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APPLICATION NOTE

Automating PathHunter® Cell-Based PD-1 Bioassay on the Tecan® Fluent Automation Workstation

Bringing Higher Throughput Efficiency with Improved Assay Consistency and Reproducibility for Easy Implementation in Quality Testing Programs

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INTRODUCTION

Therapeutic candidates require physiologically relevant functional assays as characterization tools that attest to their therapeutic activity through the stages of their development. Their mechanism-of-action (MOA) via biological activity is a critical attribute that can be accurately measured using cell-based potency assays. Moreover, analyzing potency is a key measurement for the final market release of a therapeutic product such as a biologic or biosimilar. Cell-based assays add value when implemented in quality controlled (QC) lot-release testing programs as they serve as indicators of any altered potency or stability or the presence of impurities in the manufactured drug products intended for clinical use. Furthermore, regulators accept data that is highly consistent and reproducible as they can offer precision, accuracy, and reduced assay variability, all needed to drive and influence scaling up capabilities and/or method transfers Therefore, it is important to understand the value of cell-based potency assays employed in programs that demand highly consistent and reproducible data enabling final drug product market release.

There are specific requirements associated with implementing cell-based potency assays (in QC lot-release therapeutic potency testing) including cell culture, skilled pipetting, and precise plate layout. These requirements directly contribute to the assay performance during QC testing including key parameters such as consistency, accuracy, precision, and variabilities among days and plates. If done correctly, the introduction of assay automation can bring this consistent method of execution. This is particularly valuable when assays are transferred between different groups or testing sites with automation capabilities. For these reasons, bringing automation to cell-based assays can provide better assay consistency and increased throughput, while decreasing the long term operational costs.

Assay workflows, especially for potency testing conducted at biopharma, contract research organizations (CROs), or contract development and manufacturing organizations (CDMOs) need to be designed to achieve desired assay consistency and stability that not only meet stringent QC requirements, but also exert minimal impact on assay performance, variability, sensitivity, linearity, etc. By integrating automation (e.g., through Tecan's Fluent® Automation Workstation) into Eurofins DiscoverX's bioassay workflows, we demonstrate via this PD-1 signaling bioassay application note, as well as in a CNR1 application note (discoverx.com/CNR1-Appnote), the retention of characteristics of good assay performance that includes:

- Functionality (reflective of therapeutic's MOA)
- Therapeutic MOA
- Stability
- Reproducibility
- Assay Throughput
- Transfer of Assay to Other Sites

Our collaborative work with Tecan on automating the PathHunter® PD-1 Signaling Bioassay shows highly consistent and reproducible results with minor optimization needs. In addition, the results obtained from the assay performed with automation were nearly the same as those obtained by an experienced QC scientist.

PathHunter PD-1 SIGNALING BIOASSAY

Eurofins DiscoverX bioassays have been implemented in over a hundred programs globally at pharma, biotech, CROs, and CDMOs. The assays are ready-to-use (RTU), MOA-reflective, functional cell-based assay kits that contain thaw-and-use cryopreserved cells (derived from passage tested stable cell lines) with all necessary reagents together with optimized assay protocols. These RTU assays do not require cell culture and are intended to reduce assay development and validation times by >18 months, thereby accelerating QC lot-release testing. A subset of bioassays are qualified with innovator drugs (per International Council for Harmonisation (ICH) guidelines) to ascertain they are fit-for-purpose for potency and stability testing in good manufacturing practice (GMP) settings.

The PathHunter PD-1 (SHP1) Signaling Bioassay used for this study is a simple cell-based assay that measures drug potency and can also be used to detect neutralizing antibodies. The assay principle is based on Eurofins DiscoverX's enzyme fragment complementation (EFC) technology (discoverx.com/EFC) and is shown in Figure 1. In this assay, a tagged full-length PD-1 receptor is co-expressed with the tagged regulator protein SHP1 (or SHP2 using a different PathHunter cell line) in Jurkat cells. The PD-1 receptor is tagged with a fragment of the EFC β-galactosidase (β-gal) enzyme termed the Enzyme Donor (ED) at the receptor C-terminus (cytoplasmic tail), while the SHP1 protein is tagged with the larger fragment of the β-gal enzyme termed as the Enzyme Acceptor (EA). In the presence of a ligand-presenting cell, engagement occurs through the PD-L1/PD-L2 and PD-1 receptors binding. This binding and subsequent activation results in the SHP recruitment to PD-1, bringing the EA and ED fragments together forming an active β-gal enzyme. The active β-gal enzyme can now hydrolyze its substrate in a dose-responsive manner generating a chemiluminescent signal that can be measured on any standard luminometer. However, when a therapeutic agent such as an antagonist antibody blocks the interaction between the PD-1 receptor and its ligand (PD-L1/PD-L2 receptor), SHP recruitment does not occur, resulting in loss of signal.

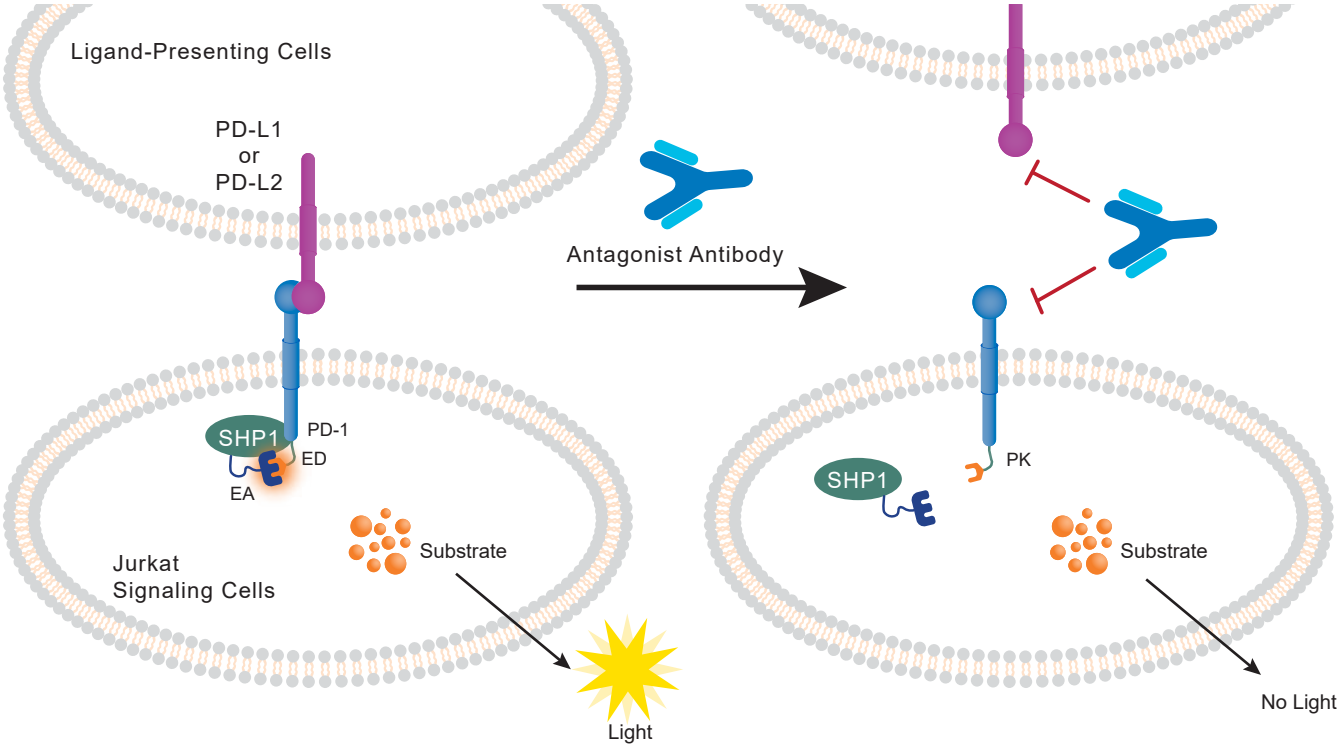


Figure 1. PathHunter PD-1 Signaling Assay Principle. The assay involves the signaling cell line with over-expressed ED-tagged PD-1 receptor and EA-tagged SH1 domain protein. Upon activation of the PD-1 receptor by its ligand (e.g. PD-L1/PD-L2 receptor on the ligand-presenting cell) or an agonist antibody, recruitment of the SH1 domain protein to the phosphorylated receptor tail leads to production of an EFC signal. In the presence of an antagonist (e.g. antagonist antibody), the receptor interaction is blocked, and the EFC signal is decreased. For a complete list of checkpoint assays, please visit discoverx.com/checkpoint.

MATERIALS

Assay Components

PathHunter® Jurkat PD-1 Bioassay Kit (Cat. No. 93-1104Y19), containing RTU PathHunter Jurkat PD-1 and U2OS PD-L1 bioassay cells, 96-well assay plate, AssayComplete™ Cell Plating (CP) Reagent 0, anti-PD-1 antibody, protein dilution buffer, PathHunter Bioassay Detection Kit, and an optimized assay protocol.

INSTRUMENTATION

Automation Workstation

Experiments were conducted on a Tecan Fluent® 780 system that includes an eight-channel Air Flexible Channel Arm™ (Air FCA), a Multiple Channel Arm™ (MCA) with an extended volume adapter for pipetting up to 500 µL with 96 tips in parallel, and a long Robotic Gripper Arm™ (RGA) to reach below the Dynamic Deck™ (Figure 2. A.). A vertical laminar flow HEPA hood with UV light (Bigneat) was integrated with the Fluent system to ensure a clean environment. Liquid handling tasks were conducted with the Air FCA using 1000 µL and 200 µL filtered disposable tips and the MCA using 150 µL filtered disposable tips. Sterile 15 mL Falcon™ tubes and 2 mL Eppendorf tubes were placed into tube runners for pipetting into sterile 96-well white, clear flat-bottom, TC treated microplates with lid and 96-well green, V-bottom microplates (Eurofins DiscoverX, Cat. No. 92-0011). A Cytomat 2 C450-LiN Automated Incubator with two stackers – for 21 and 10 microplates, respectively – was integrated with the Fluent system, enabling the storage and incubation of Jurkat PD-1 cells in microplates during the 24 hour incubation period (Figure 2. B.). The automated incubator's parameters were set at 37°C and 5% CO_2 to provide an ideal and stable environment for the cryopreserved and RTU cells.

Individual scripts were developed using the FluentControl™ software for cell plating on Day 1 and sample preparation of the agonist's serial dilutions with the subsequent addition of the detection reagents on Day 3. The chemiluminescence signal was measured with Tecan's Spark® Cyto 600 multimode reader.

Figure 2. Tecan's Fluent Automation system. The Fluent Automation Workstation (A.) was integrated with the Cytomat 2 C450-LiN Automated Incubator (B.) to automate the PathHunter PD-1 Signaling Bioassay. The measurement of the chemiluminescent signal was performed with a Spark® Cyto 600.

METHODS

The automation and manual experiments were performed independently. The protocol for experiments performed on the Fluent automation system is outlined in the protocol schematic below (Figure 3). For the manually performed experiments, the protocol in the bioassay user manual (PathHunter® PD-1 Signaling Bioassay user manual) was followed.

Cell Plating

The PathHunter PD-1 bioassay cells were manually thawed and plated in AssayComplete™ CP Reagent 0 (CP0) at 4,000 cells per well in a 96-well plate and incubated overnight at 37°C and 5% CO_2.

Antagonist Preparation

From a 1 mg/mL stock of anti-PD-1 antibody (supplied in the kit), a top dose of 50 µg/mL was prepared in CP0 in the dilution plate (well A1) followed by ten 1:3 fold dilutions made in CP0 (wells A2-A11). 20 µL of this dilution series was added to the assay plate in replicates. This resulted in a top dose of 10 µg/mL as the final volume in the assay plate would be 100 µL.

PathHunter PD-1 Assay

PathHunter PD-1 bioassay cells were treated with increasing concentrations of PD-1 antibody for 60 minutes at 37°C and 5% CO_2. Subsequently, cells expressing PD-L1 were added to the assay to stimulate PD-1 receptors for 60 min. The antibody is expected to block the binding of PD-1 to PD-L1 in the assay preventing SHP recruitment in a dose-dependent manner. This event is captured in the form of a loss in EFC chemiluminescent signal with increasing antibody concentration. The activity was measured by the addition of the chemiluminescent PathHunter Bioassay Detection Kit.

PROTOCOL SCHEMATIC

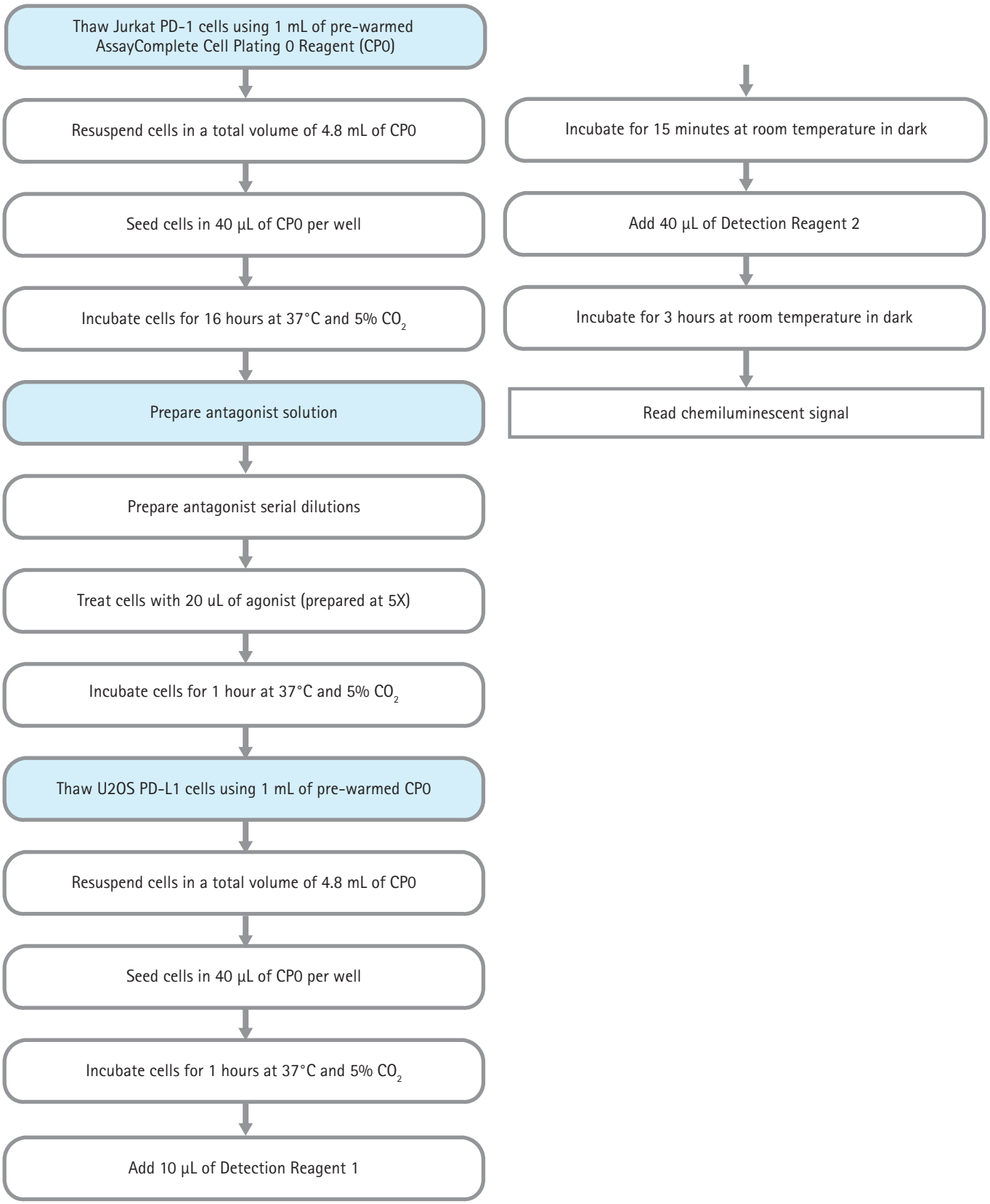


Figure 3. Protocol schematic for the PathHunter® Jurkat PD-1 Signaling Bioassay performed on Tecan's Fluent 780 System. Steps highlighted in blue required minimal human intervention.

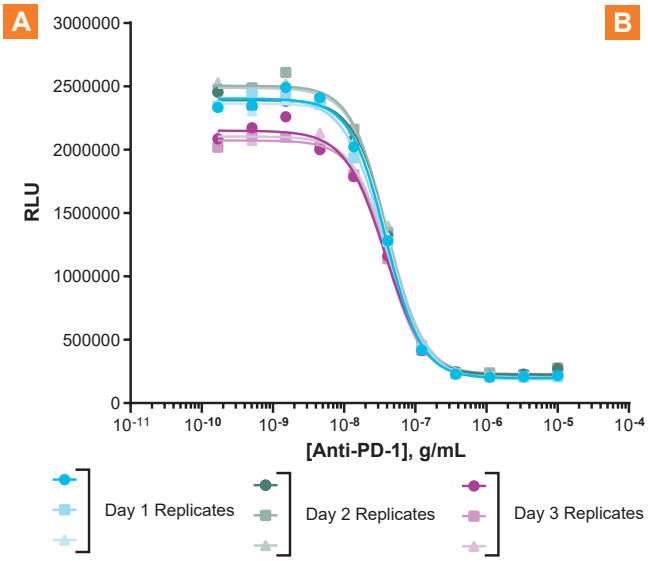
RESULTS

Automated *vs.* Manual Anti-PD-1 Receptor Activity

The automated Fluent® automation workstation experiments data for the Anti-PD-1 antibody shows blocking in PD-1 activation in a dose-dependent manner (Figure 4. A.). The antibody binds to the PD-1 receptor, thereby blocking it from binding to the ligand presented by the U2OS PD-L1 ligand cells, resulting in a decreased chemiluminescent signal. Inter-day experiments conducted thrice on three separate days show results with comparable EC_{50} values with an intermediate precision of 6.5% (Figure 4. B.). Each experiment was performed in triplicate and each replicate was plotted as a separate curve as shown below in Figure 4. A. For every experiment, all three curves show a high degree of overlap, thereby demonstrating high intra-day intermediate precision.

To evaluate inter-day replicate consistency, the raw luminescence units (RLU) from all three experiments were analyzed. As shown in Figure 4. C., the replicates for each data point are visibly tight with an average %RSD of less than 3%, demonstrating excellent inter-day replicate consistency.

A



B

Day	Replicate	S:B	EC_{50}	Mean EC_{50}	%RSD	Mean EC_{50} (3 days)	%RSD (3 days)
1	1	12.2	1.30E-09	1.30E-09	7.69%	1.32E-09	6.50%
	2		1.20E-09				
	3		1.40E-09				
2	1	11	1.40E-09	1.37E-09	4.22%		
	2		1.30E-09				
	3		1.40E-09				
3	1	10	1.20E-09	1.30E-09	7.69%		
	2		1.30E-09				
	3		1.40E-09				

B

Day 1

[Anti-PD-1 Antibody], M	Mean	%RSD
0	3.20E+06	2.40%
1.69E-10	2.30E+06	0.50%
5.08E-10	2.40E+06	2.90%
1.52E-09	2.40E+06	1.90%
4.57E-09	2.40E+06	1.10%
1.37E-08	2.00E+06	3.10%
4.12E-08	1.30E+06	1.30%
1.23E-07	4.40E+05	4.50%
3.70E-07	2.30E+05	1.80%
1.11E-06	2.10E+05	2.40%
3.33E-06	2.10E+05	0.90%
1.00E-05	2.10E+05	2.90%
	Avg. %RSD=	2.10%

Day 2

Mean RLUs (n=3)	%RSD
3.20E+06	1.50%
2.50E+06	1.60%
2.40E+06	3.30%
2.50E+06	4.60%
2.40E+06	0.60%
2.10E+06	2.00%
1.40E+06	2.90%
4.60E+05	3.40%
2.40E+05	1.70%
2.30E+05	6.40%
2.30E+05	2.80%
2.70E+05	4.40%
Avg. %RSD=	2.90%

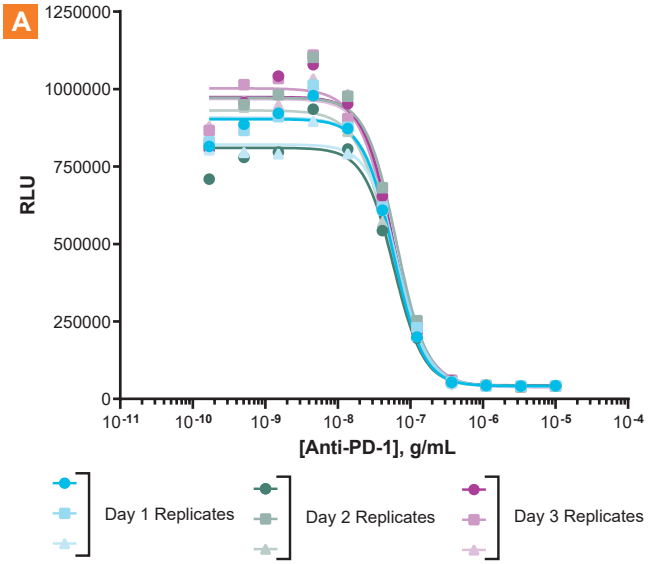
Day 3

Mean RLUs (n=3)	%RSD
2.80E+06	2.1%
2.10E+06	2.2%
2.10E+06	2.4%
2.20E+06	4.3%
2.10E+06	3.4%
1.80E+06	0.5%
1.20E+06	3.3%
4.20E+05	3.2%
2.40E+05	0.3%
2.20E+05	2.1%
2.20E+05	1.7%
2.30E+05	5.2%
Avg. %RSD=	2.6%

Figure 4. Results from the Tecan's Fluent 780 Automation Workstation. A. Dose response curves for anti-PD-1 antibody demonstrating blockage of SHP1 recruitment to PD-1 receptor in a dose-dependent manner were conducted across three days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across three days. C. Table illustrating %RSD of raw RLUs for each day. RSD = Relative Standard Deviation; RLU = Relative Luminescence Units

To compare assay consistency and reproducibility, the same set of experiments were performed manually by a QC scientist with more than six years of experience running PathHunter EFC-based assays. These experiments were also performed across three different days in triplicates. Data for each replicate per experiment is plotted as an individual curve (Figure 5). Upon analyzing the data, the results obtained were very close to the automation results with an intermediate precision for EC_{50} being 6.2%, compared to that obtained via automation being 6.5%.

A



B

Day	Replicate	S:B	EC_{50}	Mean EC_{50}	%RSD	Mean EC_{50} (3 days)	%RSD (3 days)
1	1	21.4	5.8E-08	6.17E-08	6.55%	5.90E-08	6.20%
	2		6.1E-08				
	3		6.6E-08				
2	1	21.6	5.9E-08	5.83E-08	10.30%		
	2		6.4E-08				
	3		5.2E-08				
3	1	23.5	5.8E-08	5.70E-08	1.75%		
	2		5.6E-08				
	3		5.7E-08				

Figure 5. Results from the manual experiments. A. Data for anti-PD-1 antibody demonstrating blockage of SHP1 recruitment to PD-1 receptor in a dose-dependent manner conducted across three days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across three days.

CONCLUSION

Eurofins DiscoverX PathHunter Bioassays constitute the most comprehensive menu of RTU MOA-based, functional cell-based bioassays (discoverx.com/bioassays) and are developed using stable cell lines. They are qualified with marketed innovator drugs, reference standards, or ligands following ICH guidelines. Each bioassay is designed to reflect the target biology providing direct read-out of the target receptor specific to the drug, in contrast to reporter/phenotypic-based assays. Additionally, these assays offer homogenous easy-to-run protocols that yield results within 24 hours. Their high inter-lot and intra-lot reproducibility enables seamless method transfer for faster implementation at CMOs, CDMOs, or CROs. Furthermore, integrating automation within functional, cell-based potency assays for drug discovery and development will not only aid in obtaining highly reproducible and reliable data, but also reduce time and the cost to implement within any given QC lot-release program

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For more information on bioassays, visit discoverx.com/bioassays.