Reporter cells to generate a sensitive assay for RANK activation by RANKL. C. The PathHunter U2OS RANK-NFκB Pathway Reporter Assay was used to analyze antagonist activity of the therapeutic RANKL inhibitor, Prolia® (registered trademark of Amgen) and shows a robust and sensitive response with a large signal-to-background ratio. [Prolia + 10ng/mL RANKL exhibited an IC_{50} of 23 ng/mL and an S/B ratio of 25.9, while RANKL alone resulted in an EC_{50} of 7.7 ng/mL and an S/B ratio of 29.1.]

PathHunter Jurkat NFAT Pathway Reporter Assay Monitors T-Cell Activation

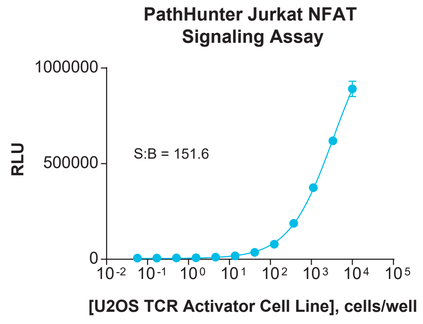

Figure 3.The PathHunter Jurkat NFAT Pathway Reporter Assay monitors T-cell activation signaling through T-cell receptors and results in changes in NFAT-regulated reporter gene expression. Jurkat NFAT reporter cells were co-cultured with increasing numbers of U2OS cells expressing a molecule that activates Jurkat T-cell receptors to initiate T-cell activation. The ePL-tagged reporter protein is expressed when a sufficient number of U2OS T-cell activator cells engage endogenous Jurkat T-cell receptors to activate the pathway and drive NFAT-dependent transcription and translation of the ePL-tagged reporter protein.

Signaling Pathway for PD-1 Inhibition of T-Cell Receptor Activation

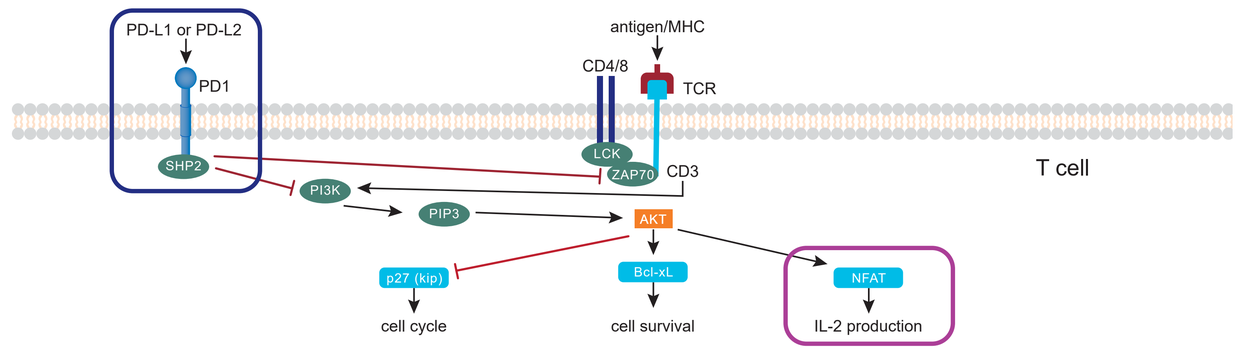

Figure 4. Graphical depiction of proximal and distal PD-1 signaling events that result in T-cell receptor inhibition. Upstream events (boxed in blue) are measured with the PathHunter PD-1 Signaling Assay (Figure 6), while the PathHunter PD-1 Pathway Reporter Assay measures NFAT-regulated events that are much further downstream of PD-1 receptor activation (boxed in purple).

Reporter-Based, Inhibitory Checkpoint Receptor Assay Principle

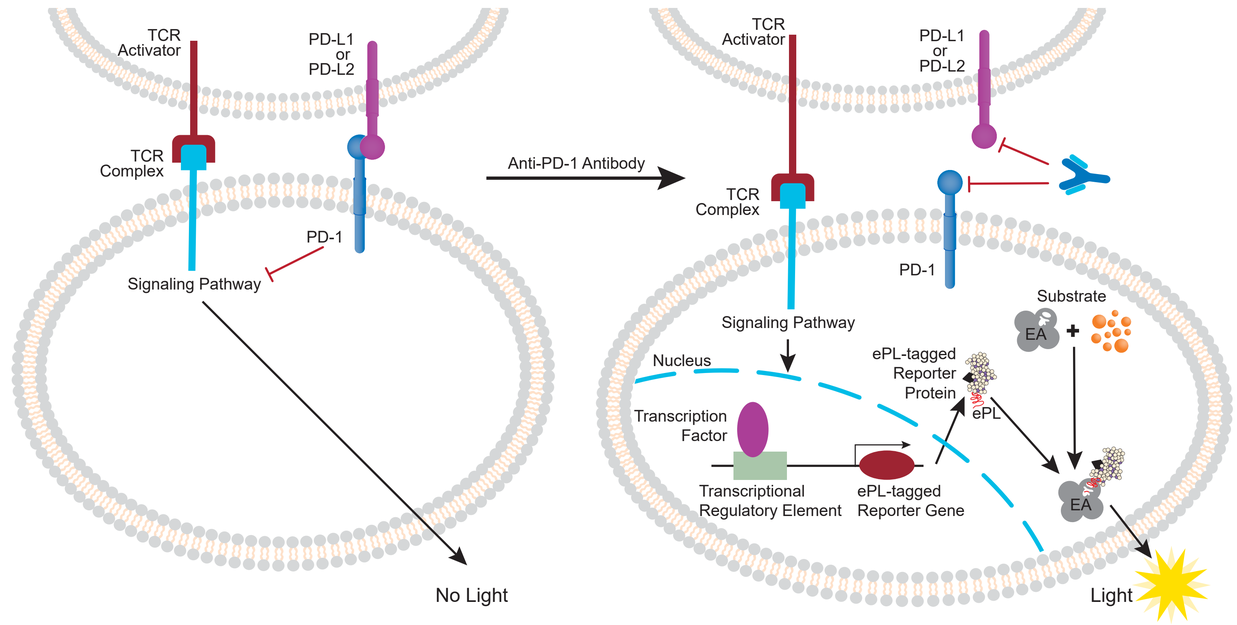

Figure 5. The PathHunter PD-1 Pathway Reporter Assay Principle. This assay monitors effects of PD-1 inhibitors by measuring T-cell activation resulting in increased NFAT-dependent expression of a reporter gene tagged with the ePL enzyme donor fragment. PD-1 is heterologously expressed in the PathHunter Jurkat NFAT reporter cell line, and co-cultured with U2OS PD-L1 ligand cells co-expressing a TCR activator, resulting in attenuated TCR activation. Pre-incubation of Jurkat PD-1 cells with an antagonist PD-1 antibody releases PD-1 inhibition of TCR signaling, and NFAT-controlled reporter expression induced by the TCR activator is detected.

Non-Reporter-Based, Inhibitory Checkpoint Signaling Assay Principle

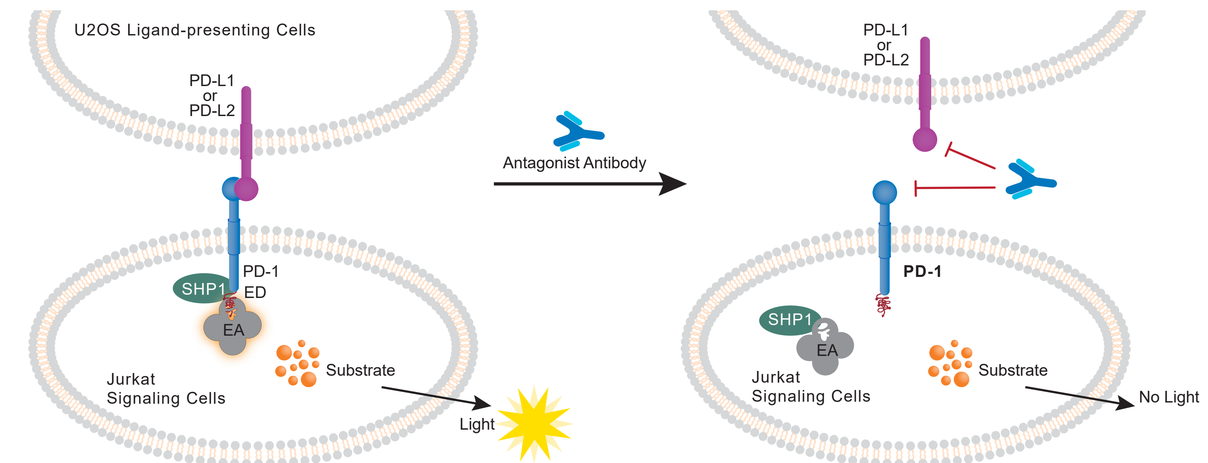

Figure 6. Assay principle of the non-reporter based PathHunter PD-1 Signaling Assay for comparison with the reporter assay principle (Figure 5). This PD-1 assay measures SHP1 recruitment to phosphorylated tyrosines found in PD-1 ITIM motifs. When U2OS PD-L1 Ligand Cells are co-cultured with Jurkat PD-1 Signaling Cells, PD-L1 activates the PD-1 receptor and the SHP1 SH2-EA enzyme fragment fusion protein is recruited to PD-1 tagged with the complementary enzyme donor (ED) tag. Antagonist antibody addition disrupts PD-1 interaction with PD-L1, inhibits PD-1 signaling, and results in a loss of chemiluminescent signal.

Complementary PathHunter Assays for PD-1 Pathway Analysis

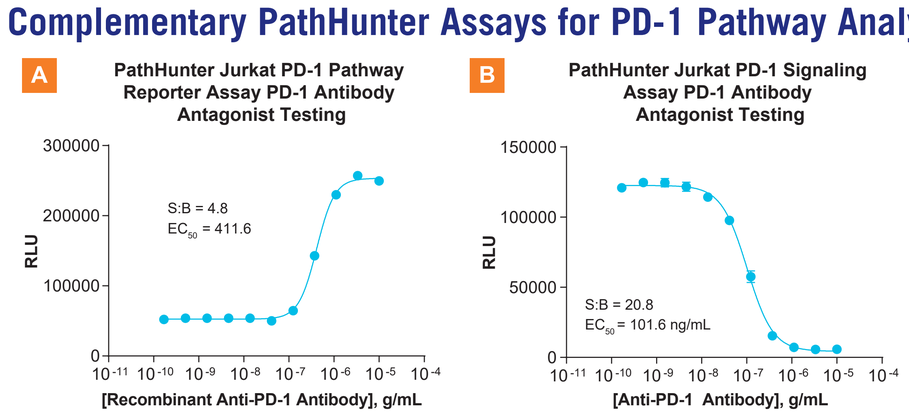

Figure 7. Comparison of antagonist testing results from the two different PathHunter PD-1 Assays. A. The PathHunter PD-1 Pathway Reporter Assay was tested with an Anti-PD-1 Antibody. Reporter assay cells co-cultured with the PathHunter U2OS PD-L1/TCR activator cell line results in increased reporter expression due to blocking PD-1 inhibition of TCR activation, resulting in increased NFAT-regulated gene expressionwhich is measured by this reporter assay. Conversely, B. represents results from the PathHunter Jurkat PD-1 SHP2 Signaling Assay. An Anti-PD-1 Antibody was used to block PD-1 activation mediated by PathHunter U2OS Ligand Cell Line co-culture. This assay measures proximal PD-1 signaling events independent of TCR activation. Both assays are robust and measure inhibition with sensitive responses, from either distal or proximal events.

Summary

- PathHunter Pathway Reporter Assays by Eurofins DiscoverX are presented here. These assays can be further modified for specific targets activating or inhibiting a pathway. For example, the PathHunter Jurkat NFAT Pathway Reporter Assay was used to develop an assay for PD-1 checkpoint receptor inhibition of T-cell activation.
- The PathHunter Jurkat PD-1 Pathway Reporter Assay offers an additional option for testing PD-1 therapeutics to complement results from our PathHunter Jurkat PD-1 Signaling Assay.
- The PathHunter Jurkat PD-1 Signaling Assay captures a proximal PD-1 signaling event (SH2-recruitment), while the PathHunter Jurkat PD-1 Pathway Reporter Assay reads out further downstream PD-1 effects (T-cell activation). Both assays produce robust and sensitive responses when tested with PD-1 antagonist antibodies. To learn more, visit discoverx.com/reporters.