APPLICATION NOTE

Evaluating the specificity of PD-1 and PD-L1 blocking antibodies using AlphaLISA human and mouse PD-1/PD-L1 binding kits.

Authors

Lauren Berstler
Cat Lautenschlager
Dawn Nida
Jeanine Hinterneder

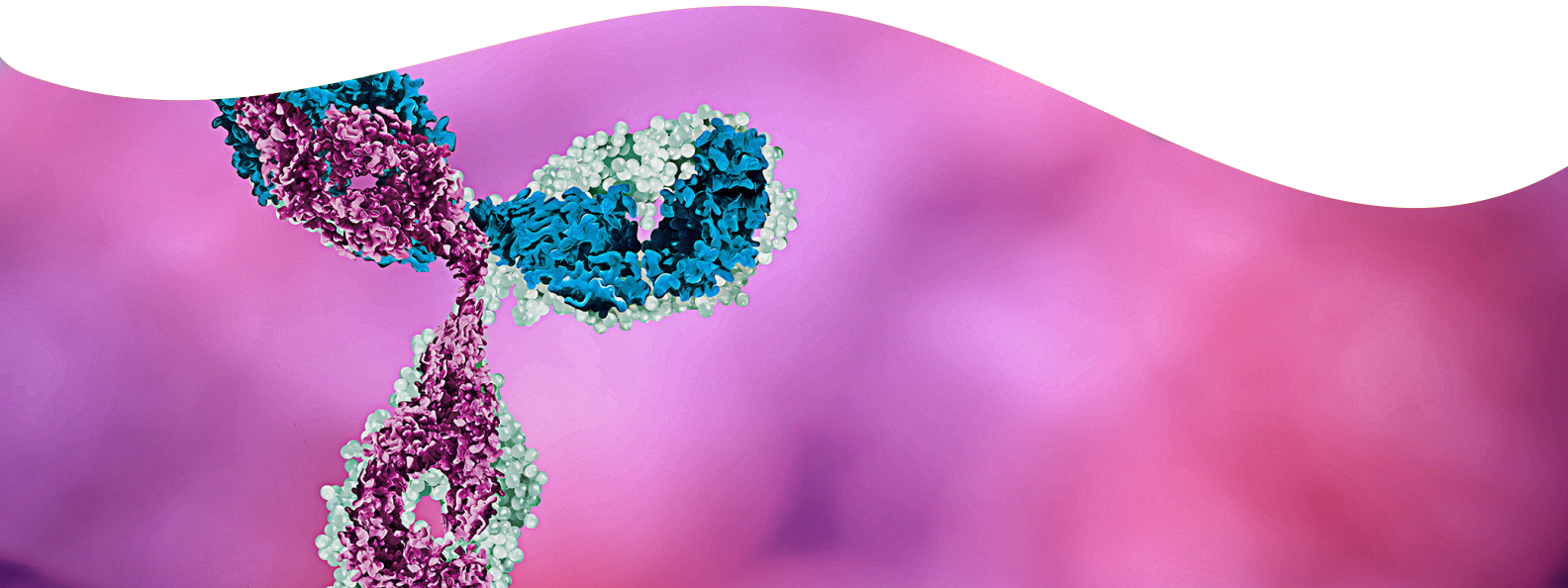
Revvity, Inc.
Hopkinton, MA

Introduction

Cancer immunotherapy utilizes the components of the immune system to treat cancer patients. These therapies are designed to work with a patient's immune system to increase native anti-tumor responses. One type of immunotherapy relies on antibodies to bind to and inhibit the function of proteins expressed by the cancer cell.[1,2] In order to investigate and develop immunotherapies in mice, syngeneic models must be used instead of xenograft models that lack a native immune system and often use human cell lines. Syngeneic mouse models, using tumor grafts derived from immortalized mouse cancer cell lines, allow the study of cancer therapies in the presence of an intact immune system.[3,4] However, working in mouse systems can often require the development of separate mouse reagents, if the therapeutic agent of interest does not cross-react with mouse.

The AlphaLISA™ mouse PD-1/PD-L1 binding kit enables researchers to develop and characterize anti-mouse PD-1 or anti-mouse PD-L1 reagents for *in vitro* and *in vivo* studies (Figure 1). This includes the characterization of putative binding ligands (such as PD-L2) or protein sub-regions for their ability to block the PD-1/PD-L1 binding interaction. Most importantly, this assay allows for the fast and easy characterization of anti-human PD-1 or anti-human PD-L1 blocking reagents for cross-reactivity with mouse PD-1 and PD-L1. The specificity of a therapeutic agent can be directly compared *in vitro* using the AlphaLISA human PD-1/PD-L1 and AlphaLISA mouse PD-1/PD-L1 binding kits and can be used to enable *in vivo* pharmacology studies.

For research use only. Not for use in diagnostic procedures.



Here, we show a number of mouse-specific antibodies (anti-mouse PD-1 and anti-mouse PD-L1) disrupt the mouse PD-1/PD-L1 interaction with the AlphaLISA mouse PD-1/PD-L1 binding kit, but do not affect binding of human PD-1 to human PD-L1. We perform the similar study with human specific antibodies and show their activity in the AlphaLISA human PD-1/PD-L1 binding kit. Further, we demonstrate a subset of antibodies which cross-react and affect both the human and mouse PD-1/PD-L1 interactions. Using a convenient homogeneous assay format, these AlphaLISA assays provide a simple and robust assay alternative to traditional wash-based ELISAs.

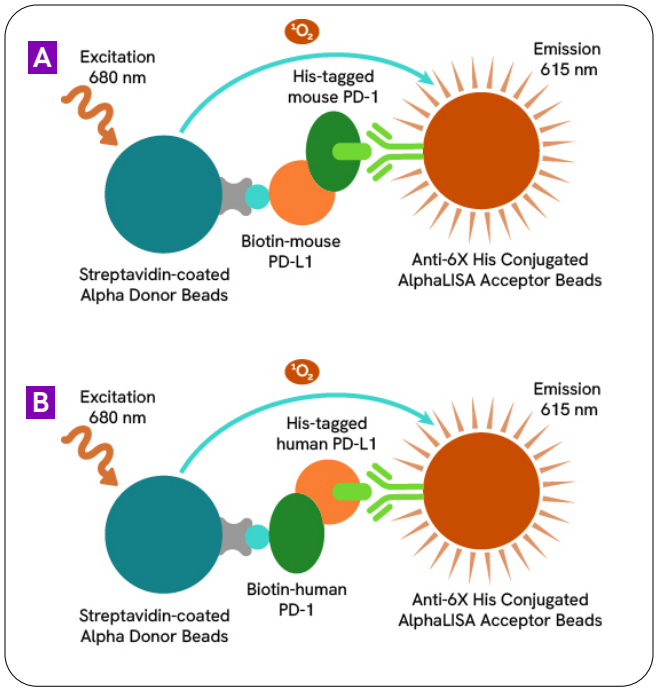


Figure 1. **Assay principle for (A) the AlphaLISA mouse PD-1/PD-L1 binding assay, and (B) the AlphaLISA human PD-1/PD-L1 binding assay.** In the mouse PD-1/PD-L1 binding assay, biotinylated mouse PD-L1 binds to Streptavidin-coated Alpha Donor beads, while His-tagged mouse PD-1 is captured by Anti-6X-His AlphaLISA Acceptor beads. When PD-1 binding to PD-L1 occurs, Donor beads and Acceptor beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm. In the human PD-1/PD-L1 binding assay, biotinylated human PD-1 and His-tagged human PD-L1 are used in combination with the same detection reagents as the mouse kit.

Materials and methods

Reagents

AlphaLISA mouse PD-1/PD-L1 binding kits (#AL580) and AlphaLISA human PD-1/PD-L1 binding kits (#AL356) were from Revvity. All tested blocking antibodies are listed in Tables 1 and 2. Assays were run using a 20 µL final reaction volume (Figure 2) with AlphaLISA Immunoassay Buffer (Revvity, #AL000, provided in each kit) in shallow-well 384-well AlphaPlates™ (Revvity, #6008350). Briefly, 5 µL of sample was distributed to the plate, followed by a 5 µL addition of 4X tagged PD-L1, then a 5 µL addition of 4X tagged PD-1. A 4X mix of Acceptor and Donor beads was then added to the plate under subdued lighting for a final volume of 20 µL. TopSeal-A™ plate seal (Revvity, #6050185) was used to prevent evaporation during the 90 minute incubation step and was removed before reading.

Table 1. Blocking antibodies used for PD-1/PD-L1 binding assays.

Antibody	Isotype	Specificity (provided by supplier)	Supplier
Anti-mouse PD-1	Rat IgG2a, K	Mouse	BioLegend®, #135204
Anti-mouse PD-L1	Rat IgG2b, K	Mouse	BioLegend®, #124304
Anti-mouse CD274 (PD-L1)	Rat IgG2a, K	Mouse	BioLegend®, #153602
Nivolumab-like, Anti-human PD-1-Ni-hIgG1	Human IgG1	Human	InVivoGen, #hpd1ni-mab1
Nivolumab-like, Anti-human PD-1-Ni-hIgG4 (S228P)	Human IgG4 (S228P)	Human	InVivoGen, #hpd1ni-mab114
Anti-human PD-L1	Mouse IgG2b, K	Human	BioLegend®, #329709
Atezolizumab	Human IgG1	Human and Mouse	InVivoGen, #hpdl1-mab1
Anti-PD-L1 neutralizing antibody	Human IgG1, K	Human, cross-reacts with mouse	ACRO Biosystems, #PDL-NA002

Table 2. Control antibodies.

Antibody	Supplier
Rat IgG2a K Isotype Ctrl	BioLegend®, #400515
Rat IgG2b K Isotype Ctrl	BioLegend®, #400621
Anti-ß-Gal-hIgG1	InVivoGen, #bgal-mab1
Anti-ß-Gal-hIgG4	InVivoGen, #bgal-mab14
Mouse IgG1, K Isotype Ctrl	BioLegend®, #401404

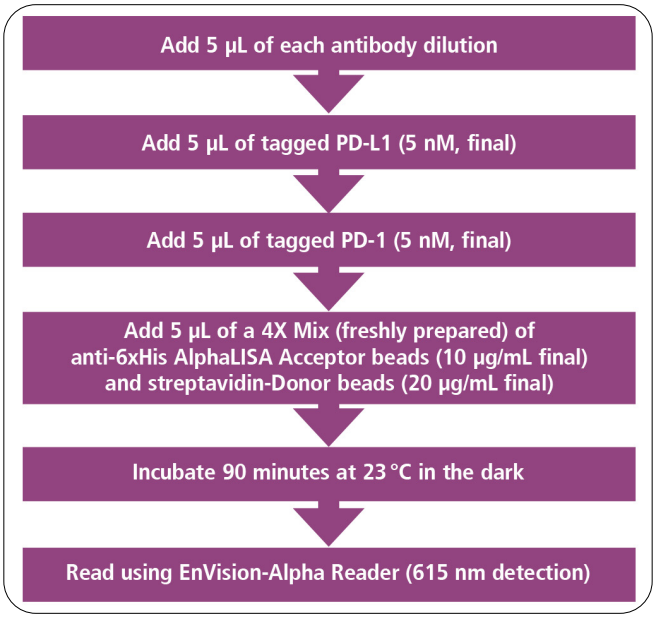


Figure 2. Workflow for no-wash AlphaLISA mouse and human PD-1/PD-L1 binding assays.

Instrumentation

All plates were measured using the EnVision™ multimode plate reader (Figure 3) using standard settings for Alpha detection. The EnVision system offers fast, sensitive Alpha detection technology, in addition to fluorescence, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence measurements.
The incorporation of unique temperature controls within the system ensures accurate, reproducible results for Alpha assays.

Figure 3. EnVision multimode plate reader.

Data analysis

Inhibition curves were plotted and IC_{50} values calculated using GraphPad Prism® (version 7.0) according to a nonlinear regression using the four-parameter logistic equation sigmoidal dose-response curve with variable slope. All data shown is the average of triplicate measurements (three wells) unless otherwise specified.

Results

Characterization of anti-mouse blocking antibodies

We first used the AlphaLISA mouse PD-1/PD-L1 binding kit to characterize commercially available anti-mouse PD-1 and anti-mouse PD-L1 blocking antibodies. Blocking antibodies were titrated from 10 µg/mL to 10 pg/mL (final concentration). Appropriate isotype controls were also run. Each mouse blocking antibody specifically inhibited the mouse PD-1/PD-L1 binding assay (Figure 4). Calculated IC_{50}s for each blocking antibody are indicated in the graphs. The anti-PD-1 antibody was the most potent at blocking the PD-1/PD-L1 interaction with an IC_{50} value of 0.031 µg/mL, whereas the anti-PD-L1 antibodies give slightly higher IC_{50}s at 0.077 µg/mL and 0.098 µg/mL. As expected, isotype controls did not show binding inhibition. The assay gives a robust signal window of 1400.

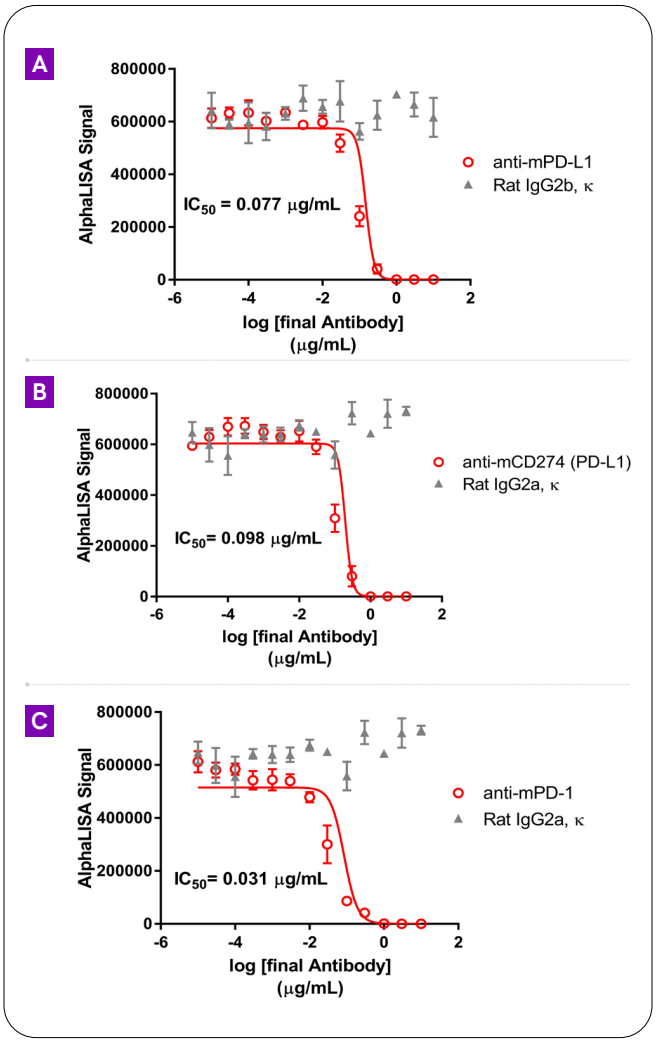
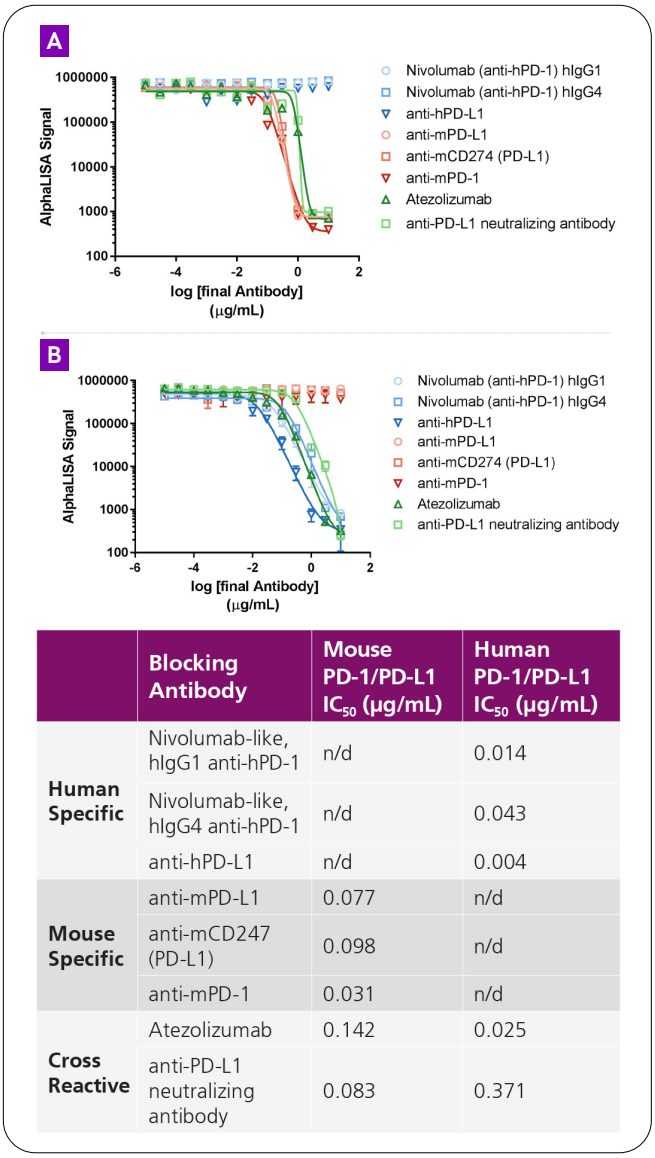


Figure 4. Mouse blocking antibody titration curves. (A) anti-mPD-L1 antibody blocks the PD-1/PD-L1 interaction with a calculated IC_{50} value of 0.077 µg/mL. (B) anti-mPD-L1 antibody blocks the PD-1/PD-L1 interaction with a calculated IC_{50} value of 0.098 µg/mL. (C) anti-mPD-1 antibody blocks the PD-1/PD-L1 interaction with a calculated IC_{50} value of 0.031 µg/mL. For each of these, an appropriate rat isotype antibody was measured as the negative control.

Determination of cross-reactivity of test antibodies using AlphaLISA mouse and human PD-1/PD-L1 binding assays

Next, we used both the AlphaLISA mouse PD-1/PD-L1 binding assay and the AlphaLISA human PD-1/PD-L1 binding assay to assess the cross-reactivity of eight blocking antibodies. For these experiments, three mouse-specific blocking antibodies (anti-mouse PD-1, anti-mouse PD-L1, and anti-mouse CD274/PD-L1), three human-specific blocking antibodies (two Niovlumab-like anti-human PD-1 antibodies and anti-human PD-1), and two blocking antibodies that are known to block both human and mouse PD-1/PD-L1 binding (Atezolizumab and anti-PD-L1) were used. As expected, the mouse-specific blocking antibodies inhibited the AlphaLISA mouse PD-1/PD-L1 binding assay but not the AlphaLISA human PD-1/PD-L1 binding assay (Figure 5A). Human-specific blocking antibodies inhibited the AlphaLISA



	Blocking Antibody	Mouse PD-1/PD-L1 IC_{50} (µg/mL)	Human PD-1/PD-L1 IC_{50} (µg/mL)
Human Specific	Nivolumab-like, hIgG1 anti-hPD-1	n/d	0.014
	Nivolumab-like, hIgG4 anti-hPD-1	n/d	0.043
	anti-hPD-L1	n/d	0.004
Mouse Specific	anti-mPD-L1	0.077	n/d
	anti-mCD247 (PD-L1)	0.098	n/d
	anti-mPD-1	0.031	n/d
Cross Reactive	Atezolizumab	0.142	0.025
	anti-PD-L1 neutralizing antibody	0.083	0.371

Figure 5. Determination of cross-reactivity using (A) AlphaLISA mouse PD-1/ PD-L1 and (B) AlphaLISA human PD-1/PD-L1 binding kits. Mouse specific blocking antibodies are shown in red, human specific blocking antibodies are shown in blue, and cross reactive antibodies are shown in green. Calculated IC_{50}s are indicated in the table. n/d= not determined.

human PD-1/PD-L1 binding assay but not the AlphaLISA mouse PD-1/PD-L1 binding assay (Figure 5B). The two antibodies known to cross-react with both human and mouse proteins were shown to inhibit in both mouse and human assays. The table in Figure 5 shows the calculated IC_{50}s for the blocking antibodies for each assay. Isotype control antibodies were also run in each assay and showed

no inhibition of either the AlphaLISA mouse PD-1/PD-L1 or AlphaLISA human PD-1/PD-L1 binding assays (data not shown).

One advantage of cross-reactivity (especially with similar IC_{50}) is that it is easier to generate preclinical research datasets in support of therapeutic development with the human-specific reagent rather than using a "surrogate" or mouse-specific reagent. While the human and mouse IC_{50}s can vary, trends and threshold potencies for each blocking protein can be evaluated to assess on-target activity. The two cross-reactive blocking antibodies tested in this study showed varying potencies in the human and mouse PD-1/PD-L1 binding assays (Figure 6).

Atezolizumab competes for PD-L1 binding to disrupt the assay signal and shows greater potency in the human PD-1/PD-L1 AlphaLISA assay (0.025 µg/mL human IC_{50} vs 0.142 µg/mL mouse IC_{50} mouse). As Atezolizumab has been optimized and clinically approved for the treatment of non-small cell lung cancer, it therefore is expected to show an increase in binding to the human PD-L1.[2] The anti-PD-L1 neutralizing antibody has the inverse potency order, where it blocks the PD-1 binding with a calculated

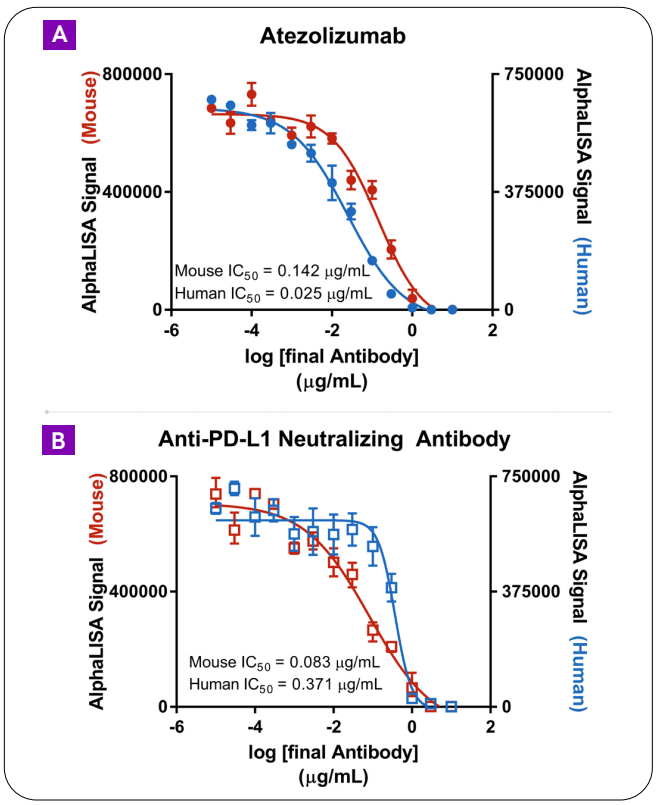


Figure 6. Inhibition of mouse PD-1/PD-L1 and human PD-1/PD-L1 binding by cross-reactive blocking antibodies: (A) Atezolizumab and (B) anti-PD-L1 neutralizing antibody. Data for the mouse PD-1/PD-L1 kit are shown in red (left y-axis), while data for the human PD-1/PD-L1 kit are shown in blue (right y-axis).

IC_{50} of 0.083 µg/mL in mouse compared to 0.371 µg/mL in human. This correlates well with the manufacturer's reported human PD-1/PD-L1 ELISA IC_{50} of 3.75 nM (~ 0.562 µg/mL).

Conclusion

We validated the AlphaLISA mouse PD-1/PD-L1 binding assay by characterizing binding inhibition with commercially available blocking antibodies against mouse PD-1 and PD-L1 proteins. Mouse pharmacological models continue to play a large role in the study of human disease, and mouse tool reagents have shown high utility in immunology and cancer research for decades. It can often be quicker to learn about immunology and the regulation of immune responses using a syngeneic mouse model. Highly valuable *in vitro* mouse PD-1 and PD-L1 binding data can be obtained by using the AlphaLISA mouse PD-1/PD-L1 binding kit which is a convenient, easy-to-use, quick assay to aid in the development of tools for basic research.

We further used the AlphaLISA mouse and human PD-1/PD-L1 binding assays to calculate IC_{50}s for known blocking antibodies. For blocking antibodies with published IC_{50}s, the AlphaLISA results correlated closely with ELISA chemiluminescent results. The AlphaLISA assay offers significant workflow advantages over ELISA assays in that it provides similar sensitivity with no tedious wash or separation steps, and results in less than two hours.

We also used the AlphaLISA mouse PD-1/PD-L1 binding kit in conjunction with the AlphaLISA human PD-1/PD-L1 binding kit to assess blocking antibody cross-reactivity. To qualify potential candidate drugs, pre-clinical testing in appropriate cellular and *in vivo* models is necessary. One advantage of a therapeutic candidate's broad specificity among species is that it is easier to generate preclinical research datasets in support of therapeutic development with the actual human-specific reagent. This limits the need for development of mouse-specific reagents. Determining the specificity with the AlphaLISA human PD-1/PD-L1 and AlphaLISA mouse PD-1/PD-L1 binding assays is a robust and simple approach to obtain binding potencies from potential novel drug candidates.

References

1. Del Paggio JC et al. Immunotherapy: Cancer immunotherapy and the value of cure. Nature Reviews Clinical Oncology (2018); 15: 268-270.
2. Farkona S et al. Cancer immunotherapy: the beginning of the end of cancer? BMC Medicine (2016); 14: 73-90.
3. Barnes, S. The Rise of Syngeneic Models in Cancer Immunotherapy. Drug Discovery & Development (2015).
4. Mosely SI et al. Rational selection of syngeneic preclinical tumor models for immunotherapeutic drug discovery. Cancer Immunology Research (AACR) (2016); 5(1): 1-13.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com