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Immuno-Oncology

Accelerating Immune Checkpoint Drug Discovery through Functional Cell-Based Assays

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ABSTRACT

Immune checkpoint receptors avert immunopathology, play a key role in maintaining antitumor immunity, and modulate overall immune homeostasis. However, cancer cells have adopted clever ways to hijack the modulation of these receptors, thus evading immune surveillance and successfully proliferating. To counter this takeover, targeted immune-modulators, such as monoclonal antibodies directed against checkpoint modulators, present a promising approach to cancer therapy, often referred to as immunotherapy. These antibodies have the ability to selectively block or stimulate specific checkpoint receptors and activate immune effector cells.

In the past decade, various clinical studies have shown that such immune checkpoint modulators check tumor growth and result in stable clinical responses. Today, there are thousands of immunotherapy drug candidates in various stages of development. From discovery to market release, these drug candidates require mechanism-of-action (MOA) based functional cell-based assays as characterization tools that can attest to their desired therapeutic activity in combating cancer. However, developing such cell-based assays has proved to be quite challenging. They are often difficult to establish in quality control (QC) labs, and typically have long and complicated assay protocols or use primary human cells. In this paper, we focus on accelerating the pace of immunotherapy drug discovery through Eurofins DiscoverX's off-the-shelf, MOA-based functional cell-based assays designed to support the drug discovery process up to late-stage development and post-market surveillance. To complement this paper, read the supplementary case studies (discoverx.com/checkpoint-case-studies) that demonstrate the assay suitability in a QC environment for specific checkpoint signaling axes.

INTRODUCTION

Immune checkpoint receptors and their role in cancer pathology have gained overwhelming traction in the last decade[1]. Prof. Tasuko Honjo and James P. Allison — winners of the Nobel Prize in 2018 for Physiology or Medicine — conducted pivotal studies on the immune checkpoint modulators, PD-1 and CTLA4 respectively, which led to significant advances in the field of cancer immunotherapy. Their pioneering work elucidated how tumor cells ingeniously evade the immune system by exploiting some key checkpoint receptors[2]. Continued research thereafter led to the development of the first FDA-approved monoclonal antibody, Yervoy® (ipilimumab) in 2011,

Yervoy (ipilimumab) is the first FDA-approved drug (2011) targeting the checkpoint receptor CTLA4 for the treatment of melanoma.

as a therapeutic agent targeting the checkpoint receptor CTLA4 for the treatment of melanoma. Since then, the development pace of immune checkpoint modulators has increased exponentially with thousands of checkpoint modulators in various stages of the drug development cycle. Overall, the field of cancer immunotherapy is projected to have a significant impact on the global drug market with an estimated market value of over $35 billion by 2023. The Cancer Research Institute estimates that over 2,900 active trials are ongoing globally for evaluating PD-1/PD-L1 immuno-oncology therapies alone, representing an ~98% increase since 2017[3]. To be able to meet this enormous pace, there is a pressing need for developing robust and reliable characterization tools to affirm the desired biological activity of these immunotherapy drug candidates.

Drug discovery programs for checkpoint modulators typically need phase-appropriate assay solutions. The early stages involve screening a large number of molecules using rapid methods in a high-throughput format. Biochemical assays are often utilized during this early phase as they tend to be less complex. However, drug candidates can exert their effects on a living organism through multiple mechanisms that biochemical assays fail to capture. Such limitations have contributed to an increased use of biologically relevant cell-based assays that offer a more representative measure of the organism's response to a drug. Cell-based assays can also be miniaturized and adapted in a high-throughput format for primary screening programs.

For a biotherapeutic drug such as a monoclonal antibody, an MOA-reflective, physiologically-relevant assay is essential to accurately report a drug product's potency and stability. Thus, as the drug moves towards the pre-clinical development phase, the criteria for selecting a cell-based assay becomes more stringent. There is an increasing trend of selecting cell-based assays for immunogenicity studies (as a part of the clinical trial) to determine if anti-drug antibodies produced in the subjects receiving treatment are neutralizing the drug. Development of cell-based assays for characterization, potency testing, and neutralizing antibodies detection to meet regulatory expectations is well-recognized as a challenging and time-intensive process due to its nature. As noted by regulatory agency's reviewers, delaying the development of cell-based assays to later stages, i.e clinical development, could potentially delay the release of the drug into the market[4].

To overcome such development challenges and accelerate drug development programs, implementing commercially-available pre-qualified assay platforms can save a significant amount of time and resources. Eurofins DiscoverX addresses this need with the industry's most extensive portfolio of optimized, MOA-reflective cell-based assays for immune checkpoint modulators that are available in phase-appropriate formats.

Cell-Based Assays for Evaluating Checkpoint Modulators

ASSAY DESIGN TO REFLECT ACCURATE MECHANISM-OF-ACTION

According to industry guidelines published by the FDA, it is essential for a potency assay to truly represent the MOA of the drug candidate. The assay readout should measure a molecular event that occurs as a consequence of the drug action, that is, the molecular event should be directly associated with the relevant therapeutic activity or intended biological effect of the drug. Thus, assays measuring physiological events that are proximal to the site of drug action are at times more desirable than reporter gene assays, particularly for biologic drugs where the site of action is extracellular at the receptor level. In the context of immune checkpoint modulators, these are the events that occur directly as a result of ligand-mediated checkpoint receptor activation. Eurofins DiscoverX's PathHunter® assays for checkpoint receptors are specifically designed to measure the proximal molecular events occurring directly as a consequence of receptor activation.

The PathHunter Jurkat PD-1 Signaling Assay is a co-culture model that is based on the molecular MOA of the receptor and is ideal for evaluating both small molecule and biologic drug candidates targeting the programmed cell death-1 receptor (PD-1) signaling pathway. *In vivo*, most immune checkpoint receptors function via a signaling axis, where the receptors and their respective ligands are expressed on different cell types. PD-1 is expressed on immune cells (such as T-cells) and its activating ligands (PD-L1 and PD-L2) are on antigen-presenting cells, such as macrophages and dendritic cells. Therefore, to accurately reflect the MOA of a checkpoint receptor signaling axis, it is advantageous to develop cell-based assays in co-culture formats with two different cell types, one expressing the receptor and the other expressing the ligand. PathHunter checkpoint assays are developed and optimized as co-culture models with signaling cells (expressing the checkpoint receptor and the SH2 domain protein) and the ligand-presenting cells, as described below for the PD-1 Signaling Assay. PD-1 is one of the key inhibitory immune checkpoint receptors found on the surface of T-cells, and plays an important role in tumor immune resistance. Under normal conditions, PD-1 signaling prevents activation of T-cells upon binding with its ligands, PD-L1 or PD-L2, expressed on the surface of antigen presenting cells (APCs) or macrophages, and thereby applies "brakes" on the immune system. PD-1 signaling also promotes apoptosis of self-reactive T-cells. Thus, binding of PD-1 to PD-L1/2 ensures that the body's immune system is activated only at an appropriate time to minimize the chances of chronic autoimmune inflammation. However, tumor cells exploit the PD-1 signaling pathway by overexpressing PD-L1/L2, thereby deactivating the T-cells. Unsurprisingly, therapeutics blocking the PD-1 signaling axis have garnered strong attention in the past decade. In 2014, the FDA approved the first anti-PD-1 drug, Pembrolizumab, for clinical use[3].

Pembrolizumab was the first FDA-approved anti-PD-1 drug for clinical use (2014).

It has been well documented that the interaction of PD-1 receptor with PD-L1/L2 results in phosphorylation of the intracellular tail of PD-1, which in turn leads to the recruitment of SH2 domain-containing proteins, SHP-1 and SHP-2 (protein tyrosine phosphatases) in B-cells and Jurkat cells[4]. The PathHunter Jurkat PD-1 Signaling Assay interrogates this early receptor-proximal event following ligand-mediated receptor activation. Using the industry-validated Enzyme Fragment Complementation (EFC, discoverx.com/efc) technology, this assay (see Figure 1.) measures SHP recruitment to the cytoplasmic tail of PD-1 receptor and the response generated is proportional to the potency of the drug. This simple and homogenous assay captures a physiologically-relevant MOA that is amenable to miniturization. It is available in both cell line (continuous culture) and bioassay (ready-to-use) formats and can be easily implemented in the drug development program from screening to characterzation and potency testing in lot release.

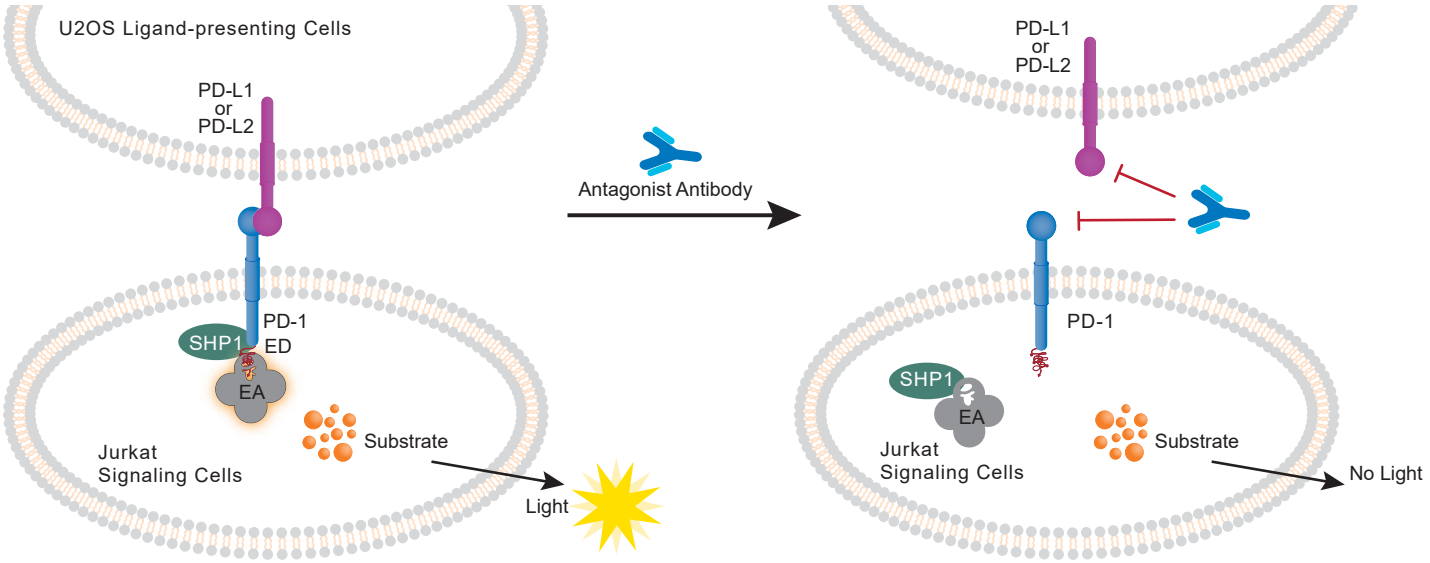


Figure 1. Assay principle of the PathHunter® PD-1 Signaling Assay: Full-length PD-1 receptor was co-expressed with SHP1 (or SHP2) in Jurkat cells. PD-1 receptor was tagged with an Enzyme Donor (ED, shown in red) fragment of β-galactosidase (β-gal) at the C-terminus (cytoplasmic tail), and SHP1 tagged with Enzyme Acceptor (EA, shown in grey) of β-gal. Ligand engagement through PD-L1/PD-L2 with PD-1 results in SHP recruitment, bringing the EA and ED fragments together. ED and EA are inactive β-gal fragments, but when brought together, their complementation (termed as *enzyme fragment complementation* or EFC) forms an active β-gal enzyme that hydrolyzes a substrate to generate a chemiluminescent signal. However, when a therapeutic agent blocks the interaction between PD-1 and its ligand, SHP recruitment does not occur, resulting in loss of EFC signal.

In addition to PD-1 signaling assay, Eurofins DiscoverX has many other cell-based assays for other key checkpoint receptors, including BTLA, CTLA4, and SIRPα signaling cell lines. For a complete list of all available assays for checkpoints, visit discoverx.com/checkpoint.

To gain a comprehensive understanding of the drug molecule's MOA, the receptor proximal drug response using the above-mentioned signaling assays can be used with a complementary downstream (transcription/translational) reporter-based assay. PathHunter Signaling Pathway Reporter assays provide a downstream read-out that is dependent on the activation/inhibition of specific signaling pathways utilized by various checkpoint modulators. These assays (see Figure 2. A.) offer a simple, robust, and sensitive cell-based screening tool that is an orthogonal method to receptor proximal read-outs. Using these two types of assays helps to better elucidate the drug molecule's MOA. Refer to Figure 2. B. and C. for a comparison of antagonist testing results from these two different PathHunter PD-1 assays, both being robust and measure inhibition sensitively. Visit discoverx.com/reporters to learn more

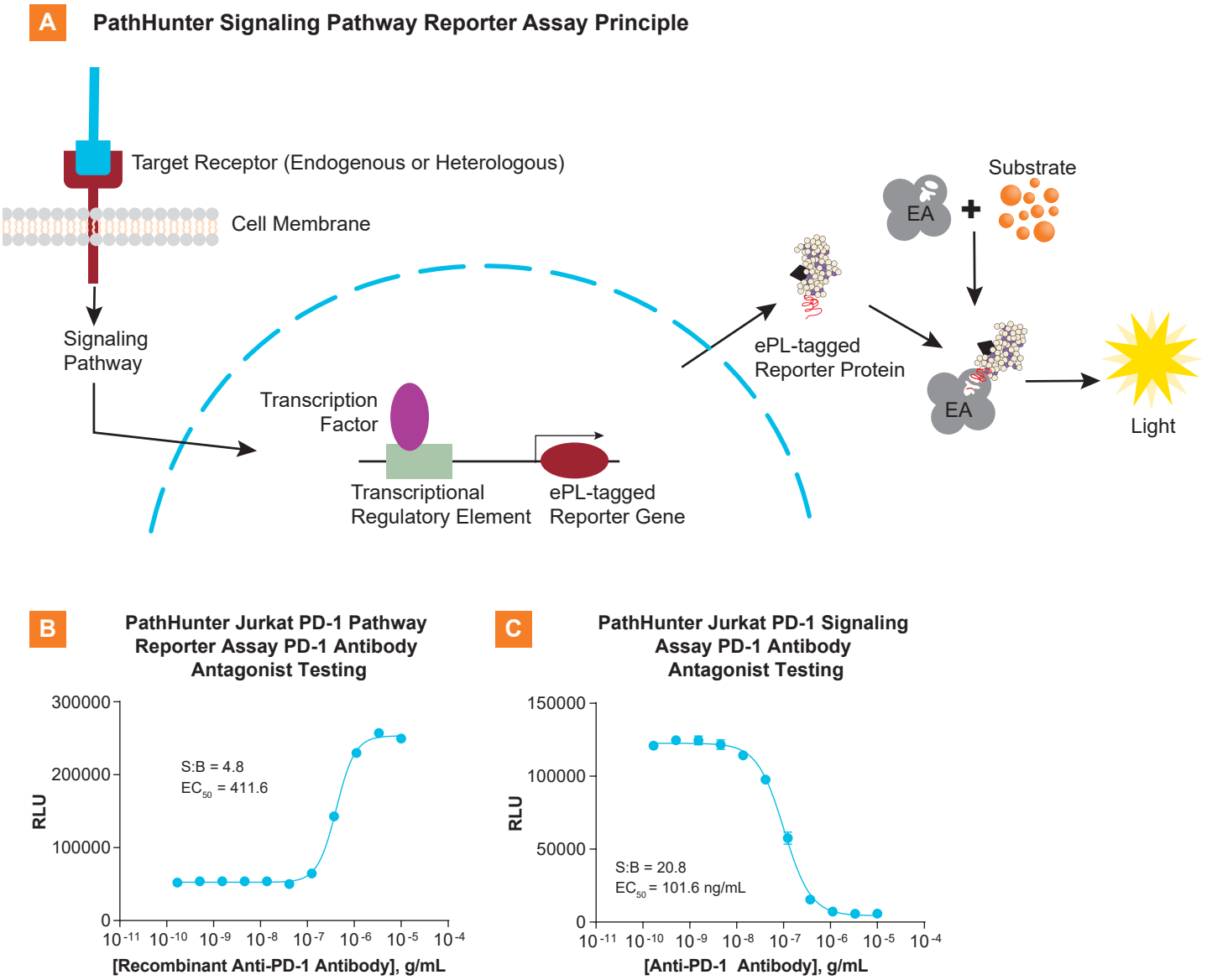


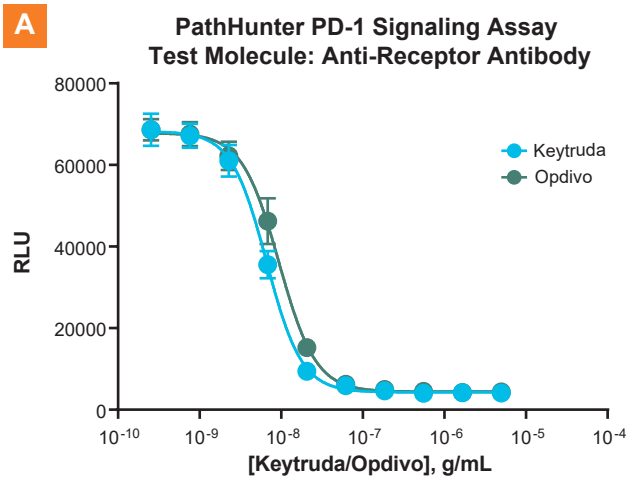
Figure 2: PathHunter® Signaling Pathway Reporter Assay prinicple and comparative assay antagonist analysis. A. Reporter assays detect target pathway signaling through the activation of endogenous receptors or receptors introduced into cells with a reporter gene construct. Ligand-mediated receptor activtion initiates pathway signaling and subsequent activation of transcription factors, which bind to a regulatory transcriptional element controlling reporter gene expression. The activated signaling pathway drives the expression of the reporter protein tagged with the small enhanced ProLabel® (ePL) β-gal enzyme donor fragment. Reporter activity is measured by lysing reporter pathway cells with a detection reagent containing the complementary EA fragment and luminescent enzyme substrate. The enzyme activity is then detected as a result of EFC. B. and C. Comparison of antagonist testing results from the PathHunter PD-1 Pathway Reporter Assay and the Jurkat PD-1 SHP2 Signaling Assay. 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, leading to increased NFAT-regulated gene expression. The reporter assay readout measures downstream effects of PD-1 receptor signaling by resulting in NFAT-regulated reporter protein expression. Conversely, in the PathHunter Jurkat PD-1 SHP2 Signaling Assay, an anti-PD-1 antibody was used to block PD1 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.

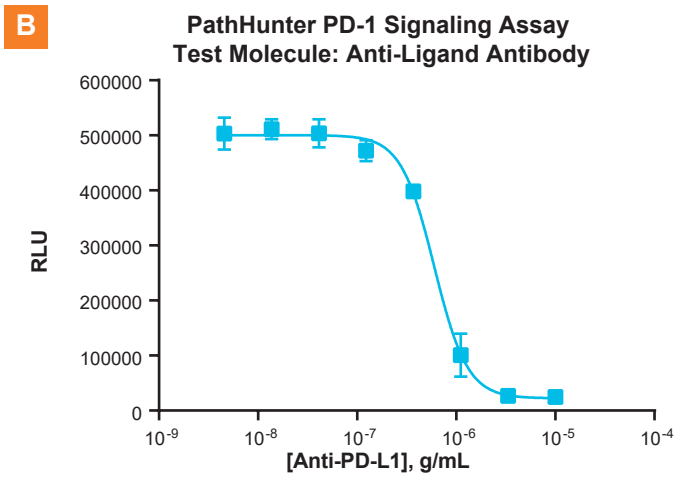
ASSAY VERSATILITY AND FLEXIBILITY

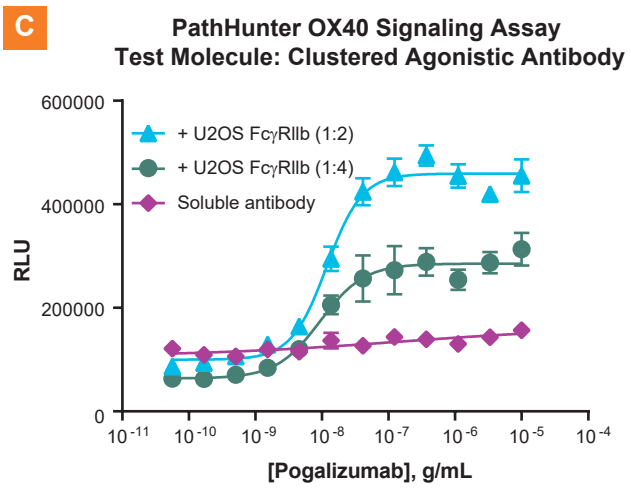
There are several different ways that immune-checkpoint pathways operate. Thus, it is essential that the assay platforms interrogating them provide the necessary flexibility to accommodate different MOAs. For instance, blockade of co-inhibitory checkpoint receptors such as PD-1 and SIRPα (Signal Regulatory Protein α) leads to the activation of T-cells and macrophages, respectively. However, unlike co-inhibitory receptors, co-stimulatory checkpoint receptors are required for proper T-cell activation. In the past several years, there have been significant improvements for developing agonistic antibodies and other biologics targeting these co-stimulatory checkpoint receptors such as OX40 and ICOS. Hence, cell-based assays for checkpoint receptors should be flexible to include such opposite receptor classes as well.

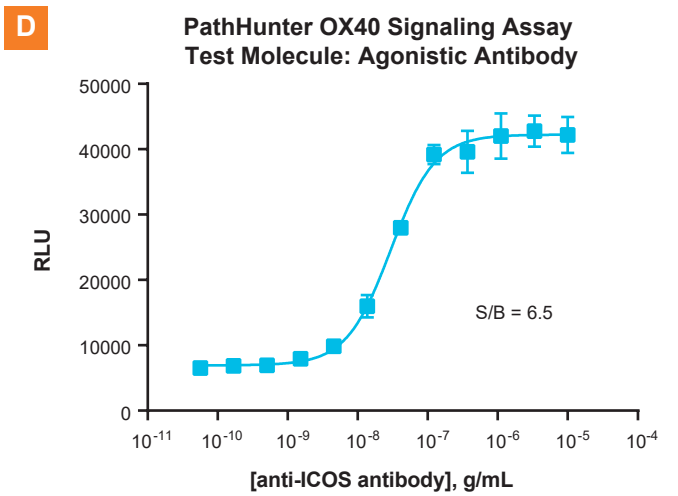
Cell-based assays also need to be adaptable for characterizing both anti-ligand and anti-receptor molecules targeting the checkpoint signaling axis. They must also enable testing of both biologic and small molecule drug candidates since there are many kinase inhibitors approved by the FDA for cancer treatment[5] and several hundred are currently under development.

The PathHunter® checkpoint assays are suitable for all these classes of checkpoint modulators mentioned above due to their flexibility, adaptability, and versatility. A few key examples included in Figure 3. show datasets for an anti-receptor and an anti-ligand antibody for PD-1 and PD-L1. In both cases, the PathHunter assays proved to be robust. Figures 3. C. and D. show datasets for agonistic antibodies for OX40 and ICOS checkpoint receptors. Interestingly, when the OX40 antibody Pogalizumab was cross-linked with FcγRIIB expressing cells, the agonistic response was highly pronounced. Figure 3. E. shows that the PathHunter SIRPα Signaling assay can be modified to run with a plate-bound CD47 ligand from a more conventional cell-presented ligand format. Apart from biologics, PathHunter checkpoint assays can also be used for small molecules, as shown in Figure 3. F., where SRC family inhibitors show a dose-dependent inhibition of PD-1 signaling.









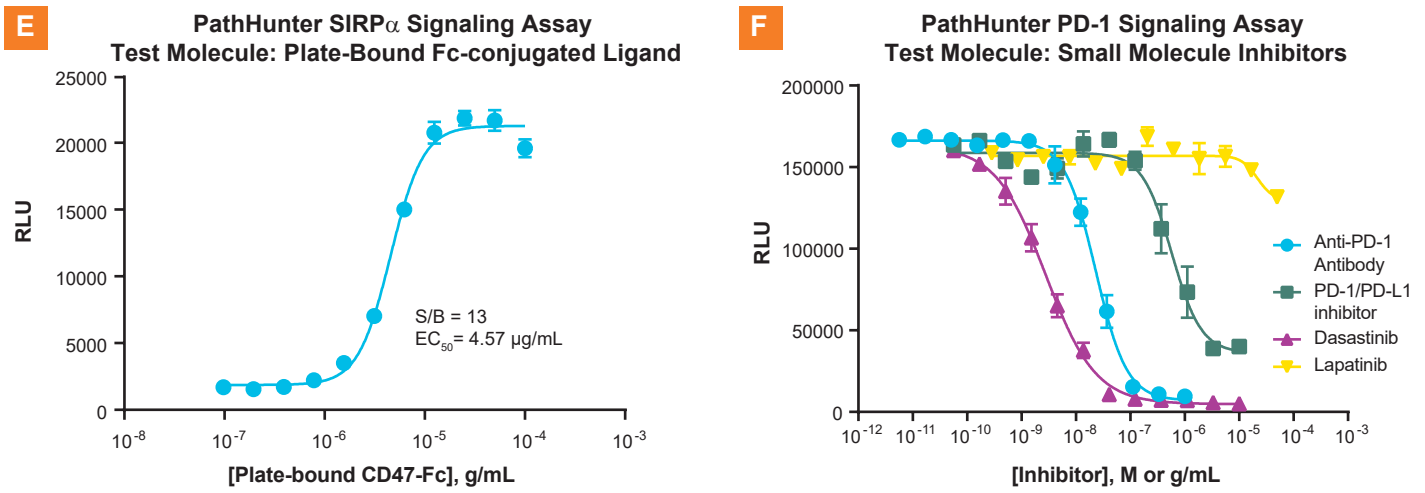


Figure 3. PathHunter® Checkpoint Assays are versatile and adaptable. A. Anti-receptor antibody and B. anti-ligand antibody experiments show robust inhibition dose-response curves for anti-PD-1 antibodies Keytruda® and Opdivos® and an anti-PD-L1 antibody, respectively. C. and D. Agonistic antibody Pogalizumab-mediated stimulation of OX40 using the PathHunter OX40 Signaling Assay. Soluble Pogalizumab was unable to activate OX40 cells alone. However, when co-cultured with human FcγRIIB cells, Pogalizumab produced a robust dose response curve. Similarly, the ICOS signaling assay quantitates the agonistic activity of an antibody targeting the ICOS receptor. E. Plate bound assay experiment using the cell-presented ligand. The dose-response curve was similar to the ICOS signaling assay, but a superior assay window was observed when the ligand-presenting cells were used. F. The PD-1 signaling assay was used to quantitate small molecule inhibitors of Src family kinases, and a low molecular weight inhibitor of PD-L1 interaction with PD-1 (PD-1/PD-L1 inhibitor). These results indicate that PathHunter assays are flexible and allow characterization of drug candidates with several different MOAs. Keytruda and Opdivo are registered trademarks of Merck and BMS, respectively.

Phase-Appropriate Formats Ensure Seamless Transitions In Drug Development Programs

The process of drug discovery and development is sufficiently long, complex, and expensive. It is of high value if an assay platform implemented in the discovery and early development stages can be used for further characterization, and ideally, the assay also proves to be fit-for-purpose in late-stage drug release programs (such as QC lot release). Therefore, we developed different phase-appropriate formats of our cell-based assays to meet the requirements of specific development stages (see Figure 4). One of the assay formats is the recombinant stable cell lines that provide a continuous culture format. The other key assay format, the bioassay, is derived from the qualified stable cell line and provides a thaw-and-use approach that alleviates any assay variability associated with cells derived from continuous culture (passage-to-passage variability). Bioassays are further optimized with a ready-to-use protocol where cells are thawed and plated directly into assay plates, and thus do not require cell culture. These bioassays are ideal for comparability, QC lot release testing, and are critical for accelerating the drug release into the market, as will be discussed in following sections.

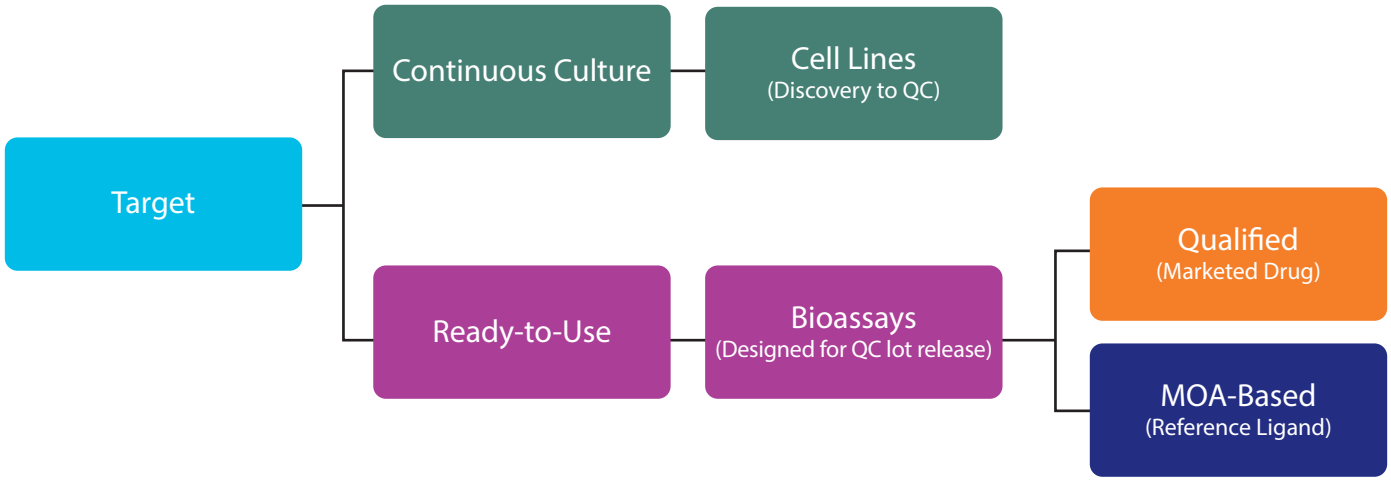
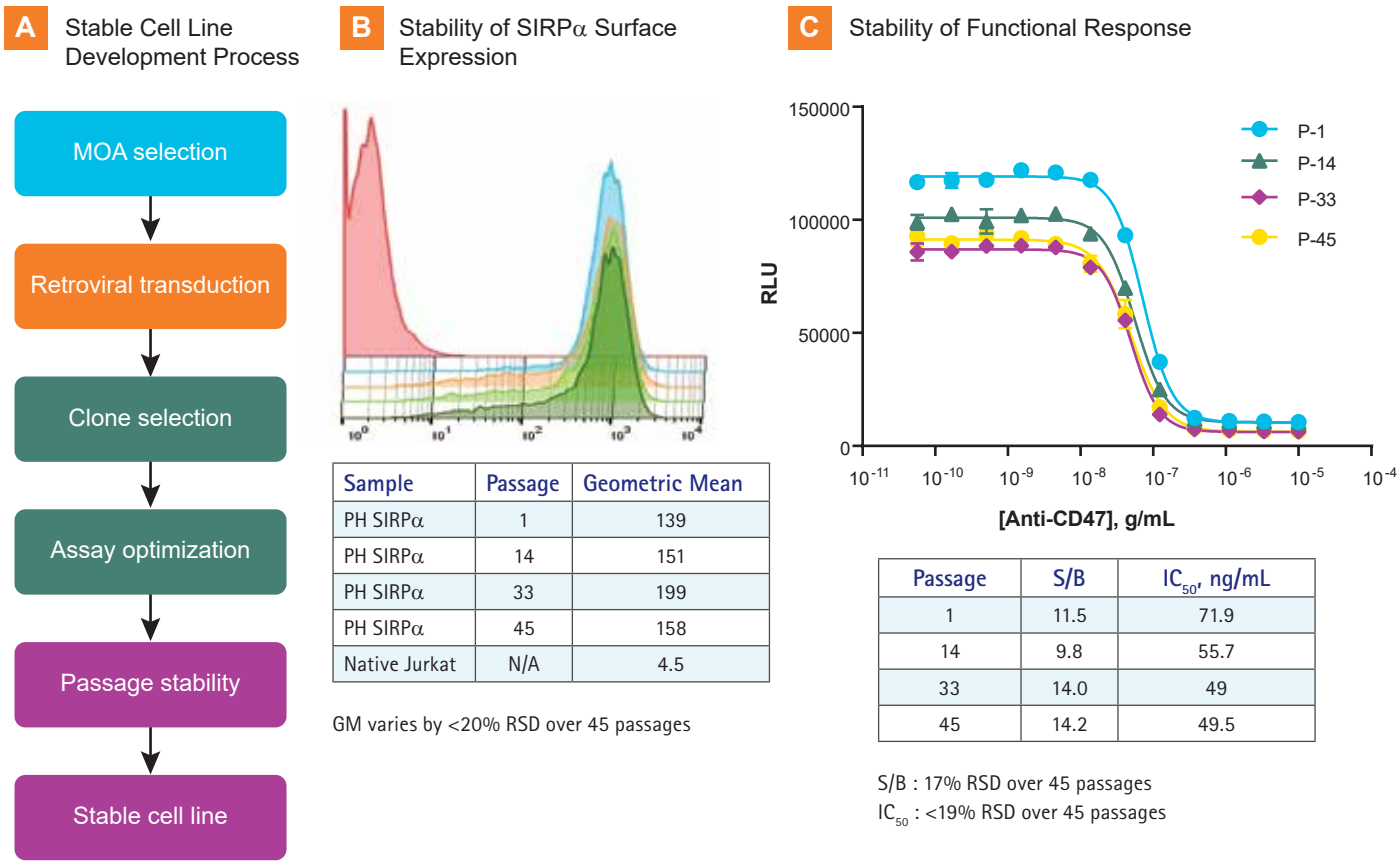


Figure 4: Eurofins DiscoverX phase-appropriate solutions for drug discovery and development program.

CELL LINES FOR SCREENING AND CHARACTERIZATION

Recombinant stable cell lines are the most frequently used format for high-throughput screening, target validation, lead optimization, and further characterization in a drug discovery program. In contrast to transient expression, stable cell lines produce repeatable results. In the Eurofins DiscoverX recombinant stable clonal cell lines, the gene of interest is introduced into the target cell background using retroviral transduction, resulting in stable genomic integration of the expression cassette. Such stable genomic integration of the expression cassette ensures high passage stability and reproducible assay performance, as shown in Figure 5. In addition, we use CRISPR technology together with the well-established EFC system to create recombinant stable cell lines for the evolving class of checkpoint therapeutics.

In Figure 5., the SIRPα cell line was analyzed for stability of cell surface expression of the SIPRα receptor up to 45 passages. It is greatly desirable to have such high passage stability in recombinant cell lines to ensure a consistent assay performance and obtain reliable results. The flow cytometry data shows a very stable expression of the receptor across these passages. Furthermore, the cell line was also functionally tested for assay performance where inhibition of SIRPα signaling with a commercially-available anti-CD47 antibody was analyzed using cells derived from different passages. Overall, a robust dose-dependent decrease in signal with a consistent assay window was observed.



Sample	Passage	Geometric Mean
PH SIRPα	1	139
PH SIRPα	14	151
PH SIRPα	33	199
PH SIRPα	45	158
Native Jurkat	N/A	4.5

GM varies by <20% RSD over 45 passages

Passage	S/B	IC_{50}, ng/mL
1	11.5	71.9
14	9.8	55.7
33	14.0	49
45	14.2	49.5

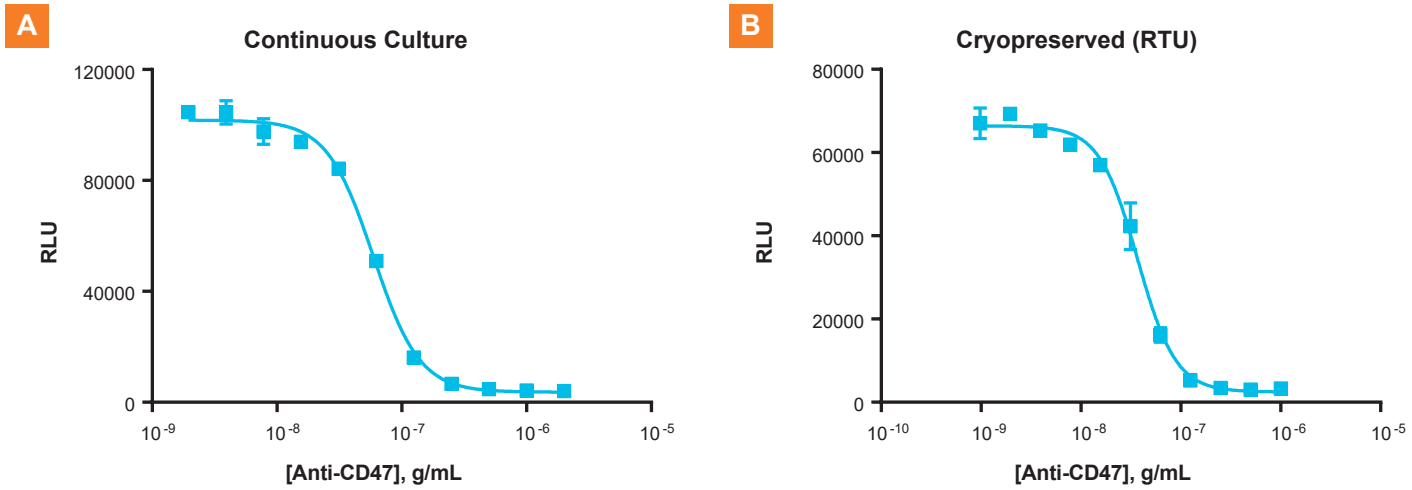
S/B : 17% RSD over 45 passages
IC_{50} : <19% RSD over 45 passages

Figure 5: Eurofins DiscoverX stable cell line generation and stability testing. A. The process outline for cell line generation. After ascertaining the assay's MOA, the gene of interest is tagged with an appropriate β-gal tag and introduced in the cells using retroviral transduction followed by pool and clone selection. The assay is optimized with the reference ligand, and the selected clone is tested for passage stability. B. Evaluation of the clone for cell surface expression of the transduced receptor. Cell surface expression was tested for extended passages to ensure a stable expression of the receptor. Cell surface expression of SIRPα receptor was analyzed up to 45 passages and was found to be highly consistent. C. Passage stability of the SIRPα cell line was analyzed functionally. Inhibition of SIRPα signaling with a commercial anti-CD47 antibody presented a robust dose-dependent decrease in signal, where a consistent assay window was observed.

READY-TO-USE BIOASSAY KITS

Stable cell lines, though critical for expediting discovery and characterization of drug candidates, may not be suitable for later stages of a drug development program. This is mainly because cells derived from continuous culture introduce passage-to-passage variabilities in the assay. Experiments run on different days sometimes do not align well since the cells were derived from different passages. In addition, it also becomes critical to constantly monitor passage stability as cells are progressively passaged. On the contrary, ready-to-use bioassays, where the cells are derived from a single passage, eliminate passage-to-passage variability and produce highly robust and consistent assay responses. These ready-to-use assays offer a high degree of inter-day reproducibility that is essential to enable potency and stability testing of the drug candidate in a QC environment.

Bioassay kits from Eurofins DiscoverX maintain the same MOA as the cell line and produce highly consistent data with excellent assay accuracy and precision (To learn more, read the case study focused on PathHunter® PD-1 and SIRPα signaling assays at discoverx.com/checkpoint-case-studies). These kits include ready-to-plate cryopreserved cells and contain all the necessary reagents optimized for the assay. Cryopreserved cells included in the kit are not required to be maintained in continuous culture, thus significantly reducing the assay run time and saving cell culture costs. Figure 6. draws a comparison between the PathHunter SIRPα assay performance of cells in continuous culture and bioassay cryopreserved cells. The results generated were highly comparable, deeming cryopreserved cells suitable for such a bioassay.



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Format	Hill Slope	IC_{50} (ng/mL)	S/B
Continuous Culture	-2.337	59.1	28
Cryopreserved (RTU)	-2.264	36.8	20

Figure 6. Assay performance with cells derived from a stable cell line maintained in a continuous culture and cryopreserved bioassay cells. The two graphs reflect the dose-response curves generated when a commercial anti-CD47 was incubated with cells in A. continuous culture and B. cryopreserved cells. C. The IC_{50} values and assay windows obtained are comparable.

PathHunter® Bioassays accelerate QC lot release testing with easy-to-implement assays using a homogenous protocol and provide results within 24 to 48 hours. The simple, add-and-read protocol enables easier assay adoption, facilitating global method transfer, and can be successfully implemented at multiple sites for potency testing. Figure 7. illustrates a thaw-and-use protocol for the PD-1 Bioassay Kit. Other bioassays within the portfolio use similar steps for testing samples.

Accelerate QC lot release testing with easy-to-implement assays using a no-wash, homogenous protocol and obtain results within 24-48 hours.

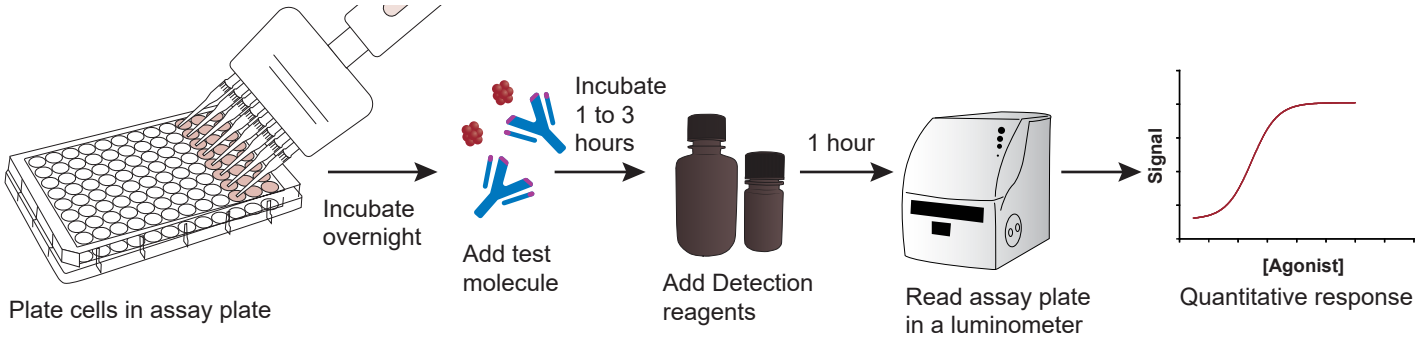

Figure 7. Protocol for PathHunter ready-to-use bioassay kits with PD-1 signaling assay as an example. The Jurkat-PD1 cells are thawed, plated, and incubated for 16 hours, after which the antibody is added. Following an incubation time of 1 hour, the ligand-presented cells are added to the assay plate. After incubating the plate for approximately 2 hours, detection reagents are added with an additional incubation time of 1 hour, after which the plate can be read on any standard luminometer. The assay delivers results in less than 24 hours.

CONCLUSION

With an unprecedented pace of developing immune checkpoint therapeutics, thousands of drug candidates are currently under clinical development. These therapeutics require fit-for-purpose assays for their characterization and lot release processes. By making such assays available for key immune checkpoint targets, Eurofins DiscoverX helps remove serious time constraints in the therapeutic candidates' market release.

Here we have shown that the PathHunter® Checkpoint Signaling Assays offer phase-appropriate formats suitable for different drug development stages, from early screening to late-stage QC lot testing. In particular, the bioassay format offers commercial ready-to-plate cryopreserved cells in a qualified kit that enables drug manufacturers to skip complex and time-consuming method development processes, thus accelerating drug release programs to move a candidate faster into assay validation for potency and stability testing. For biosimilar development programs, the availability of rigorously tested assays that are qualified with originator drugs or reference standards can be of high value in establishing drug comparability.

To ensure the best outcome for your therapeutic drug, Eurofins DiscoverX, with its staff of trained scientists, will aid in the global method transfer process when these assays are implemented in QC lot release programs to ensure successful adoption of the methodology. Such programs can be established either in-house or you can take advantage of one of the many CROs certified by Eurofins DiscoverX to implement these programs on your behalf (discoverx.com/cro-certification). The CRO Certification Program was developed and implemented to ensure that specific CROs receive hands-on workshops at their site(s) and provide comprehensive training on the PathHunter Bioassays to make the method transfer to get your programs up and running faster.

The PathHunter Checkpoint Assays save a valuable amount of time in the development of assays for immune checkpoint modulators. These assays are:

- **Physiologically-Relevant – Reflect a molecular event proximal to the drug's site of action and built using a co-culture format**
- **Rapid, Easy-to-Use – Simple, homogenous protocol yielding results between 24-48 hours**
- **Phase-Appropriate – Available as cell lines or ready-to-use bioassay kits**
- **Robust Assays – Designed to meet requirements of accuracy, precision, linearity, and reproducibility**

Eurofins DiscoverX also understands that your target of interest may be different and not available as an off-the-shelf assay. For that reason, we offer custom assay development capabilities to meet your specific assay needs with the same level of robustness as off-the-shelf assays.

RESOURCES

Case Study (discoverx.com/checkpoint-case-studies): Evaluating Checkpoint Receptor Bioassays: Fit-for-Purpose Assays Intended for Potency Testing of Therapeutic Candidates in QC Lot Release

Knowledge-Based Videos (discoverx.com/knowledge-videos)

- Novel Cell-Based Assays to Enable Immunotherapy Drug Development for Checkpoint Receptors
- A Novel MOA-reflective Bioassay for Quantifying Potency of Therapeutics Targeting the SIRPα|CD47 Signaling Axis
- Cell Banking for Bioassays
- Reporter Assays for Therapeutics Targeting Signaling Pathway

Webinar (discoverx.com/webinars): Establishing Qualified Bioassays for Checkpoint Receptors to Implement in QC Lot Release: Case Studies on PD-1 and SIRPα

Cell Banks for Bioassays (discoverx.com/cell-banks)

CRO Certification Program (discoverx.com/cro-certification)

Custom Assay Development (discoverx.com/cad)

Immuno-Oncology Solutions (discoverx.com/IO)

Checkpoint Assays (discoverx.com/checkpoint)

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