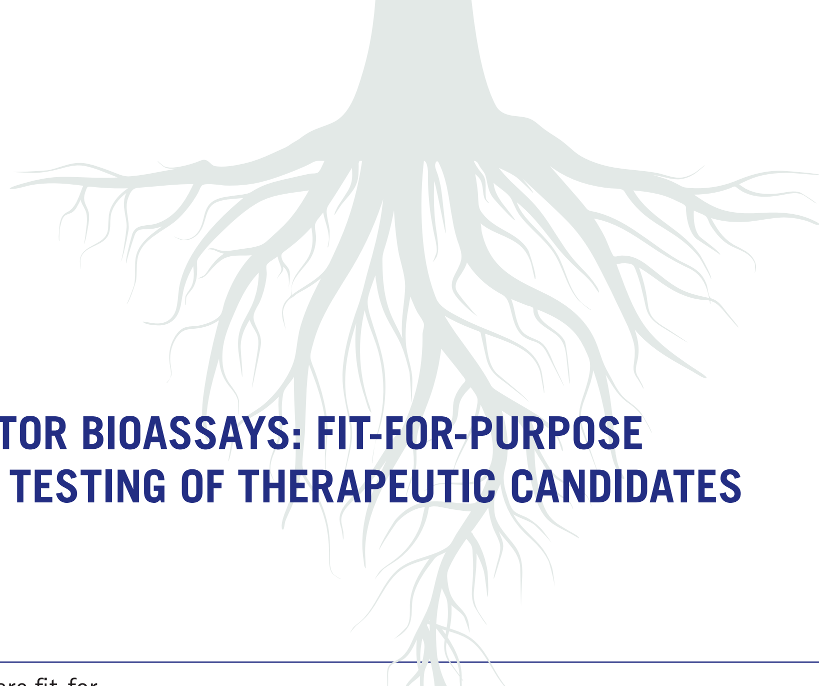
eurofins | DiscoverX

The Eurofins Discovery PRODUCTS COMPANY



CASE STUDY

EVALUATING CHECKPOINT RECEPTOR BIOASSAYS: FIT-FOR-PURPOSE ASSAYS INTENDED FOR POTENCY TESTING OF THERAPEUTIC CANDIDATES IN QC LOT RELEASE

ABSTRACT

Ready-to-Use qualified PathHunter® checkpoint bioassays are fit-for-purpose for potency testing for implementation in downstream development phases and Lot release of the biologic drugs at sponsors' testing sites or their partner Contract Research Organizations (CRO)/Contract Development and Manufacturing Organization (CDMO). These bioassays are developed and optimized following systematic qualification studies to meet regulatory expectations. During these qualification studies, we evaluate the key attributes desired in a functional bioassay as defined by The International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use and United States Pharmacopeia (USP) guidelines. These include accuracy, precision, linearity, range, specificity, robustness, and stability-indicating properties. These attributes are deemed critical in a bioassay when measuring drug potency and stability during drug manufacturing and release under quality control (QC) conditions. Therefore, qualification studies are key in demonstrating that the assay performance is robust and sensitive to measure manufacturing consistency, and measurements can be correlated with clinical outcomes of the drug candidate. Here, we present case studies on two PathHunter bioassays—the PD-1 Signaling Bioassay and the SIRPα Signaling Bioassay. Refer to the complementary whitepaper titled "Accelerating Immune Checkpoint Drug Discovery through Functional Cell-Based Assays" (discoverx.com/checkpoints-whitepaper) for additional information.

Ready-to-use qualified bioassays can accelerate your immune checkpoint drug discovery program.

- Use an MOA-relevant assay to assess your drug's biological activity
- Obtain results with high accuracy, precision, and linearity
- Implement bioassays in potency and stability testing programs

CASE STUDY 1: PathHunter PD-1 SIGNALING BIOASSAY

PD-1 (programmed cell death 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.

ASSAY PRINCIPLE

The PathHunter® Jurkat PD-1 Signaling bioassay is designed to evaluate biologic and small molecule therapeutics that aim to block PD-1 signaling. The assay captures an early event following ligand-mediated receptor activation. 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[1]. Using the industry-validated Enzyme Fragment Complementation (EFC; discoverx.com/efc) technology, this assay measures SHP recruitment to the cytoplasmic tail of PD-1 and therefore captures a physiologically relevant mechanism-of-action (MOA). In this assay (visit discoverx.com/checkpoints), full-length PD-1 receptor was co-expressed with SHP1 (or SHP2) in Jurkat cells. The PD-1 receptor was tagged with a small enzyme donor (ED) fragment of β-galactosidase (β-gal) at the c-terminus (cytoplasmic tail), and SHP1 tagged with a larger enzyme acceptor (EA) 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 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.

Assay qualification study with PathHunter PD-1 Signaling Bioassay was performed with two FDA-approved therapeutic anti-PD-1 antibodies, Keytruda® and Opdivo® (Figure 1.).

Keytruda is a registered trademark of Merck Sharp & Dohme Corp., and Opdivo is a registered trademark of Bristol-Myers Squibb Company.

PathHunter ASSAYS HAVE HIGH ACCURACY, PRECISION, AND LINEARITY

> Results from the PathHunter PD-1 Signaling Bioassay qualification study suggest that the assay is easy to implement, and produces comparable results with low variability and good reproducibility between different analysts over multiple days, and across sites.

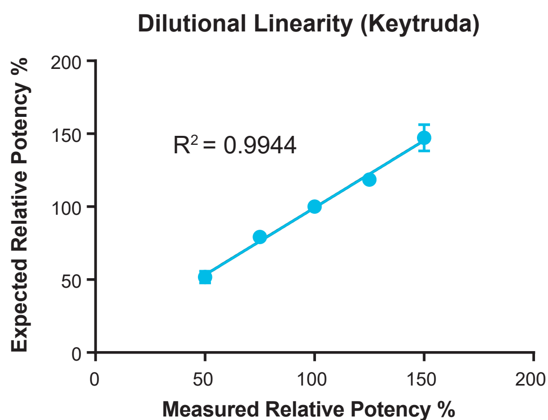
The first two critical aspects, assay accuracy and precision, are key qualification parameters for a bioassay intended for drug potency and stability studies. Assay accuracy reflects the closeness of the experimental results to the expected value. A high degree of assay accuracy here suggests that the bioassay can measure relative potency with high confidence. Conversely, assay precision is a direct measure of assay repeatability between assays performed on different days and different analysts. In these set of experiments (Figure 1.), anti-PD-1 samples were tested at varying potencies (ranging from 50% to 150%) relative to a reference sample of 100% potency. The potency range was selected based on ICH guidelines[2, 3]. The experiments were performed in triplicates in four independent experiments by two analysts over four days. As Figure 1. C. shows, the assay was found to be very accurate (99.7%) and precise (4.4%). The assay was highly repeatable when performed on different days with two different analysts. In addition, the assay showed a minimal inaccuracy or relative bias (0.7%). This suggests that the assay does not have any significant systemic pattern in over or underestimating relative potencies (RP). In perspective of relative bias, the overall accuracy was within 5.3% across the entire potency range of 50% to 150%.

To demonstrate dilutional linearity of anti-PD-1 sample measurements, we analyzed "measured" against "expected" relative potencies (Figure 1. B.) and calculated the slope of the curve and the associated R^2 values, arriving at an almost perfect R^2 value of 99.4%. R^2 values closer to 100% indicate that the measured values in the assay method are in high alignment to the predicted values. Dilutional linearity of the assay reflects if the measured potency of the drug remains proportional to the expected concentration in the assay and thus constitutes an important parameter. Similar assay performance results were obtained in a separate qualification study with Opdivo® as summarized in Figure 1. C. and D.

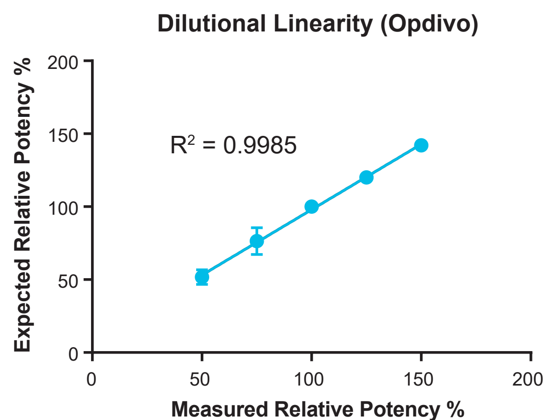
A

Expected RP (%)	Experiment No.	Analyst No.	Measured RP (%)	Average RP (%)	RSD (%)	Accuracy (%)	Relative Bias (%)
150	1	1	143	147	4.6	98	-1.8
	2	1	159				
	3	1	138				
	4	2	149				
125	1	1	115	119	2.6	95.2	-4.7
	2	1	117				
	3	1	122				
	4	2	120				
75	1	1	82	79	2.6	105.3	5.3
	2	1	79				
	3	1	77				
	4	2	79				
50	1	1	54	52	7.8	104	4.0
	2	1	56				
	3	1	47				
	4	2	50				

B



C



D

Drug	Accuracy	Precision	Linearity
Keytruda®	100.6%	4.4%	99.44%
Opdivo®	100.5%	6%	99.85%

Figure 1: PathHunter® PD-1 Signaling Bioassay shows accuracy, precision and linearity. A. The PathHunter PD-1 Signaling Bioassay was qualified using Keytruda. The experiments were performed in triplicates in four independent experiments generated by two analysts over four days over a potency interval range of 50% to 150%. Data was analyzed using Restricted Mode; 4PL fit in PLA software (PLA, Stegmann Systems GmbH, Rodgau, Germany). B. and C. The average measured relative potency was plotted against the expected relative potency for Keytruda and Opdivo respectively. These assay linearity graphs quantify data generated by two analysts using this bioassay over four days. D. The PD-1 signaling bioassay demonstrates excellent accuracy, precision, and linearity for both Keytruda and Opdivo. Precision values are expressed as a percentage of Relative Standard Deviation (RSD).

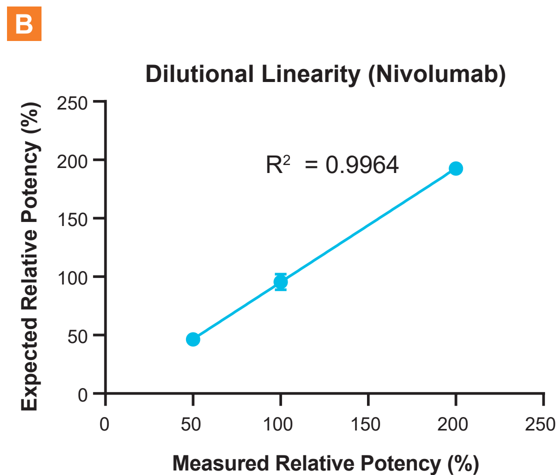
PathHunter ASSAYS CAN BE READILY TRANSFERRED AND IMPLEMENTED IN POTENCY AND STABILITY PROGRAMS

One of our primary goals when developing bioassays is to make them easy to transfer and implement in QC lot release programs that typically happen under GxP or "good practice" conditions at a CRO site. Thus, as an exercise to demonstrate a successful assay transfer, we partnered with BioOutsource at Sartorius Stedim Biotech, a reputable CRO, where an independent phase-appropriate qualification study with our PD-1 Bioassay was performed. The qualification study analyzed the FDA-approved anti-PD-1 antibody nivolumab, over a potency range of 50% to 200% with five different analysts. Figure 2. summarizes the results. The study produced high accuracy within 7.33% across the entire 50% to 200% range. In addition, the intermediate precision (inter-day) was within 7.1%. The R^2 value for dilutional linearity across the range was 99.64% (Figure 2. B). These results suggest that the assay is easy to implement, and produces comparable results with very low variability and good reproducibility between different analysts over multiple days, and across sites.

A

Expected RP (%)	Experiment No.	Analyst No.	Measured RP (%)	Average RP (%)	RSD (%)	Accuracy (%)	Relative Bias (%)
200	1	2	197.1	192.67	1.97	100.03	7.33
	2	3	190.6				
	3	3	190.4				
100	1	1	88.0	95.43	7.02	100.34	4.57
	2	1	97.3				
	3	2	101.1				
50	1	1	46.1	46.43	2.04	100.03	3.57
	2	2	45.7				
	3	3	47.5				

B



C

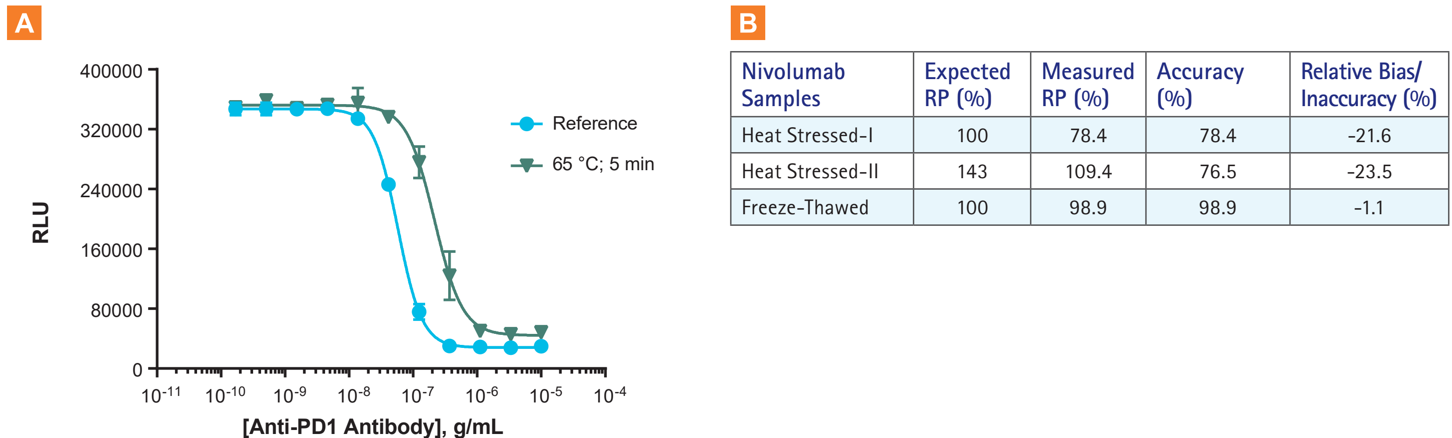
Drug	Accuracy	Precision	Linearity
Nivolumab	100.1%	3.67%	99.64%

Figure 2: Independent PD-1 Signaling Bioassay qualification study performed at a CRO site. A. A potency interval range of 50% to 200% was analyzed using restricted mode; 4PL fit in PLA software. B. The average measured relative potency was analyzed against the expected relative potency to obtain dilutional linearity. C. The table summarizes key assay parameters suggesting the PD-1 signaling assay demonstrate excellent accuracy, precision, and linearity for the anti-PD-1 antibody nivolumab. Data courtesy BioOutsource, Sartorius Stedim; used with permission.

PathHunter PD-1 SIGNALING ASSAY IS STABILITY-INDICATING

In addition to potency, a physiologically-relevant assay is needed to determine the stability of therapeutic drug candidates, preferably through an assessment of the drug's biological activity. The need for stability testing is to ascertain how the drug quality is affected by a repertoire of environmental factors. The most common factors are freeze-thaw cycles, long-term storage temperatures, solubility in a given matrix, humidity, exposure to light (photostability), likely molecule-specific degradation pathways (e.g. oxidation, deamidation, etc.), and other attributes of the drug. These factors can vary during storage and are likely to affect drug stability, safety, and potency. Analyzing drug stability under induced stress conditions is a common practice for establishing suitable analytical methods. Heat-stressed samples of an anti-PD-1 antibody were tested to evaluate if the PathHunter® PD-1 Signaling Bioassay will differentiate the stressed material from the reference sample. As demonstrated in Figure 3. A, a marked difference between the two response curves of the heat-stress and reference samples was observed, demonstrating that the PathHunter PD-1 Signaling Bioassay is stability indicating.

To further demonstrate that the PathHunter PD-1 Bioassay is stability-indicating, an additional set of experiments were performed as a part of a qualification study at BioOutsource with nivolumab. Nivolumab samples were stressed either by exposing them to high temperatures for 2 hours in a 60°C water bath, or frozen and thawed five times (frozen at -90°C and thawed at room temperature). The effects of these treatments were first analyzed using ion-exchange chromatography. The chromatogram profile indicated that only the heat-treated, and not freeze-thawed sample, showed clear indications of structural differences (data not shown). Thereafter, the treated samples were analyzed alongside untreated material (at 100% potency) in the PathHunter® PD-1 Signaling Bioassay. The results were in-line with the chromatography outcomes, and only the heat-treated sample showed significant reduction activity, while the freeze-thawed sample displayed no differences with respect to the untreated reference material. To further confirm these results, the heat-treated samples were run at 143% RP to assess the sensitivity to the assay when run at a different potency interval. At 143%, the results returned with a similar shift in the potency of the heat-treated sample as at 100% (Figure 3. B.).



Nivolumab Samples	Expected RP (%)	Measured RP (%)	Accuracy (%)	Relative Bias/ Inaccuracy (%)
Heat Stressed-I	100	78.4	78.4	-21.6
Heat Stressed-II	143	109.4	76.5	-23.5
Freeze-Thawed	100	98.9	98.9	-1.1

Figure 3. PD-1 Signaling Bioassay is stability-indicating. A. Anti-PD-1 antibody Keytruda was heat-treated at 65°C for 5 minutes and tested for potency alongside an untreated reference sample. A marked difference between the treated and untreated sample was observed. B. Anti-PD-1 antibody nivolumab samples were stressed as indicated in the table and evaluated in PD-1 Signaling Bioassay. Samples that were heat-stressed (2 hours at 60°C) showed a reduced activity at different relative potency intervals. The sample that was stressed with repeated freeze-thawing (five freeze-thaw cycles -90°C to room temperature) showed no significant difference in potency (data courtesy BioOutsource, Sartorius Stedim; used with permission).

CASE STUDY 2: PathHunter SIRPα SIGNALING ASSAY

The PathHunter Jurkat SIRPα Signaling Bioassay Kit is the first commercially available bioassay specifically designed for measuring the potency of anti-CD47 or anti-SIRPα therapeutic drug candidates. Like the PD-1 signaling bioassay, it demonstrates high accuracy, precision, and dilutional linearity.

Another emerging checkpoint receptor is SIRPα (signal regulatory protein α). SIRPα is a cell surface receptor predominantly found on macrophages and dendritic cells of the innate immune system. Unlike PD-1, SIRPα is involved in an innate immune response, where it binds with its ligand, CD47, a cell surface glycoprotein expressed on a variety of cells. This interaction facilitates the inhibition of phagocytosis of CD47-expressing cells. This cell surface marker, CD47, is often referred to as the "do not eat me" signal, which prevents phagocytosis of normal, healthy cells. For instance, a high expression of CD47 on hematopoietic stem cells ensures they are able to migrate in the body without being phagocytosed[4]. However, a loss of CD47 on aged red blood cells leads to macrophage-mediated phagocytosis. Tumor cells are also shown to overexpress CD47 in various cancers such as prostate, breast, and liver cancer. Overexpression of CD47 help cancerous cells evade the primary immune surveillance by suppressing macrophage-mediated phagocytosis via SIRPα signaling in macrophages. Thus, the blockade of SIRPα-CD47 signaling axis presents a promising approach for the treatment of these cancers.

The PathHunter® Jurkat SIRPα Signaling Bioassay Kit is the first commercially available bioassay specifically designed for measuring the potency of anti-CD47 or anti-SIRPα therapeutic drug candidates. Much like the assay principle of the PathHunter PD-1 Signaling Bioassay, Jurkat cells were engineered to express SIRPα tagged with the small and inactive β-gal fragment (ED), and the SHP1 protein is tagged with the larger complementary β-gal fragment (EA). Naïve Jurkat cells are used as the ligand-presenting cells as they express high levels of CD47. Incubation of these two cell types results in an active signaling axis that causes the recruitment of the SHP1 protein to the receptor. As a result, ED and EA are brought into close proximity and form an active β-gal enzyme. In the absence of a blocking antibody, CD47 will activate SIPRα, and hence SHP1 recruitment produces a quantitative EFC signal. However, in presence of a blocking antibody for SIRPα or CD47, a loss of signal is observed.

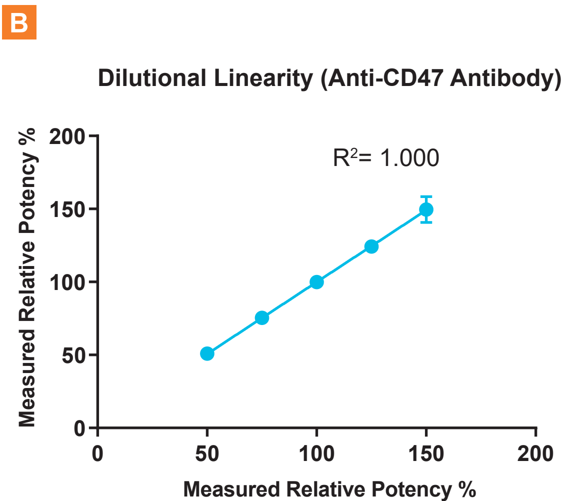
PathHunter SIPRα SIGNALING ASSAY HAS HIGH ACCURACY, PRECISION, AND DILUTIONAL LINEARITY

A comprehensive qualification study was designed for evaluating the suitability of the PathHunter SIRPα Bioassay for use in potency and stability testing of anti-CD47 antibodies. Two separate analysts performed relative potency assays across a 50 % to 150% range, spanning three days to determine assay accuracy, precision, and linearity. The results of the qualification study are summarized in Figure 4. The assay was found to be highly accurate, with less than 2% relative bias/inaccuracy and excellent intermediate precision (inter-day) within 7% CV. Overall, the dilutional linearity was shown to be excellent in this qualification study (Figure 4. C.).

A

Expected RP (%)	Experiment No.	Analyst No.	Measured RP (%)	Average RP (%)	RSD (%)	Accuracy (%)	Relative Bias (%)
150	1	1	164	149.5	5.96	99.7	-0.3
	2	1	144				
	3	1	145				
	4	2	140				
	5	2	148				
	6	2	156				
125	1	1	123	124.2	3.16	99.4	-0.6
	2	1	125				
	3	1	124				
	4	2	119				
	5	2	123				
	6	2	131				
100	1	1	102	99.8	3.66	99.8	0.2
	2	1	95				
	3	1	103				
	4	2	104				
	5	2	98				
	6	2	97				
75	1	1	75	75.3	5.15	100.4	0.4
	2	1	73				
	3	1	79				
	4	2	73				
	5	2	81				
	6	2	71				
50	1	1	55	50.8	6.51	101.6	1.6
	2	1	52				
	3	1	53				
	4	2	51				
	5	2	48				
	6	2	46				

B



C

Antibody	Accuracy	Precision	Dilutional Linearity
Anti-CD47 Antibody	100.02%	6.5%	1.000

Figure 4. PathHunter® SIRPα Bioassay shows high accuracy, precision and linearity. A. SIRPα Signaling Bioassay was qualified using a mouse monoclonal anti-CD47 antibody. The experiments were performed in triplicates with six independent experiments by two different analysts, spanning three days, over a potency interval range of 50% to 150%. Data was analyzed using Restricted Mode; 4PL fit in PLA software. B. The average measured relative potency was plotted against the expected relative potency. The assay linearity graph quantifies data generated by both analysts using this bioassay over three days. C. Similar to the PD-1 signaling assay, the SIRPα signaling bioassay qualification testing results demonstrated excellent accuracy, precision, and linearity.

PathHunter SIRPα SIGNALING ASSAY IS STABILITY-INDICATING

Heat-stressed anti-CD47 antibody sample (heat-treated at 65°C for 17 hours) was analyzed to assess if the PathHunter® SIRPα Bioassay is stability indicating. Compared to an untreated reference, the stressed anti-CD47 showed a decreased potency as depicted by a right-shifted response as shown in Figure 5.

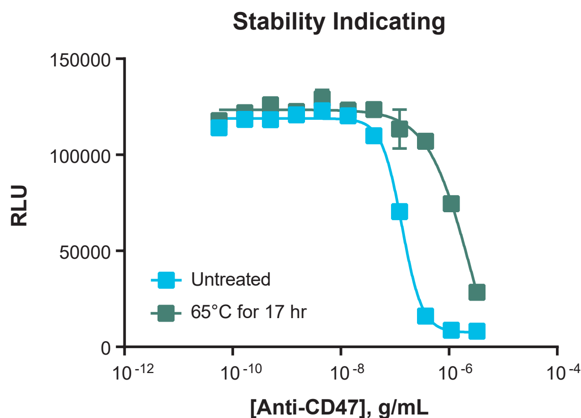


Figure 5. Stability-indicating property of the SIRPα signaling bioassay. Anti-CD47 antibody was subjected to stress by heat-treatment at 65°C for 17 hours. The assay was run with this stressed alongside untreated antibody reference. The assay showed a reduced potency for the heat-treated samples suggesting that the Bioassay is stability indicating.

CONCLUSION

Immune responses are tightly regulated through a balance of co-stimulatory and inhibitory checkpoint receptors, often exploited by cancer cells. For this reason, therapeutics that block inhibitory receptors or activate immuno-stimulatory checkpoint receptors are proving to be powerful agents to restore and strengthen anti-tumor immune responses. However, developing drugs targeting these checkpoint receptors has proved to be very challenging, as *in vitro* cell-based assays needed for screening and characterization for functional drugs are often difficult to create, involve the use of human primary cells, and have long, complicated assay protocols. Here, we present datasets for assays targeting PD-1, and an emerging IO target, the SIRPα/CD47 signaling axis, both of which are clinically relevant co-inhibitory receptors. The PathHunter signaling MOA-reflective, cell-based assays measure receptor activation and signaling using the industry-validated EFC technology. They facilitate the development of relevant therapeutic drugs, enabling rapid and sensitive screening of biologics and small molecules. These assays do not require human primary cells, and provide a highly sensitive response, with an easy-to-use protocol that delivers results in a day.

The data presented here shows excellent assay robustness and reproducibility, thus demonstrating suitability of these assays in QC lot release programs for drug candidates. With their easy-to-use homogenous protocol and chemiluminescent readout, the assays can be read on any standard plate reader and implemented rapidly at labs globally. The assays were qualified using the marketed innovator drug molecules through a multi-day qualification exercise with different analysts, and they were demonstrated to have high accuracy and precision with excellent dilutional linearity over the tested range of 50% to 150%. Additionally, these assays have been developed with frozen ready-to-use cells, which have proven to be advantageous operationally and technically over traditional continuous culture assays.

Biopharmaceutical companies developing biologics and biosimilars are increasingly engaging and partnering with CRO/CDMOs that can support their therapeutic programs with implementation of robust bioassays for the therapeutic. To this end, we have designed the CRO Certification Program, where we organize hands-on workshops at CRO/CDMO sites and provide comprehensive training on our assays. Furthermore, we also provide dedicated end-to-end support for the bioassays for the programs being transferred to CRO sites through a streamlined method transfer process that has already ensured fast and successful implementation.

ADDITIONAL RESOURCES

Whitepaper (discoverx.com/checkpoints-whitepaper): Accelerating Immune Checkpoint Drug Discovery through Functional Cell-Based Assays

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

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)

REFERENCES

1. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol.* 2007; 19(7): 813-824. doi:10.1093/intimm/dxm057
2. ICH Q6B: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products. US Fed. Reg. 18 August 1999: 44928; www.ich.org/ fileadmin/Public_ Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/ Q6B_Guideline.pdf.
3. ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology. US Fed. Reg. 1 March 1995: 11260; www.ich.org/fileadmin/Public_Web_Site/ ICH_Products/ Guidelines/Quality/Q2_R1/Step4/Q2_R1__ Guideline.pdf. This piece was published as a peer reviewed article with the following reference - *BioProcess International*, 2016: 14(1), 36-44.
4. Zhang W, Huang Q, Xiao W, *et al.* Advances in Anti-Tumor Treatments Targeting the CD47/SIRPα Axis. *Front Immunol.* 2020; 11:18. Published 2020 Jan 28. doi:10.3389/fimmu.2020.00018

AUTHORS

Gaurav Agrawal, Ph.D., Manisha Pratap, Dana Haley-Vicente Ph.D., Jane Lamerdin Ph.D.

© 2021 Eurofins DiscoverX, a Eurofins Discovery Company. All Rights Reserved. 21219 042721