Improved Cell-Based Assays To Assess Therapeutic Molecules Against Immune Checkpoint Receptors Such As PD-1, PD-L1 And PD-L2

Sean Deacon, Jennifer Lin-Jones, Hanako Daino-Laizure, Mimi Nguyen, Jason van der Tuig, Abhi Saharia, and Jane E. Lamerdin

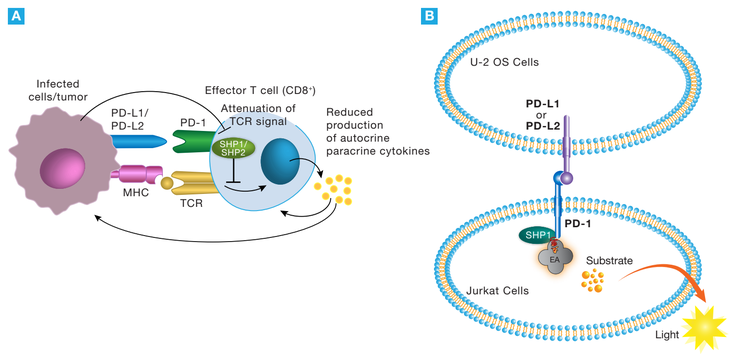
DiscoverX Corporation, Fremont, CA 94538-3142

Abstract

Regulation of immune responses is tightly controlled through a balance of co-stimulatory and inhibitory checkpoint receptors, often exploited by many cancers. Therefore, therapeutics that block inhibitory receptors have proved to be powerful agents to restore anti-tumor immune responses. One key inhibitory checkpoint receptor that is the target of several therapeutic agents in the clinic is programmed cell death 1 (PD-1). PD-1 is expressed on T-cells, while it's ligands, PD-L1 or PD-L2, are expressed on the surface of tumor cells or antigen presenting cells. Like many other immunoglobulin receptors, PD-1 harbors immunoreceptor tyrosine inhibitory motifs (ITIMs) in it's cytoplasmic tail that are important signaling motifs. When its ligand, e.g. PD-L1, binds to PD-1, Src family kinases phosphorylate the ITIM motif, resulting in the recruitment of SH2-domain containing phosphatases, SHP-1 and SHP-2, which are involved in inhibiting the T-cell response.

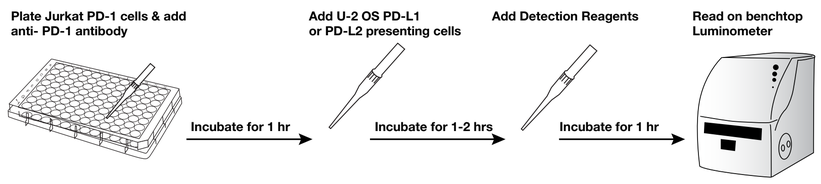
Here, we present a robust assay for quantifying SHP recruitment to PD-1 using our proprietary enzyme fragment complementation (EFC) technology. Jurkat cells expressing the PD-1 and SHP-1 proteins, each fused to a fragment of our EFC system, are co-incubated with ligand-presenting cells. This results in PD-1 activation and SHP-1 recruitment to the PD-1 receptor, bringing together the two EFC fragments and generating a light signal. We demonstrate the suitability of the assay for quantifying pathway activation as well as inhibition of PD-1 signaling by both anti-ligand (anti-PD-L1) and anti- receptor (anti-PD-1) antibodies. The assay is rapid (<4 hours), extremely robust, and has an excellent assay window (>20-fold) with unparalleled sensitivity. In summary, the EFC-based PD-1 assay provides a valuable tool for both drug screening & characterization assays, with a possible role in lot release testing and stability studies during drug manufacture. This also provides a proof of concept for developing assays for other therapeutically relevant checkpoint receptors, such as TIGIT and CD47.

SH2 Recruitment Assay For PD-1 Checkpoint Target: Assay Concept

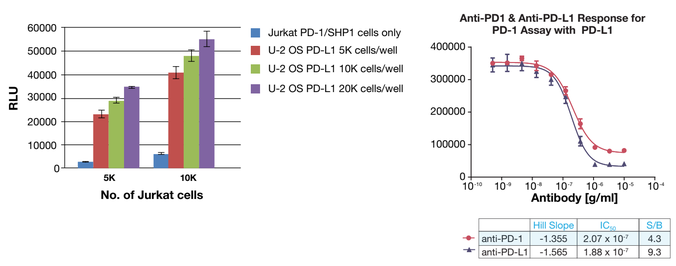


A. Many inhibitory checkpoint receptors (e.g. PD-1, TIGIT) harbor immunoreceptor tail tyrosine (ITT)-like and ITIM motifs in their cytoplasmic tails (motifs that recruit SH2 domain proteins to phosphorylated tyrosines). B. Full-length PD-1 receptor was engineered with a small β-gal fragment (PK in red in figure B) fused to its C-terminus, and the SH2-domain of SHP-1 was engineered with the complementing β-gal fragment (EA). These constructs were stably expressed in Jurkat cells, while untagged full length PD-L1 or PD-L2 were stably expressed in U-2 OS cells (ligand-presenting cells). Ligand engagement, through co-culture with ligand-presenting cells, results in phosphorylation of PD-1-PK fusion protein, leading to the recruitment of SHP-1-EA which forces complementation of the EFC components to create an active β-gal enzyme. This active enzyme hydrolyzes substrate to create chemiluminscence as a measure of receptor activity. Figure A derived from Okazaki *et al*., 2013. Nature Immunology 14, 1212-1218.

Assay Outline



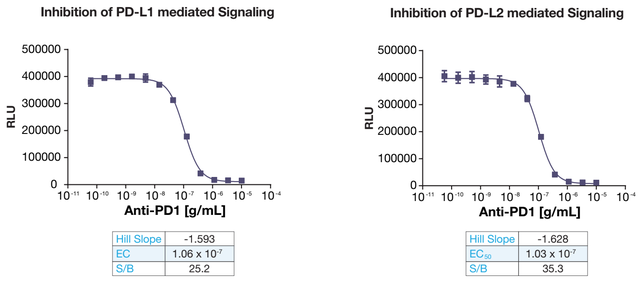
Robust Recruitment Of SHP-1 To PD-1 Receptor In Jurkat Cells



	Hill Slope	IC_{50}	S/B
anti-PD-1	-1.355	2.07×10^{-7}	4.3
anti-PD-L1	-1.565	1.88×10^{-7}	9.3

(Left) Stably transduced Jurkat cells expressing PD-1/SHP-1 are stimulated by co-culture with U-2 OS cells stably expressing PD-L1, with increasing cell numbers, keeping the PD-1 receptor cell line constant. This assay indicates a robust PD-1 response to the presence of PD-L1. (Right) Stimulation of the PD-1 assay is inhibited by blocking with anti-ligand (anti-PD-L1) or anti-receptor (anti-PD-1) antibodies.

Anti-PD-1 Antibody Demonstrates Strong Inhibition Of PD-L1 And PD-L2-Dependent Responses

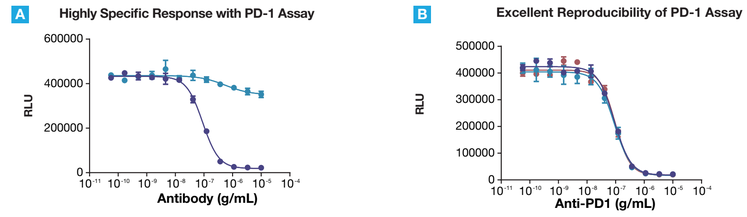


Hill Slope	-1.593
EC	1.06×10^{-7}
S/B	25.2

Hill Slope	-1.628
EC_{50}	1.03×10^{-7}
S/B	35.3

A commercial anti-human PD-1 antibody disrupts the signaling stimulated by co-culturing the Jurkat PD-1 cell line with an adherent U-2 OS cell line over-expressing either PD-L1 (left panel) or PD-L2 (right panel). The assay has a robust and sensitive response to anti-PD-1 dependent receptor inhibition.

PD-1 Assay Generates A Highly Specific And Reproducible Response

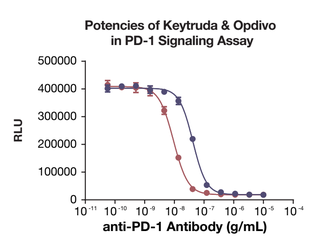


	Hill Slope	IC_{50}	S/B
Anti-PD1	-1.532	9.0×10^{-8}	20.2
anti-TIM3			

	Hill Slope	IC_{50}	S/B
R1	-1.513	9.01×10^{-8}	20.9
R2	-1.490	8.97×10^{-8}	19.8
R3	-1.667	9.53×10^{-8}	22.5

A. Jurkat PD-1 cells were plated and anti PD-1 (blue curve) or anti-TIM3 (teal) antibodies were prepared in dose response format and added to cells for 1hr at 37°C. Cells were then stimulated with a U-2 OS PD-L2 cells for 2hr at RT prior to addition of detection reagents. Response is highly specific to the anti-PD-1 antibody. B. Reproducibility of the PD-1 assay was assessed by preparing three independent dose response curves with a commercial anti-PD-1 antibody (each dose run in duplicate), and running the assay on the same plate. Assay demonstrates excellent intra-assay precision, with a relative standard deviation (% RSD) of 3.39% for calculated IC_{50} values.

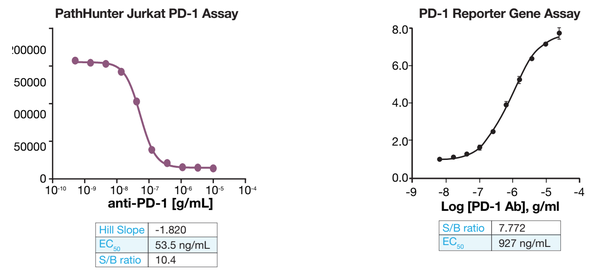
Highly Sensitive And Robust Assay Performance With Keytruda® And Opdivo®



	Hill Slope	IC_{50}	S/B
Keytruda	-1.913	4.20×10^{-8}	22.7
Opdivo	-1.775	9.34×10^{-9}	22.6

Jurkat PD-1 cells were treated with serial dilutions of Keytruda (blue) or Opdivo (maroon) for 1hr prior to stimulation with U-2 OS PD-L2 cells for 2hr at RT. Expected rank order of the two therapeutic antibodies (e.g. Opdivo more potent than Keytruda) is observed in the assay with low ng/ml sensitivity for the two marketed drugs. Keytruda® and Opdivo® are registered trademarks of Merck and BMS, respectively.

PathHunter PD-1 Assay Has 15X Greater Sensitivity Than Reporter Gene Assay



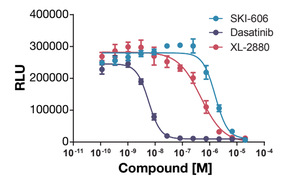
Hill Slope	-1.820
EC_{50}	53.5 ng/mL
S/B ratio	10.4

S/B ratio	7.772
EC_{50}	927 ng/mL

Assay	EC_{50}
PathHunter PD-1 Assay	53.5 ng/mL
Reporter Gene Assay	927 ng/mL

Comparison of PathHunter Jurkat PD-1 signaling bioassay to commercially available PD-1 reporter gene bioassay. Using the same commercially available anti-PD-1 antibody (BioLegend Catalog Number 329912), we were able to compare the assay performance of our PathHunter PD-1 signaling assay to a commercially available reporter gene assay, and observed that the PathHunter PD-1 assay demonstrated 17-fold better sensitivity than the reporter gene assay, with a slightly better assay window as well.

PD-1 Assay Is Suitable For Detection Of Small Molecule Inhibitors



	SKI-606	Dasatinib	XL-2880
Hill Slope	-1.901	-1.979	-1.045
IC50	1.602e-006	5.762e-009	4.740e-007
S/B	49.7	33.1	24.3

PD-1 is known to be phosphorylated with an as yet unidentified src family kinase, which causes the recruitment of the SHP-1 protein to the phosphorylated PD-1 receptor. Testing of the PathHunter PD-1 assay with small molecule inhibitors that are known to inhibit several known src family kinases has shown inhibition of the SHP-1 recruitment to PD-1. This is intriguing for multiple reasons. Firstly, this assay can be used to identify the specific src family kinase that could phosphorylate PD-1 and secondly it could be used to identify novel kinase inhibitors of this exciting immunotherapy target. Finally, this data demonstrates the ability to use the PathHunter assay for identifying both small molecule and biologic modifiers of the PD-1 receptor and its ligands PD-L1 and PD-L2.

Summary

- We have developed a PD-1 signaling assay that measures receptor activation through co-culture with ligand-presenting cells.
- Assay Highlights
  - Simple and biologically relevant assay that does not require complex activation of T-cells or T-cell receptors
  - Rapid response (<5 hrs total assay time)
  - Large assay window of >20 fold with excellent precision (%RSD <4%)
  - Highly sensitive signal (>10-fold better sensitivity than existing assays)
  - Highly specific assay response to PD-L1 and PD-L2 and their inhibitors
  - Applicable for biologics (α-PD-1, α-PD-L1 & α-PD-L2) and small molecule screening & characterization

Learn more at discoverx.com/checkpoint

