Screening for inhibitors of PD-1 and PD-L1 binding with AlphaLISA technology.

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Introduction

One important requirement of a healthy immune system is its ability to distinguish itself from foreign invaders. Under normal physiological conditions, immune checkpoints are necessary to prevent autoimmunity, protecting tissues from damage when the immune system is responding to pathogenic infection. Many tumor cells, which would normally be recognized by T cells and thereby targeted for destruction, have developed ways to evade the host immune system by taking advantage of these pathways. Among the most promising approaches to activating therapeutic antitumor immunity is through the blockade of immune checkpoints. The programmed cell death-1 (PD-1) immune checkpoint pathway is a negative regulator of T-cell immune function. When PD-1 is bound to programmed cell death-ligand 1 (PD-L1), T cell response is suppressed. Inhibitors that block PD-1/PD-L1 complex formation result in increased activation of T-cells and immune system functions, allowing the body's immune system to identify and attack tumor cells. So far, several anti-PD-1 or PD-L1 monoclonal antibodies have been developed to treat a variety of cancers, including non-small cell lung carcinoma (NSCLC), metastatic melanoma and renal cancer. The promise of therapeutically exploiting this pathway has created a need for more robust, straight-forward assays to identify and qualify potential inhibitors which interrupt PD-1/PD-L1 binding.

AlphaLISA technology provides a simple, straightforward method for detecting PD-1/PD-L1 binding in a simple, homogeneous, no-wash format. As shown in Figure 1, biotinylated PD-1 binds to streptavidin-coated Donor beads while HIS-tagged PD-L1 is bound to an Anti-HIS antibody conjugated directly to an
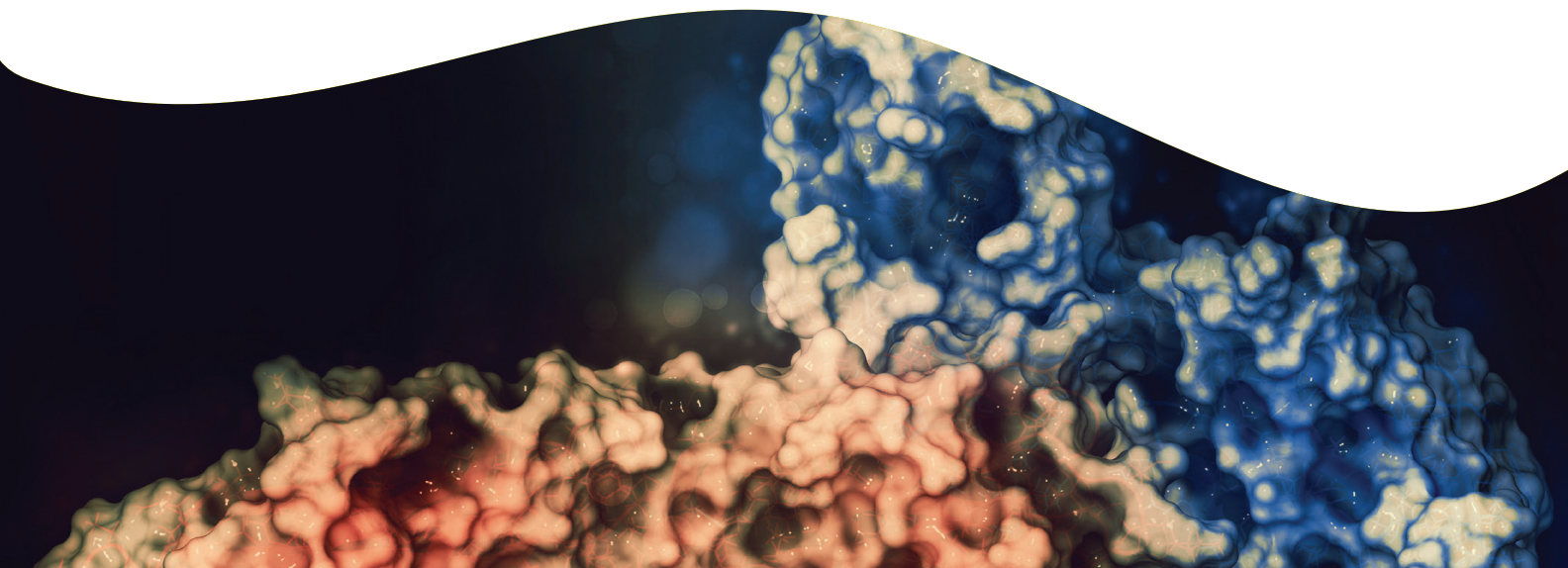
AlphaLISA Acceptor bead. When PD-1 and PD-L1 associate they bring the donor and acceptor beads into close proximity of each other. Upon excitation at 680 nm, the donor beads generate singlet oxygen molecules that diffuse in solution to activate the acceptor beads, which then emit a sharp peak of light at 615 nm. This light emission can then be detected on an Alpha- enabled reader. Alpha assays can easily be automated and miniaturized to increase assay throughput and can be used to detect samples in a variety of matrices. In this application note, we demonstrate the utility of an AlphaLISA assay for screening biotherapeutic antibodies and compounds that block binding of human PD-1 and PD-L1.

Materials and methods

AlphaLISA PD-1/PD-L1 binding assay

The AlphaLISA binding assay for the detection of human PD-1 and PD-L1 binding (#AL356C) was performed according to the recommended protocols provided in the kit manual. Unless otherwise specified, samples to test (drug, blocking antibodies, competitor or compound) were titrated at 12 different concentrations with AlphaLISA ImmunoAssay Buffer (provided in kit, #AL000). Samples were prepared at four times the final concentration and 10 µL of each sample concentration were added to a white 96-well ½-AreaPlate (#6005560). Next, 10 µL each of 20 nM HIS-tagged PD-L1 and 20 nM biotinylated PD-1 were added resulting in a final concentration of 5 nM for each. A 10 µL mixture of 40 µg/mL Anti-6xHIS AlphaLISA-conjugated acceptor beads and 80 µg/mL Streptavidin-coated donor beads were then added and the total reaction (40 µL) was incubated for 90 minutes in the dark at room temperature. To test the efficacy of all antibodies at inhibiting PD-1/PD-L1 binding in our assay, a 12-point titration of each was performed at 4X the final concentration from a top concentration of 10 µg/mL (final assay concentration). Final concentrations for all antibodies tested were converted to nM using the assumed standard MW for each IgG is 150 kDa.

Blocking antibodies, inhibitors, and other reagents

To test the utility of the assay for screening antibodies, a series of known blocking antibodies purchased from a variety of sources were assessed for their ability to block PD-1 binding to PD-L1. The anti-PD-1 blocking antibodies tested were two isotypes of Nivolumab from InvivoGen: IgG4(S228P) (#hpd1ni-mab14) and IgG1 (#hdp1ni-mab1),

and an Anti-human PD-1, mouse IgG1 (Biolegend, #329912). Two non-specific antibodies from InvivoGen, Anti-β-Gal-hIgG1 (#bgal-mab1) and Anti-β-Gal-hIgG4 (S228) (#bgal-mab14) were simultaneously tested as negative controls. Anti-PD-L1 antibodies tested were Atezolizumab (InvivoGen, #hpdl1-mab1), an anti-PD-L1 Neutralizing Antibody Human IgG1 (ACRO Biosystems, #PDL-NA002), and a non-blocking anti-PD-L1 rabbit Mab (Sino Biological, #10084-R015). For the competition assay, recombinant human PD-L1-Fc was purchased from Peprotech (#310-35). Finally, a small macrocyclic inhibitor, PD-1/PD-L1 inhibitor 3 was purchased from SelleckChem (#S8158). In order to examine sample buffer and matrix effects, AlphaLISA Universal Buffer
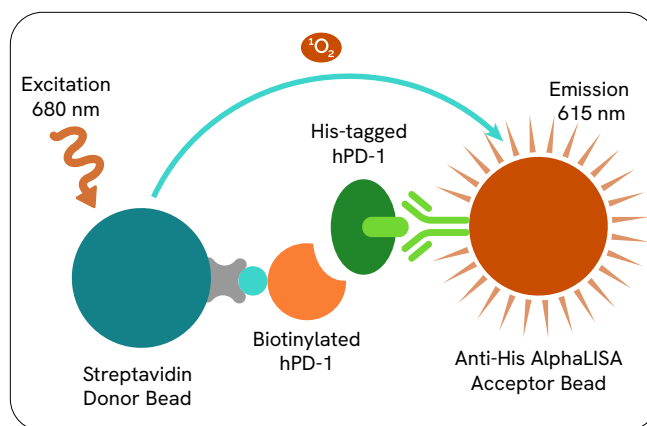

Figure 1. AlphaLISA PD-1/PD-L1 binding assay schematic.

(Revvity #AL001), 1X PBS (HyClone #SH30264.01), and Serum Free Hybridoma media (ThermoFisher #12045084) were tested. For tolerance testing, DMSO from Sigma (#D8418) was used.

Data analysis

Inhibition curves were plotted and IC_{50} values calculated using GraphPad Prism (version 6.0) according to a nonlinear regression using the 4-parameter logistic equation sigmoidal dose-response curve with variable slope. All data shown is the average of triplicate measurements (three wells) unless otherwise specified.

Instruments

For most assays, plates were mixed slowly during the 90 minute incubation on a rotating shaker (DELFIA PlateShake). All AlphaLISA assays were measured on a standard Revvity EnVision® multimode plate reader (Figure 2) using standard settings for Alpha detection. In addition to standard fluorescence, luminescence, absorbance, fluorescence

polarization, and time-resolved fluorescence detection technologies, EnVision Multilabel microplate reader systems also offer fast sensitive Alpha technology detection. They incorporate unique temperature control for sensitive Alpha and AlphaPlex assays, ensuring that experiments are both reproducible and accurate.

Results

PD-1/PD-L1 blocking antibodies

Nivolumab (marketed as the drug Opdivo) is a well-known human IgG4 monoclonal antibody that targets PD-1 and blocks its binding with PD-L1 and has been approved by the FDA to treat melanoma and metastatic non-small cell lung cancer (NSCLC). Two different isotypes are available from

Figure 2. EnVision multilabel plate reader for Alpha detection.

Invivogen that contain the variable region of Nivolumab with either the standard human IgG4 (S228P) constant region or that of human IgG1. Atezolizumab (trade name Tecentriq) is a fully humanized IgG1 (N298A) monoclonal antibody that targets PD-L1 that became the first PD-L1 inhibitor approved by the FDA for treatment of urothelial carcinoma (the most common type of bladder cancer) and is currently in clinical phase trials for treatment of a variety of other cancer types. In addition to these known therapeutics, a mouse IgG1 anti-PD-1 blocking antibody from BioLegend and an anti-PD-L1 blocking antibody from ACRO Biosystems were also examined. The negative control antibodies used in this experiment were Anti-β-Gal antibodies (IgG1 and IgG4 isotypes). To test the efficacy of all these antibodies at inhibiting PD-1/PD-L1 binding in our assay, a 12-point titration of each isoform was performed at 4X the final

concentration. Data and inhibition curves for all antibodies are presented in Figure 3 and blocking antibody potencies were rank ordered in the table below the graph from highest to lowest IC_{50}. The two different IgG isotypes of Nivolumab gave highly comparable results in the assay, though they also showed steepest slopes. Anti-PD-L1 antibodies appeared to give the lowest IC_{50}s and the most desirable Hill slopes. Both negative control antibodies demonstrated no specific activity on the binding interaction.

Non-Blocking antibodies do not interfere with PD-1/PD-L1 binding

In order to ascertain that non-blocking antibodies will not be detected by the assay, the effect of an Anti-PD-L1 specific rabbit Mab that binds to PD-L1 but does not block the binding site with PD-1 was assayed. A titration of this antibody was run alongside Nivolumab and Atezolizumab and the inhibition curves are shown in Figure 4. The non-blocking antibody to PD-L1 does not inhibit the binding to PD-1 in our assay. IC_{50}s (and Hill slopes) for Nivolumab and Atezolizumab were 757 pM (-2.99) and 326 pM (-1.58) respectively, which is consistent with the previous experimental data shown in Figure 3.

Competition with untagged human PD-L1 and a small compound inhibitor

To show the assay utility with samples other than blocking antibodies, a competition assay with untagged-human PD-L1 and a small compound inhibitor were performed. For the competition assay, recombinant human PD-L1 Fc (extracellular portion of PD-L1 fused to an Fc portion of human IgG1) was titrated into the microplate and then HIS-tagged human PD-L1 was added before the addition of biotinylated PD-1 so both ligands had equal time to compete for binding. The data from this competition assay are presented in Figure 5A and the IC_{50} value for untagged-hPD-L1 was 19.22 nM with a Hill slope of -1.003.

Though many screening programs are searching for compounds to inhibit this binding interaction, few molecules are available commercially. One macrocyclic inhibitor reported to demonstrate highly efficacious binding to PD-L1 was tested in this assay (Figure 5B). The IC_{50} value for the compound was 440 nM with a Hill slope of -1.239. These data illustrate the utility of this assay for small molecule screening.
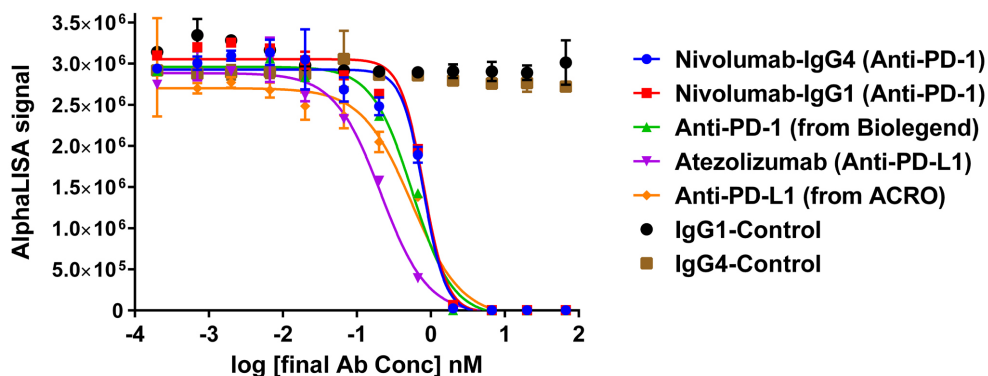
Blocking Antibody	IC_{50} (nM)	Log IC_{50}	Hill Slope	Max	Min	S:B
Nivolumab - Anti-PD1 IgG4 (S228P)	0.799	-0.097	-2.91	2,940,077	2,768	1,062
Nivolumab - Anti-PD-1 IgG1 Isotype	0.801	-0.096	-2.77	3,098,853	2,703	1,146
Anti-PD-1 IgG from Biolegend	0.561	-0.251	-1.6	2,912,277	2,136	1,363
Anti-PD-L1 from ACRO Bioscience	0.551	-0.259	-1.29	2,956,294	1,645	1,797
Atezolizumab - Anti-PD-L1	0.211	-0.675	-1.38	2,743,877	1,738	1,579

Figure 3. Inhibition curves with known blocking antibodies. A panel of antibodies were tested using a 12-point titration for their ability to block the PD-1:PD-L1 binding interaction. Binding inhibition is represented as a decrease in AlphaLISA signal and inhibition curves for seven antibodies are all graphed here. The table lists IC_{50} values for each blocking antibody with antibodies rank ordered top to bottom from highest to lowest IC_{50}.
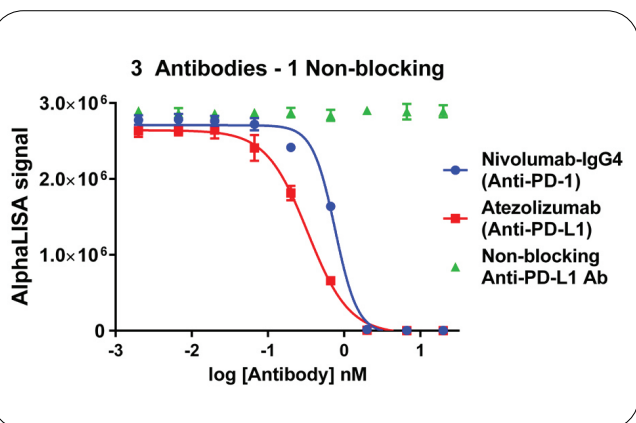

Figure 4. Non-blocking Antibody to PD-L1 does not inhibit binding. An antibody that binds PD-L1 but is known to not block binding to PD-1 (green triangles) was assayed alongside Nivolumab and Atezolizumab.

Assay variability and DMSO tolerance

To illustrate robustness of this assay, variability was assessed using three concentrations of an anti-PD-1 antibody (from Biolegend) as described: 24 wells were treated with either 2 µg/mL for achieving 100% inhibition (IC_{100}), 0.2 µg/mL to achieve 80% inhibition (IC_{80}) and 0.0841 µg/mL to give 50% inhibition (IC_{50}). Data are

graphically represented in Figure 6A and the variability calculations for IC_{80} and IC_{100} are presented in the table below. The z' value calculated for this assay was 0.76 indicating the assay is robust and suitable for screening.

To assess tolerance of this assay for DMSO, a common diluent for compound testing, assay buffer was spiked with 40% DMSO and titrated down 12 times by halves. 10 µL of these samples were tested for effect on total signal from the standard binding assay and the data is shown in Figure 6B. Even at the highest concentrations of DMSO (10% final in reaction), the drop in overall signal was no more than 20% with a 3% reduction in signal for 5% DMSO. All other concentrations tested showed no appreciable effect on overall signal.

Sample buffer and media effects

All data presented thus far contained samples diluted directly in 1X AlphaLISA Immunoassay Buffer (25 mM HEPES, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100 and 0.05% Proclin-300) which is provided at 10X in the AlphaLISA kit. In order to examine the effects of different sample buffers on signal and assay performance, samples were directly diluted

in two other common buffers: 1X AlphaLISA Universal Buffer (containing 1X PBS, 0.1% BSA and 0.01% Proclin-300) and 1X PBS. The rest of the assay components were prepared in 1X Immunoassay buffer. Resulting inhibition curves are presented in Figure 7A with the raw counts and calculations in the table below. The data illustrate that, though the highest signal is generated from samples prepared in Immunoassay buffer, other standard buffers may produce comparable or better signal to background windows.

Monoclonal antibodies may be developed and produced in hybridoma cell cultures prior to downstream modifications and purification. To demonstrate this assay is tolerant of such samples, Nivolumab was diluted and titrated directly in a serum free hybridoma media. As illustrated in Figure 7B, the overall signal is reduced, but the IC_{50} curve dynamic range (Hill slope) has improved and the signal to background is still over 250.

These data suggest that our assay can be used to examine antibodies produced in hybridoma cell culture allowing for early or initial assessment prior to purification or buffer exchange steps.

Examining order of addition effects on PD-1 blocking antibody inhibition

The slopes of the inhibition curve for anti-PD-L1 blocking antibodies and compound are within desirable ranges. However, in some cases, the slopes for anti-PD-1 antibodies in this assay are rather steep conferring a small dynamic range. To examine whether the introduction of the blocking antibody's binding partner (i.e., HIS-tagged PD-L1) first would affect the inhibition curve, the order of addition was examined for Nivolumab whereby both isotypes were titrated into two plates. In one plate, biotinylated PD-1 was added first (blue lines, Figure 8) followed by PD-L1 (within 5 minutes). In the second plate, HIS-tagged PD-L1 was added first, followed by PD-1 (red lines, Figure 8).

The order of addition does not appear to appreciably affect the IC_{50}, slope or signal to background for this assay.
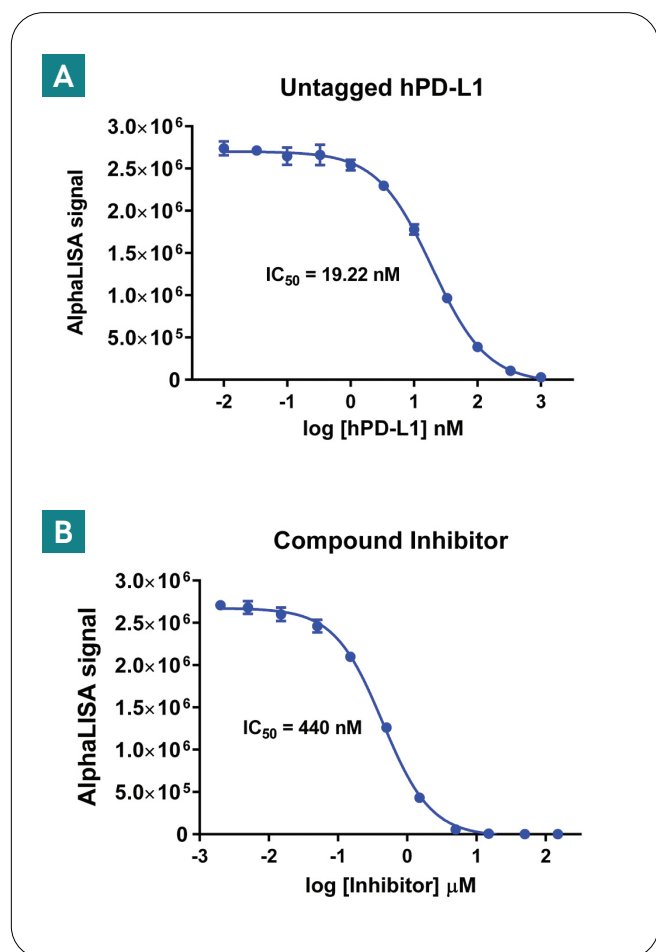

Figure 5. Competition binding curve and compound inhibitor titration. A) Untagged human PD-L1 was titrated at ½ log intervals from a top concentration of 1 µM (final) to generate a competition binding curve with the HIS-tagged PD-L1 in the assay.
B) A macrocyclic peptide inhibitor compound from SelleckChem (PD-1/ PD-L1 Inhibitor 3) was assayed.
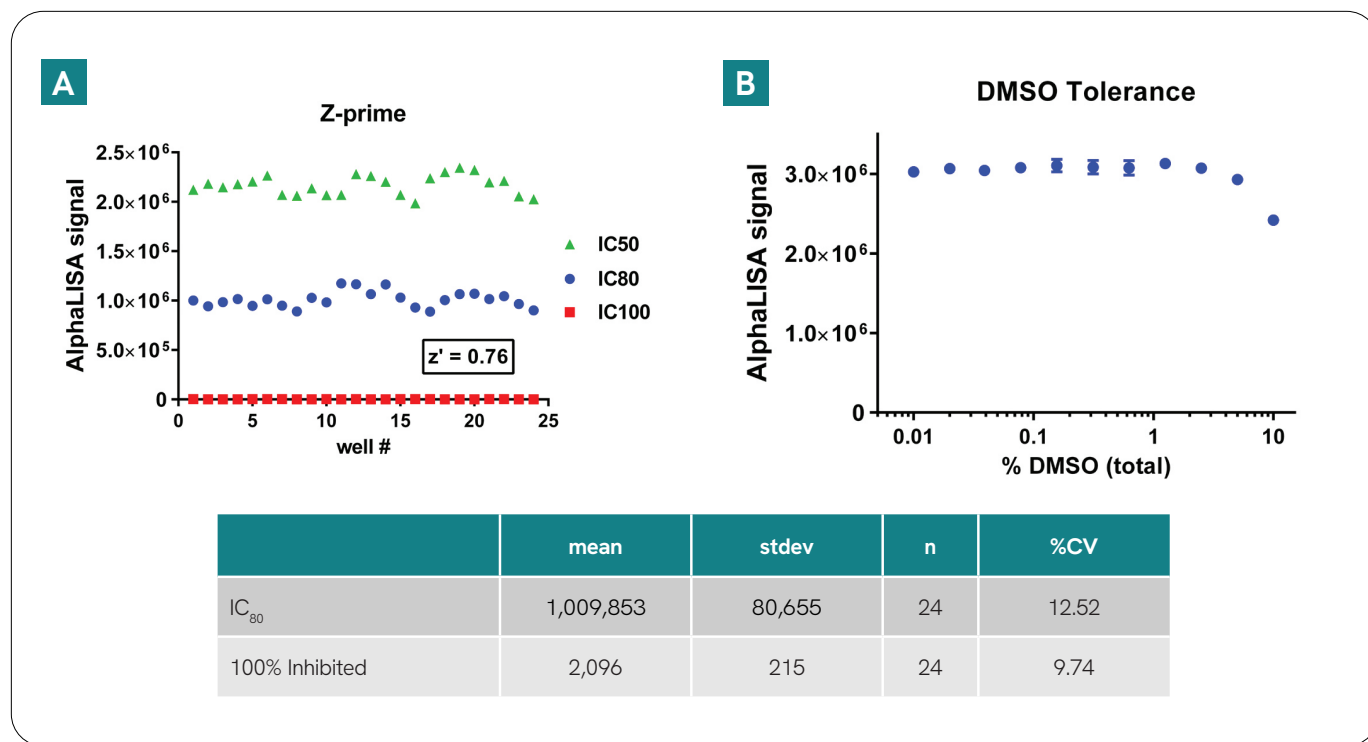
Figure 6. Variability and DMSO tolerance. A) Variability was assessed using the Anti-PD-1 antibody from Biolegend to inhibit binding at calculated IC_{50} and IC_{80} values and at concentrations to achieve complete inhibition in 24 wells per condition. The calculated Z-prime from IC_{80} and IC_{100} treated wells is 0.76. B) The assay is extremely tolerant of DMSO concentration as 10% DMSO final concentration barely affected signal.
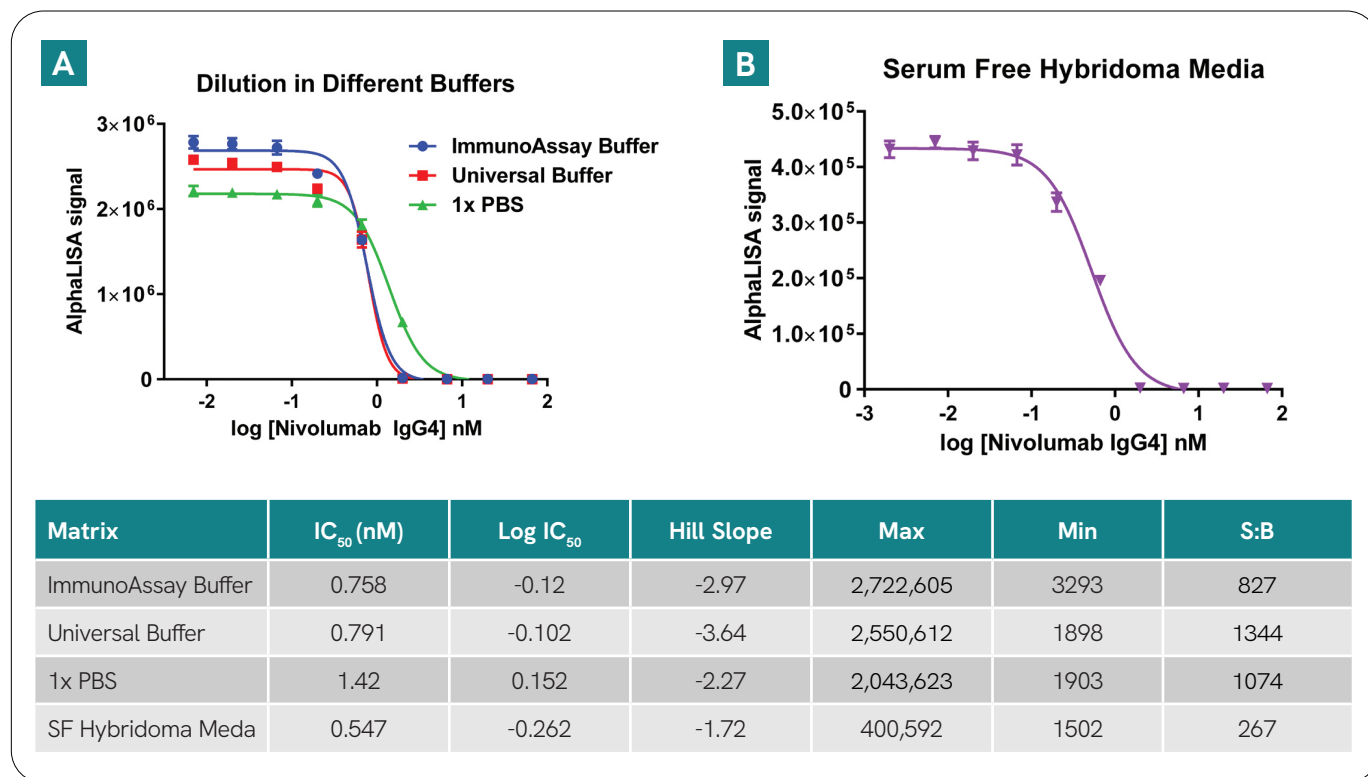

Figure 7. Tolerance for different assay buffers and hybidoma cell culture media. Nivolumab anti-PD-1 blocking antibody titrations were performed in different assay buffers (A) and in a Hybridoma Media (B) and assay performance was assessed and presented in the table.

Titration of PD-1 improves slope of inhibition curve for an anti-PD-1 blocking antibody

One thing to consider when developing an assay for the screening of antibodies is avidity, since IgG molecules contain two binding regions. Avidity, or functional affinity, is the accumulated strength of multiple affinities of individual non-covalent binding interactions. Thus, one antibody could potentially associate with more than one molecule of its binding partner in an assay, affecting the overall binding efficiency and result in a steeper inhibition curve (giving a higher Hill slope). So the steeper inhibition curves observed in our assay for Nivolumab may be due to the drug (anti-PD-1 antibody) binding two times more PD-1, thus blocking binding to PD-L1 with less drug. Since the signal to background of our assay can be as high as 1000X (or greater), it can tolerate reducing the concentration of one of the binding partners in the assay. So we examined the effect

of decreasing the concentration of PD-1 by 5 and 10 times on the inhibition curve of the anti-PD-1 antibody, Nivolumab. The inhibitory effects of titrating Nivolumab (IgG4 isotype) were tested with 5 nM, 1 nM and 0.5 nM of biotinylated PD-1 (final concentrations). Decreasing the concentration of the Nivolumab binding partner PD-1 greatly reduced the assay window (S/B) and decreased IC_{50} values (as expected), as shown in the data in Figure 9. However, it also improved the slope of the inhibition curve drastically, reducing it from -3 to -1. So, if the blocking antibodies being tested are producing inhibition curves with steeper Hill slopes, reducing the concentration of the binding partner in the assay (either PD-1 or PD-L1) can improve the dynamic range and still produce a sufficient assay window.
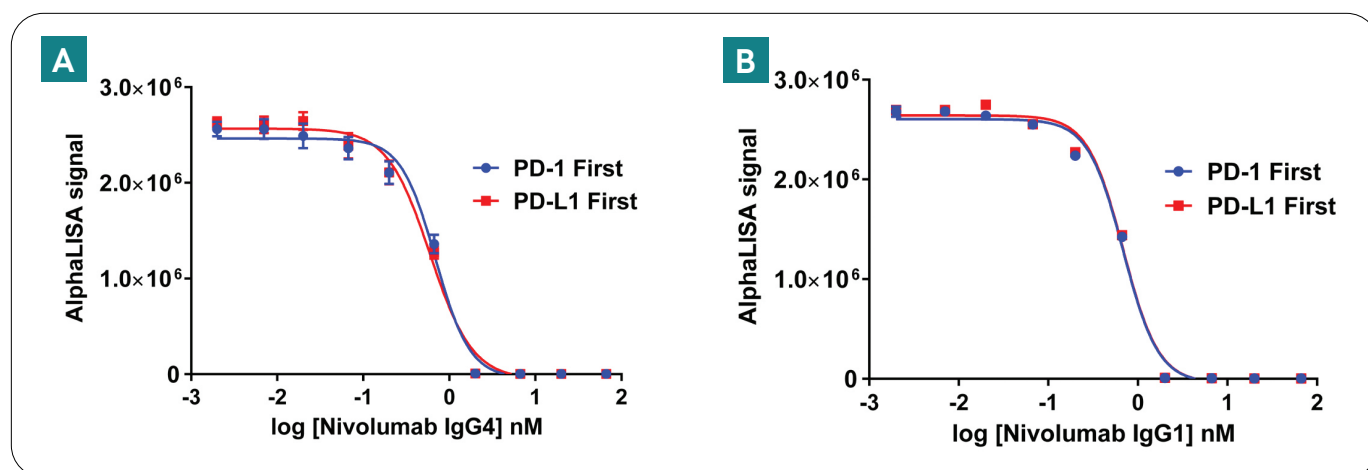

Figure 8. Examining order of addition effect of each binding partner on Nivolumab inhibition curves.
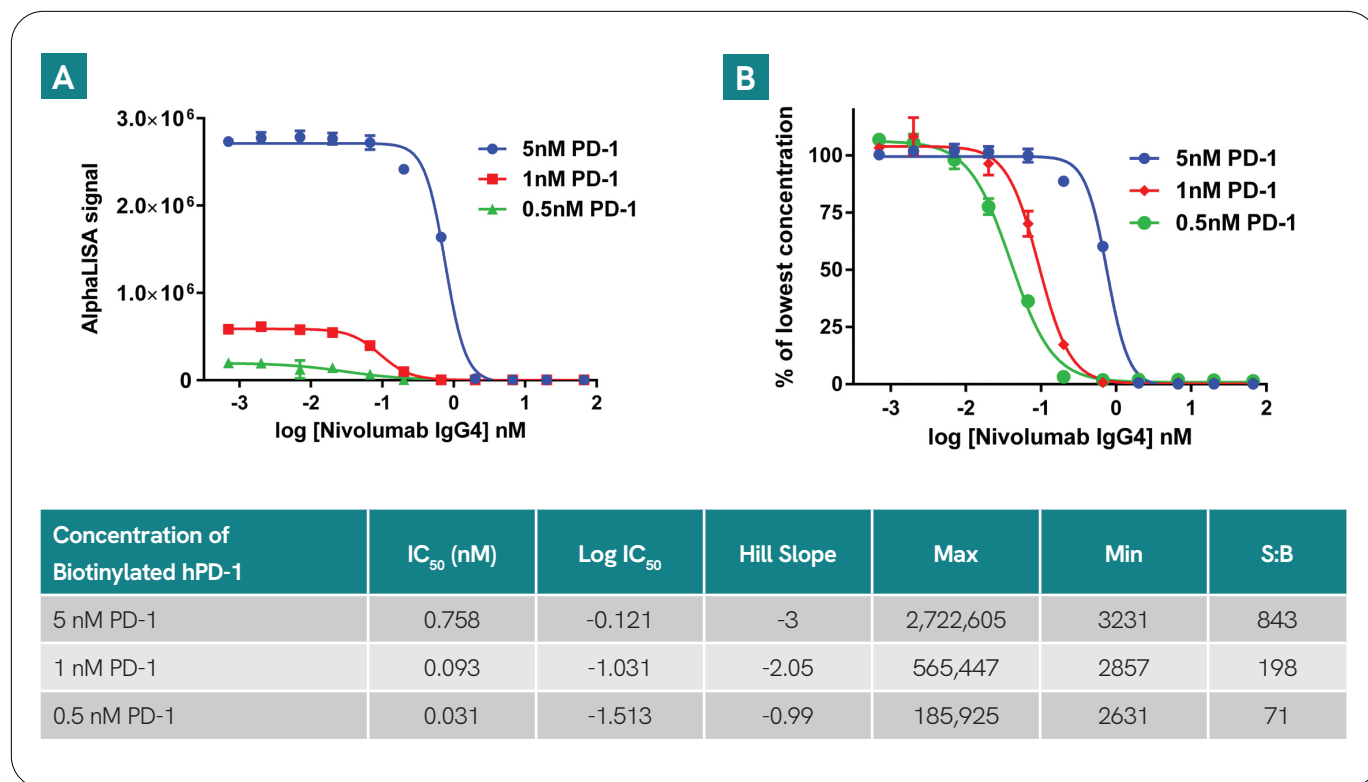
Screening for Inhibitors of PD-1 and PD-L1 Binding with AlphaLISA technology.

Figure 9. Reducing the PD-1 concentration in the assay improves the Hill slope for anti-PD-1 blocking antibodies. Nivolumab (IgG4) was titrated and IC_{50} curves analyzed with differing concentrations of PD-1 in the assay.

Concentration of Biotinylated hPD-1	IC_{50} (nM)	Log IC_{50}	Hill Slope	Max	Min	S:B
5 nM PD-1	0.758	-0.121	-3	2,722,605	3231	843
1 nM PD-1	0.093	-1.031	-2.05	565,447	2857	198
0.5 nM PD-1	0.031	-1.513	-0.99	185,925	2631	71

Conclusions

In this application note, we have demonstrated that AlphaLISA technology can be used to screen for immunotherapeutic antibodies and compounds that block the interaction between the immune checkpoint modulator PD-1 with its ligand PD-L1. A number of different PD-1 and PD-L1 blocking antibodies were titrated and rank-ordered for their potency and ability to block the PD-1 checkpoint pathway. These data illustrate how this assay provides robust and reproducible data, as both antibodies and small molecules were tested across multiple days and generated consistent results. This all-in-one well, no-wash format allows for collecting fast and reliable data from a variety of samples in a high-throughput manner.

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