APPLICATION NOTE

Developing a LANCE TR-FRET assay for screening PD-1 and PD-L1 binding inhibitors.

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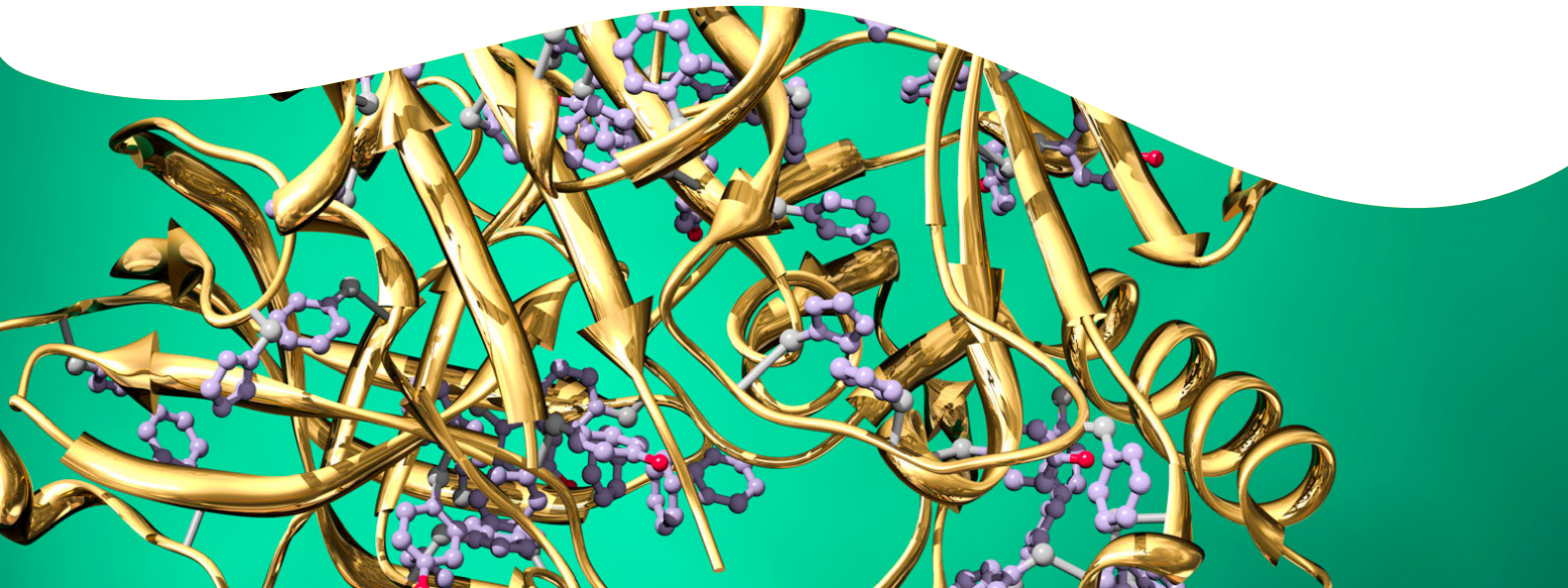
Introduction

The programmed cell death-1 (PD-1) immune checkpoint pathway is a negative regulator of T cell immune function. When PD-1 on T cells is engaged with its ligand, programmed cell death-ligand 1 (PD-L1), it transduces a signal that inhibits T cell proliferation, cytokine production and cytolytic function.[1] Many tumor cells escape anti-tumor immunity through their expression of Programmed Death Ligand 1 (PD-L1 or B7-H1), which interacts with T cell-expressed PD-1 and results in T cell apoptosis. Several anti-PD-1 or PD-L1 monoclonal antibodies have been developed so far to treat a variety of cancers by blocking PD-1/PD-L1 complex formation. However, there remains a need for more rapid, high-throughput assays to identify and qualify novel inhibitors of PD-1/PD-L1 binding. Here, we demonstrate the utility and benefits of using LANCE® *Ultra* TR-FRET assay technology for identifying and characterizing PD-1/PD-L1 binding inhibitors.

LANCE time-resolved fluorescence resonance energy (TR-FRET) technology allows for the detection of molecules of interest in a homogeneous, no-wash format. These assays utilize the unique fluorescent properties of a Europium-based chelate (Eu chelate) as the donor. Eu chelates have high quantum yield, large Stokes shift, a narrow-banded emission, and a long lifetime. These properties make Eu chelates ideal energy donors in TR-FRET assays, as they are less susceptible to interference and reduce background signal. *ULight*™ is a small, light-resistant acceptor dye that has red-shifted emission maximum at 665 nM. In a LANCE protein-protein interaction (PPI) assay, one protein



is labeled, either directly or indirectly, by the donor, and the other by the acceptor fluorophore. If the two proteins interact so that the donor and acceptor fluorophore are brought into close proximity, a signal can be generated.

Using Revvity's LANCE *Ultra* technology, we illustrate how a PPI assay for measuring modulators of PD-1 binding to PD-L1 can be developed quickly and easily. In the assay described here, biotinylated human PD-1 binds to streptavidin-labeled U*Light* while HIS-tagged PD-L1 binds to an Anti-HIS antibody labeled with Europium, thus bringing the Eu and U*Light* into close proximity (illustrated in Figure 1). Upon excitation at 320 or 340 nM, the Eu chelate activates the U*Light* through fluorescence energy transfer (FRET) to emit light at 665 nM. This light emission is detected on a TR-FRET enabled reader. The experiments in this application note demonstrate how LANCE Europium and U*Light* Reagents can be used to develop a fast and easy method for screening inhibitors of the PD-1 and PD-L1 interaction.

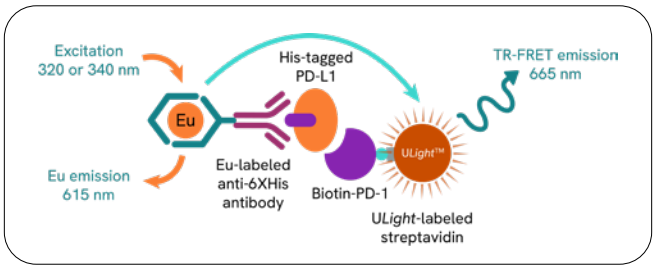


Figure 1: Schematic of the LANCE *Ultra* TR-FRET human PD-1 and PD-L1 binding assay.

Materials and methods

Assay components and biotinylation

His-tagged human PD-L1 (Revvity, #AL356S) is supplied lyophilized and was resuspended at 500 nM with 100 µL of sterile water just prior to use. Recombinant human PD-1 Fc Chimera protein (R&D Systems) was biotinylated with ChromaLink™ biotinylating reagent (Solulink, #B1001-105) using standard biotinylation and purification procedures. Briefly, 0.1 mg of PD-1 and 7.64 µL of biotinylating reagent (2 mg/mL) were mixed together at a 1:8 molar protein/biotin ratio. Purification of the biotinylated PD-1 was performed using a 0.5 mL Zeba desalting column.

The molar ratio of biotin to protein was determined from absorbance readings at 354 nM and 280 nM performed on the DropletQuant system (Revvity, #CLS146933) and calculated to be 1:3.3 (PD-1 : biotin). The concentration of biotinylated PD-1 was adjusted to a stock concentration of 4 µM with PBS pH 7.4 (HyClone, #SH30264.01) and stabilized with 0.05% NaN_3 (Sigma, #13412) and 0.1% Tween-20 (Thermo Fisher, #28320).

LANCE Eu-W1024 Anti-6xHIS (Revvity, #AD0205) and LANCE *Ultra* U*Light*-Streptavidin (Revvity, #TRF0102) were used for detection. All assays were run in white 384-well OptiPlates (Revvity, #6007290).

LANCE *Ultra* TR-FRET assay workflow

The basic protocol used for optimization is illustrated in Figure 2A. The inhibitor compounds tested (drug, blocking antibodies, competitor or compound) were titrated at 12 different concentrations in LANCE *Ultra* Hiblock Buffer (Revvity, #TRF1011). Inhibitors were prepared at four times the final concentration and 5 µL of each concentration was added first to microplate wells. Unless otherwise specified, the next two components added in order were 5 µL each of PD-1 and PD-L1 at 4X the final concentrations needed for the assay. Finally, a 5 µL mixture of Eu-anti-6xHIS and U*Light*-Streptavidin (U*Light*-SA) was added (each at 4X the final concentration) and the total reaction (20 µL) was incubated at room temperature with the plate sealed with TopSeal®-A Plus (Revvity, #6050185) and protected from light with a black microplate lid (Revvity, #6000027).

Blocking antibodies and other inhibitors

To test the utility of the assay for screening antibodies, a series of known blocking antibodies were assessed for their ability to block PD-1 binding to PD-L1 (Table 1).

Table 1: Blocking antibodies and other inhibitors used in LANCE *Ultra* PD-1 and PD-L1 binding assays.

Blocking antibody or inhibitor	Description	Supplier
Anti-hPD1-Ni-hIgG4 (S228P)	Anti-PD-1 blocking antibody (Nivolumab isotype)	*Invivo*Gen #hpd1ni-mab14
Anti-hPD1-Ni-hIgG1	Anti-PD-1 blocking antibody (Nivolumab isotype)	*Invivo*Gen #hdp1ni-mab1
Anti-ß-Gal-hIgG1	Non-specific antibody (negative control)	*Invivo*Gen #bgal-mab1
Anti-ß-Gal-hIgG4 (S228)	Non-specific antibody (negative control)	*Invivo*Gen #bgal-mab14
Anti-hPD-L1-hIgG1	Anti-PD-L1 blocking antibody (Atezolizumab isotype)	*Invivo*Gen #hpdl1-mab1
Anti-PD-L1 Neutralizing Antibody Human IgG1	Anti-PD-L1 blocking antibody	ACRO Biosystems #PDL-NA002
Rabbit Mab to Human PD-L1	Non-blocking anti-PD-L1 antibody	Sino Biological #10084-R015
Recombinant human PD-L1-Fc	Used for competition assay	Peprotech #310-35
PD-1/PD-L1 inhibitor 3	Small macrocyclic inhibitor	SelleckChem #S8158

Instrumentation

LANCE *Ultra* TR-FRET assays were measured using a Revvity EnVision® multimode plate reader (Figure 2B) equipped with a laser using default values for TR-FRET detection for both laser and flashlamp excitation. In addition to fast, sensitive LANCE technology detection, the EnVision reader detects fluorescence intensity, luminescence, absorbance, fluorescence polarization, and AlphaLISA® and AlphaPlex™ assay technologies.

Data analysis

The data generated include two readouts per well as the U*Light* acceptor emission signal at 665 nM and the Eu donor emission signal at 615 nM. This allows the reporting of either the 665 nM signal alone or the calculation of the ratiometric output of both signals. The ratiometric data (signal at 665 nM/615 nM and then commonly multiplied by a factor of 10,000) can help to reduce well-to-well variability that can arise from pipetting error or fluorescent artifacts from compounds and buffer/media additives. Inhibition curves were plotted and IC_{50} values calculated using GraphPad® Prism (version 6.0) according to a nonlinear regression using the four-parameter logistic equation sigmoidal dose-response curve with variable slope. For apparent K_d analysis, titration curves with one binding partner held constant at high concentration and titration of the second binding partner were fitted using the one-site specific binding curve function in GraphPad Prism.

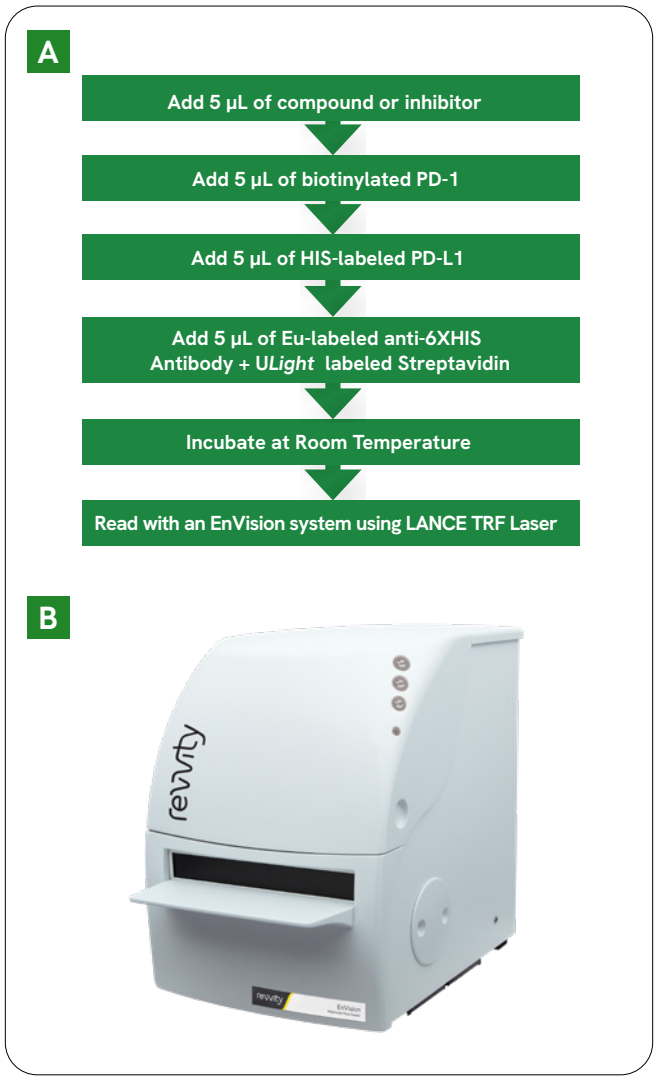


Figure 2: (A) Basic LANCE *Ultra* protein-protein interaction assay workflow. (B) EnVision multimode plate reader.

Results

Protein cross-titration

When designing a LANCE protein-protein interaction assay, the first step is to titrate several concentrations of the two proteins together using concentrations of Eu and U*Light* to optimize the signal to background in order to choose the best concentrations of each protein to use in the final assay.[2,3] The assay we describe was set up with biotinylated PD-1 and HIS-tagged PD-L1 due to the availability of a preexisting source of 6XHIS-labeled PD-L1 (Revvity, #AL356S). This had been verified for binding activity with PD-1 using an alternative assay technology (Revvity's AlphaLISA PD-1/PD-L1 binding kit, #AL356). If starting from scratch, both binding partners would be tagged with each label to test for optimal signal to background.

Several concentrations of PD-1 and PD-L1 proteins were titrated in LANCE *Ultra* HiBlock buffer at 2X dilutions and 5 µL of each were pipetted into microplate wells (in duplicate). Then, 10 µL of a 2X mix of 2 nM (final) Eu anti-6XHIS and 100 nM (final) U*Light*-SA were added to each well and the plate was incubated for 1 hour. The average ratiometric data from this experiment are presented in Figure 3A, illustrating that increasing concentrations of both binding partners increase the average U*Light* signal relative to average Eu signal.

Since we are using excess Eu and U*Light* components, we can determine apparent K_d values for each binding partner by titrating one at saturating concentrations of the other and fitting the normalized (ratiometric) LANCE signals to one-site specific binding curves using GraphPad Prism software. Saturating concentrations of PD-L1 were reached by the three top concentrations tested (shown in Figure 3B) and the calculated apparent K_d values for PD-1 ranged from 20 – 28 nM for these curves. In this same experiment, the apparent K_d calculated from the data PD-L1 titration curve generated with 200 nM PD-1 was 11 nM for PD-L1 (curves shown in Figure 3C). For subsequent experiments, 10 nM PD-L1 and 50 nM PD-1 were chosen as they produced a sufficient signal to background, were below saturating concentrations and were relatively close to K_d values.

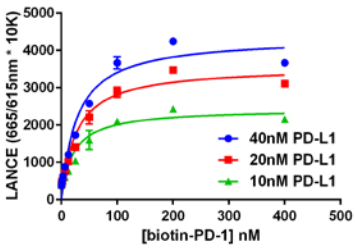
Optimization of Europium and U*Light* concentrations

The first experiment allowed the determination of optimal protein concentrations. As there are approximately three biotins per PD-1 molecule and we are using five times more PD-1 than PD-L1 for our assay, concentrations of U*Light*-SA and Eu-anti-HIS needed to be optimized. Five concentrations of Eu-anti-6XHis and U*Light*-SA were cross titrated and tested for their ability to detect 10 nM HIS-PD-L1 binding to 50 nM biotin-PD-1. Average data from this experiment, presented in tables in Figure 4, illustrate how increasing both Eu and U*Light* increase signal with and without the presence of PD-1 and PD-L1. However, when calculating signal to background, a hook point is observed (as expected with homogeneous assays) around 1 nM Eu and 40 nM U*Light* indicating that detections reagents have reached their optimal concentrations for the assay. Figure 4 also illustrates the differences observed between measuring raw signal (right side) and the normalized LANCE signal (left side). In order to optimize assay signal to background, 1 nM Eu-anti-HIS and 30 nM U*Light*-SA were chosen for subsequent inhibition assays.

A

[HIS-PD-L1] nM	[Biotinylated PD-1] nM											
	400	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0
40	3696	4196	3553	2528	1732	1202	856	641	510	448	389	355
20	3078	3410	2787	2082	1408	1013	759	572	490	439	412	399
10	2158	2456	2104	1419	1041	767	607	508	446	408	409	391
5	1481	1602	1297	957	751	583	517	455	437	428	413	387
2.5	921	963	827	658	565	484	464	430	402	405	409	400
1.25	652	643	578	523	470	444	430	419	434	411	404	411
0.63	541	535	514	475	454	441	423	409	430	403	409	403
0.31	511	485	459	462	426	418	424	415	404	413	405	381
0.16	493	469	454	443	426	414	419	407	392	394	400	395
0.08	488	464	455	432	430	427	412	405	406	404	399	399
0.04	467	470	461	438	441	423	417	436	434	414	407	381
0	461	454	454	442	422	421	417	430	435	408	411	

B



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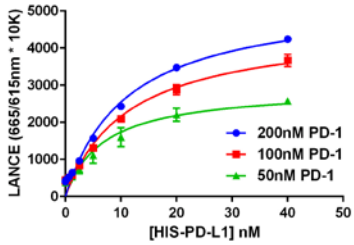


Figure 3: Initial Cross-titration of PD-1 and PD-L1. Several concentrations of each protein were titrated in LANCE *Ultra* HiBlock buffer in a 384-well plate with excess concentrations of Eu and U*Light* and incubated for one hour in duplicate. (A) The resulting ratiometric output data (665 nM/615 nM signal multiplied by 10,000) were averaged and displayed in table format with a heat map showing wells with concentrations producing the highest signals in red. Three top concentrations of PD-L1 and PD-1 were chosen and raw data are presented from (B) PD-1 titration and (C) PD-L1 titration and curves fitted using One-site specific binding curve function in GraphPad Prism.

Normalized Data (665 nm/ 615 nm x 10,000)

	50nM biotin-PD-1 and 10nM His-PD-L1				
	nm ULight				
nM Eu	80	40	20	10	5
4	2993	2575	1945	1442	1164
2	3114	2890	2114	1365	919
1	2752	2839	2283	1438	963
0.5	1828	1865	1602	1011	695
0.25	1823	1831	1581	967	639
	No PD-1 or PD-L1				
	nm ULight				
nM Eu	80	40	20	10	5
4	763	650	520	506	469
2	445	360	296	275	272
1	291	228	197	181	173
0.5	241	187	161	141	136
0.25	224	184	147	136	129
	Signal to Background				
	nm ULight				
nM Eu	80	40	20	10	5
4	3.92	3.96	3.74	2.85	2.48
2	7.00	8.02	7.14	4.97	3.38
1	9.46	**12.46**	**11.61**	7.95	5.55
0.5	7.58	9.95	9.93	7.17	5.10
0.25	8.14	9.95	10.76	7.09	4.97

Raw U*Light* Signal (665 nm only)

	50nM biotin-PD-1 and 10nM His-PD-L1				
	nm ULight				
nM Eu	80	40	20	10	5
4	83343	71754	54518	40308	32529
2	84373	79124	57842	37604	25213
1	66435	70265	56389	36076	24062
0.5	34379	35416	29782	19367	13352
0.25	20521	21809	18598	12212	7876
	No PD-1 or PD-L1				
	nm ULight				
nM Eu	80	40	20	10	5
4	20359	17451	13993	13676	12683
2	11085	9090	7468	6980	6948
1	5841	4716	4117	3808	3668
0.5	3254	2639	2341	2035	2132
0.25	1793	1602	1217	1197	1156
	Signal to Background				
	nm ULight				
nM Eu	80	40	20	10	5
4	4.09	4.11	3.90	2.95	2.56
2	7.61	8.70	7.75	5.39	3.63
1	11.37	**14.90**	**13.70**	9.47	6.56
0.5	10.57	13.42	12.72	9.52	6.26
0.25	11.44	13.61	15.28	10.20	6.82

Figure 4: Optimization of Europium and ULight concentrations. Five concentrations of Eu-anti-6XHis and U*Light*-SA were cross-titrated and tested for their ability to produce the best signal to background with 50 nM biotin-PD-L1 and 10 nM His-PD-L1, compared to control wells containing no PD-1 or PD-L1. Data are presented on the left as ratiometric data where U*Light* signal is normalized to Eu signal and on the right side, only raw U*Light* signal is shown.

Confirmation of assay functionality and optimization of incubation times

In order to determine the ability of this assay to screen for inhibitors of PD-1 and PD-L1 binding, two well-known therapeutic drugs known to bind and block the interaction were tested. Nivolumab (marketed as the drug Opdivo®) is a well-known human IgG4 monoclonal antibody that targets PD-1, blocks its binding with PD-L1 and has been approved by the FDA to treat melanoma and metastatic non-small cell lung cancer (NSCLC).[4,5] Atezolizumab (trade name Tecentriq®) is a fully humanized IgG1 (N298A) monoclonal antibody that targets PD-L1 and was the first PD-L1 inhibitor approved by the FDA for treatment of urothelial carcinoma (the most common type of bladder cancer). Twelve-point titrations of each drug were tested in the assay with final assay concentrations of each component: 50 nM biotin-PD-L1, 10 nM His-PD-L1, 1 nM Eu-anti-HIS, and 30 nM *ULight*-SA. To show stability of the LANCE signal and assay and to discern whether the binding partners had reached equilibrium, the assay plate was read multiple times at one, two, three, and four hours after addition of final assay components and again the next day (after 18 hours incubation). Data from these assays are shown in Figure 5. As the signal appeared to rise significantly after one hour of incubation and the curve shifted, it is apparent that the binding interactions required at least two hours of incubation to reach equilibrium.

Verification of assay specificity

Multiple PD-1 and PD-L1-specific antibodies were tested next to further determine the specificity of this assay for detecting binding inhibitors. Two different isotypes of Nivolumab that contain the normal variable region with either the standard IgG4 (S228P) constant region or that of human IgG1 were tested alongside anti-β-Gal antibodies (IgG1 and IgG4 isotypes as negative controls). 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 25 µg/mL (final assay concentration). Final concentrations of all antibodies tested were converted to nM using the assumed standard MW for each IgG as 150 kDa. Inhibition curves for all antibodies tested are presented in Figure 6 and IC_{50}s were calculated for each blocking antibody (Fig 6C). Both negative control antibodies and the non-blocking anti-PD-L1 antibody demonstrated no specific activity on the binding interaction.

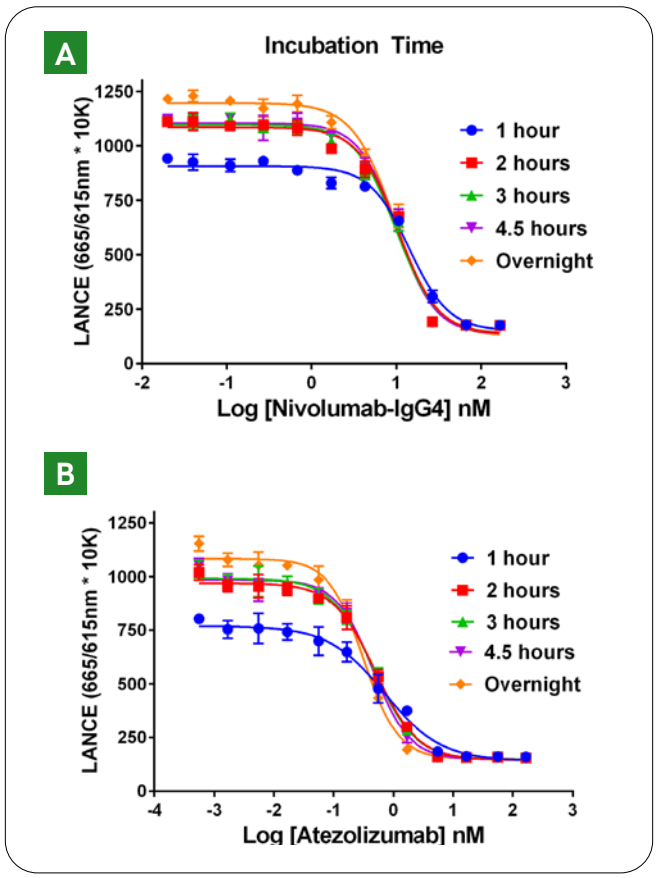
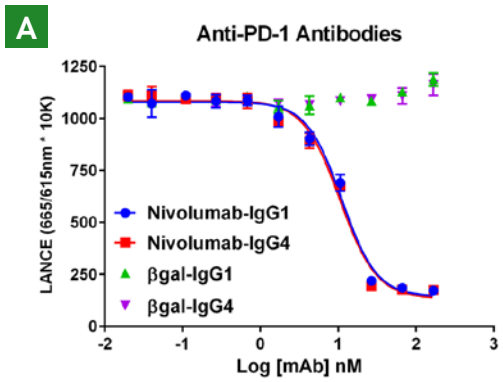
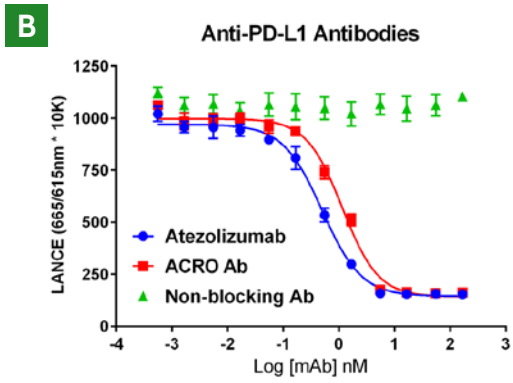


Figure 5: Testing of two different blocking antibodies and multiple incubation times. A) Nivolumab was titrated at 2.5X and B) Atezolizumab at ½ log concentrations. Plates were read over time after 1, 2, 3, 4.5 hours and overnight to determine optimal incubation times.





C

Drug/blocking antibody	IC_{50} (nM)	Hill slope	S:B
Nivolumab (Anti-PD-1) IgG1 Isotype	11.10	-1.93	6.2
Nivolumab (Anti-PD-1) IgG4 (S228P)	10.50	-1.85	6.3
Atezolizumab (Anti-PD-L1)	0.502	-1.23	6.1
Anti-PD-L1 (ACRO Bioscience)	1.157	-1.34	6.2

Figure 6: Testing of multiple blocking antibodies. A) Nivolumab isotypes and negative control antibodies were titrated at 2.5X and B) Anti-PD-L1 antibodies were titrated at ½ log concentrations. C) Inhibition curves were fitted and resulting IC_{50} values for each inhibitor are listed in the table.

To further show the specificity of the assay, 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 7A and illustrate that untagged PD-L1 does compete with HIS-tagged ligand, though the top concentration tested (stock concentration) was not sufficient to completely inhibit the interaction.

Though many screening programs search for compounds to inhibit this binding interaction, few molecules are available commercially. One macrocyclic inhibitor, PD-1/PD-L1 Inhibitor #3 (Selleckchem S8158), reported to demonstrate highly efficacious binding to PD-L1, was also titrated and tested in this assay and the data are presented in Figure 7B. These data illustrate the additional utility of this assay for small molecule screening.

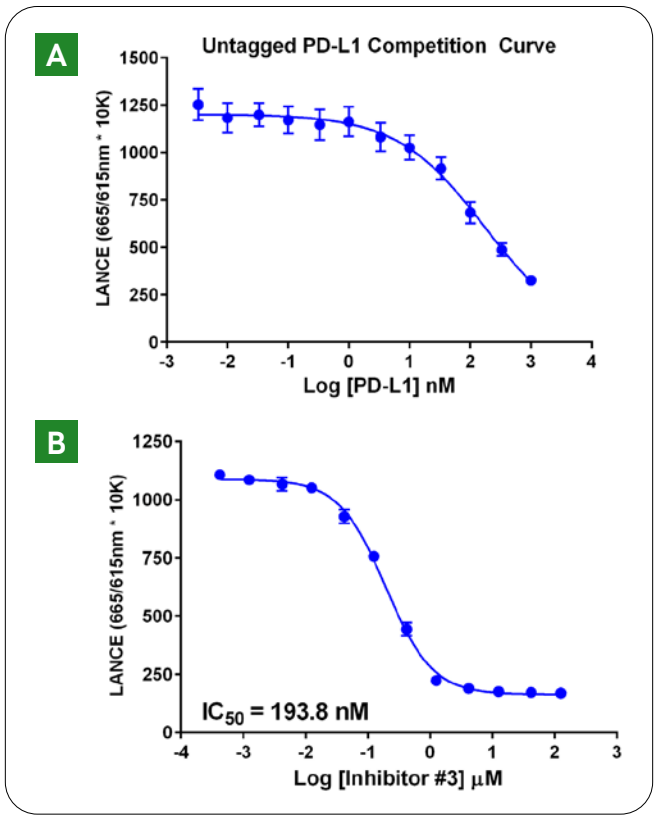


Figure 7: PD-L1 competition binding curve and compound inhibitor titration. A) Untagged human PD-L1 was titrated at ½ log intervals from a top concentration of 1 µM (final) to generate a competition binding curve with the HIS-tagged PD-L1 in the assay. B) A macrocyclic peptide inhibitor compound from SelleckChem (PD-1/PD-L1 Inhibitor 3) was assayed.

Conclusions

In this application note, we describe the development of a fast and easy LANCE *Ultra* TR-FRET assay for measuring inhibition of PD-1/PD-L1 binding in a homogeneous, no-wash format. The data illustrate how the assay rapidly provides robust, reproducible data, as both antibodies and small molecules were examined. LANCE technology provides a straightforward, stable platform for screening large and small molecule inhibitors of PD-1/PD-L1.

References

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