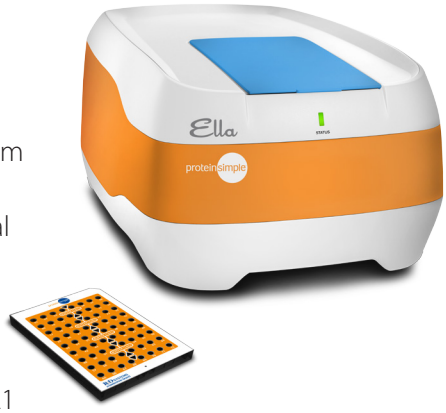
High Fidelity Detection of Endogenous PD-L1 at Low Picogram Levels with Simple Plex Assays

Introduction



Enlisting a patient's own immune system to fight cancer has been a longstanding dream for cancer biologists. Immune checkpoint inhibitors targeting molecules like programmed death-ligand 1 (PD-L1) help make the dream a reality and are now transforming today's cancer therapy. PD-L1 therapy has gained interest as a paradigm shifting approach to cancer treatment due to its durable effects, and its ability to target a broad range of cancers[1,2,3] with manageable toxicity compared to traditional chemotherapy.

Current diagnostic tests for PD-L1 use immunohistochemistry (IHC) to score the tumor microenvironment, but results can be really variable. And traditional, high-quality ELISAs don't always have the sensitivity to detect endogenous levels of PD-L1 in primary patient samples. Ella's Simple Plex™ PD-L1 assay gets rid of these downsides, letting you detect single-digit picogram levels of endogenous PD-L1 with single-digit CVs. Reproducibility at that level means you can have confidence in your data. Plus, you'll only need 25 µL of neat sample and your results are ready in an hour. For these reasons, researchers at the NIH recently used all the advantages of Simple Plex assays to monitor immunotherapy responses in humans.[4]

Simple Plex assays also give you single plex and multiplex options so you can measure levels of just PD-L1 alone or PD-L1 in a panel with three other proteins in your sample.

In this application note, we compare endogenous PD-L1 detection in PTEN mutated glioma cell supernatant and blood cells using the Simple Plex assay and a commercially available sandwich ELISA assay to demonstrate data equivalency and the added sensitivity you'll get with a Simple Plex assay.

Same or better results with the Simple Plex assay

We ran a variety of different cell supernatants with and without treatment known to affect PD-L1 protein levels using a commercially available sandwich ELISA and the Simple Plex PD-L1 assay with Ella™. Both techniques correlated well to each other, with a coefficient of 0.997 (**Figure 1**). 86.7% of the samples showed ≤20% difference in the amount of PD-L1 measured, confirming Simple Plex assays will give you the same answers as a widely accepted ELISA.

However, the Simple Plex assay was clearly more sensitive compared to the commercial ELISA. PD-L1 was measured in only eight of the 15 samples with the ELISA assay and none of the untreated cell supernatant samples. Ella's Simple Plex PD-L1 assay detected endogenous levels of PD-L1 in 14 out of 15 samples, all of which had <28 pg/mL endogenous PD-L1. The last sample was an untreated PBMC sample that presumably had levels of endogenous PD-L1 below the detection limit of both the ELISA and Simple Plex assay. Plus, all sample CVs measured with the Simple Plex assay came in at less than 5.2%. So, you'll get the reproducible data you can trust and a much better shot at detecting low endogenous levels of PD-L1 in your samples with the Simple Plex assay.

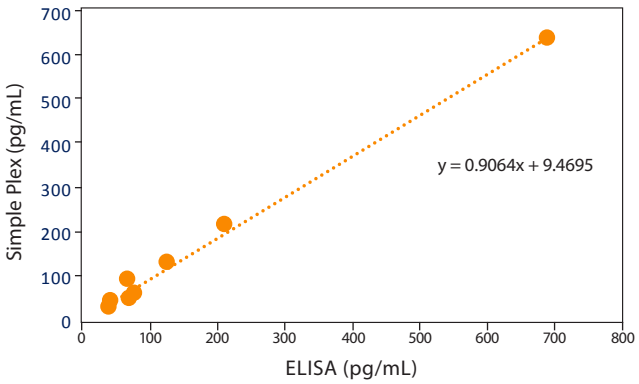


FIGURE 1. Measured levels of PD-L1 in glioma cell supernatant, PBMC, and HDLM-2 samples correlated very well between Simple Plex and ELISA assays with a correlation coefficient of 0.997. 86.7% of the samples showed <20% difference in PD-L1 detected.

SIMPLE PLEX ASSAYS DETECT LOWER LEVELS OF PMA-INDUCED PD-L1 EXPRESSION IN GLIOMA CELL SUPERNATANT

We looked at PD-L1 levels in PTEN-mutated Glioma cell supernatant. U87-MG, U251-MG, and A172 cells were treated with PMA to increase PD-L1 protein levels. Cells were grown until they were >90% confluent. They were then treated with 60 nM PMA overnight for 18 to 24 hours. Supernatant was collected the next day and centrifuged at >500 x g for 5-10 minutes before aliquoting and storing at -20 °C.

Aliquots of the frozen cell supernatant were run using a commercially available ELISA assay according to insert instructions. 100 µL of supernatant was used to run each sample, so a total of 200 µL of supernatant was needed to generate duplicate data.

An aliquot from the same frozen cell supernatant was also run with the Simple Plex PD-L1 assay on Ella. Supernatant was diluted a minimum of 2-fold with Sample Diluent by adding 30 µL of sample to 30 µL of sample diluent. Next, 50 µL of the diluted sample was added to the Simple Plex 72x1 PD-L1 assay cartridge (SPCKB-PS-999452). Cartridges come with a factory-set standard curve so we didn't have to run a standard curve — the built-in standard curve loads when the cartridge barcode is scanned right before it is placed into the Ella instrument to be run. During the run, each sample automatically generates three data points so you use 10X less sample with the Simple Plex assay too. With the conventional sandwich ELISA assay, you have to run three separate 100 µL samples to generate triplicate data.

The sandwich ELISA was only able to detect endogenous levels of PD-L1 in untreated U87-MG cell supernatant as levels in the U251-MG and A172 cell supernatants were too low to be detected (**Table 1**). We found that the lower limit of detection for the PD-L1 ELISA kit fell somewhere around 28 pg/mL. With the Simple Plex PD-L1 cartridge, Ella was able to detect endogenous levels for all untreated cell samples. The assay even detected PD-L1 in A172 cells, which had the least amount of endogenous PD-L1 at 7.4 pg/mL, 3X higher than the reported Simple Plex PD-L1 assay lower limit of quantitation at 2.35 pg/mL. The data also correlated well between the two assays. Only one sample, the U251-MG + PMA had a slightly higher percent

	ELISA		SIMPLE PLEX	
	VALUE (pg/mL)	% CV	VALUE (pg/mL)	% CV
U87-MG control	77.6	5.9	63.3	1.0
U87-MG + PMA	124	1.3	132	6.8
U251-MG control	ND	--	27.8	1.2
U251-MG + PMA	65.9	4.1	94.2	1.1
A172 control	ND	--	7.4	3.4
A172 + PMA	40.3	0.9	44.5	1.1

TABLE 1. ELISA and Simple Plex assays both measured similar concentrations of PD-L1 in glioma cell supernatant reproducibly with all CVs <6.8%. The Simple Plex PD-L1 assay detected endogenous PD-L1 in all samples tested while the ELISA assay measured protein in all but two untreated samples (ND = not detected).

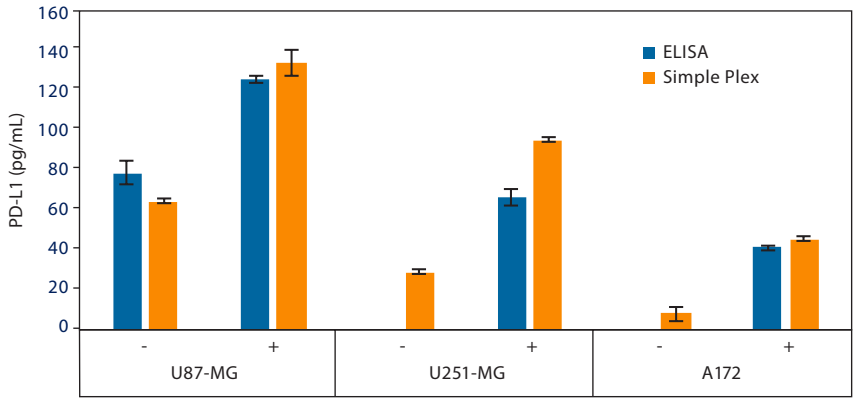


FIGURE 2. Traditional sandwich ELISA and Simple Plex assays both detect similar fold increases in PD-L1 expression after PMA treatment. The Simple Plex assay detected PD-L1 in all samples but the traditional ELISA didn't detect PD-L1 expression in control samples below its LLOQ

difference. That result could have been caused by two different operators running the sandwich ELISA versus the Simple Plex assay, potentially leading to the additional variability between the two techniques. Our result s also showed that both assays were very reproducible with ELISA assay CVs all under 5.9% and Simple Plex assay CVs all under 6.8%. As an added bonus, Simple Plex assay CVs for the untreated glioma cell supernatant samples were all under 3.4%, even at this 7.4 pg/mL level.

As expected, PD-L1 was increased in all samples treated with PMA (**Figure 2**). All samples showed between a 2 to 6-fold increase in PD-L1 protein levels after PMA treatment based on the Simple Plex assay results. There was also really good correlation between the ELISA and Simple Plex assays in the U87-MG supernatants with a 1.6- and 2.0-fold increase in PD-L1 expression, respectively.

SIMPLE PLEX ASSAYS DETECT PD-L1 IN MORE PBMC AND HDLM-2 SAMPLES

We also looked at PD-L1 levels in peripheral blood mononuclear cell samples (PBMCs) and HDLM-2 cells, a Hodgkin's lymphoma cell line. To obtain the PMBCs, blood was collected from apparently healthy donors

	ELISA		SIMPLE PLEX	
	VALUE (pg/mL)	% CV	VALUE (pg/mL)	% CV
PBMC	ND	--	ND	--
PBMC + IFNγ	ND	--	20.8	3.8
PBMC	ND	--	12.8	0.5
PBMC + IFNγ + LPS	39.0	9.6	35.4	1.4
PBMC	ND	--	9.9	2.1
PBMC + IFNγ + LPS	69.4	0	52.8	2.5
HDLM-2	210	0.7	216	0
HDLM-2 + PMA	691	2.9	631	0.3

TABLE 2. ELISA and Simple Plex assays both measured similar concentrations of PD-L1 in blood samples reproducibly with CVs for the ELISA assay <9.6% and CVs for the Simple Plex assay <3.8%. The Simple Plex PD-L1 assay detected endogenous PD-L1 in all samples except for one untreated PBMC sample. The ELISA kit only detected PD-L1 in treated PBMC samples and both treated and untreated HDLM-2 samples (ND = not detected).

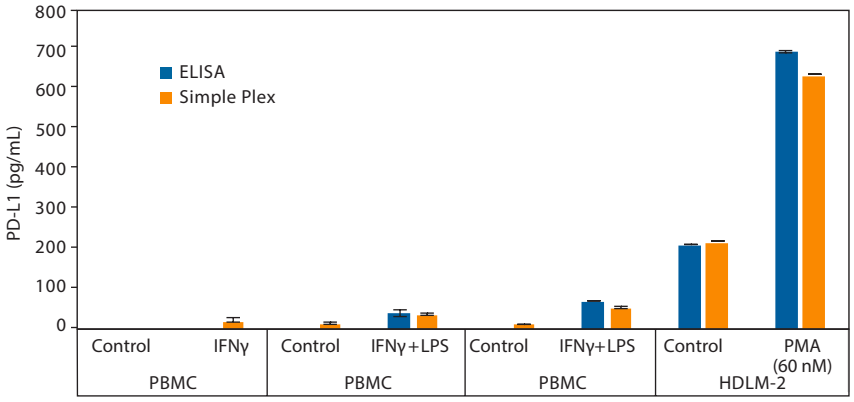


FIGURE 3. ELISA and Simple Plex assays both detect similar fold increase in PD-L1 expression after PMA, IFNγ, or IFNγ + LPS treatment. The Simple Plex assay detected PD-L1 in all but one control sample but the ELISA assay didn't detect PD-L1 expression below its LLOQ.

into heparinized tubes. PBMCs were isolated using Ficoll density gradient centrifugation by mixing the whole blood 1:1 with PBS, and then layering it on top of 20 mL Ficoll-Paque PLUS. The sample was then centrifuged 30-40 minutes at approximately 400 x g to isolate the PBMC layer. After lysis of red blood cells, PBMCs were counted and plated into two 150 mm dishes in 25 mL of media (RPMI + 10% FBS) at a concentration of 2 x 10^6 cells/mL. Cells were then treated with either 40 ng/mL rhIFNγ (R&D Systems, #285-IF/CF) overnight for 18-24 hours or 100 ng/mL IFNγ + 1 µg/mL LPS for 48 hours.

HDLM-2 cells were seeded in 25 mL of media (RPMI + 10% FBS) in T175 flasks and grown for several days until they reached exponential growth before treating with 60 nM PMA for 18-24 hours.

Samples were run using a commercial ELISA assay according to insert instructions. 100 µL of supernatant was used for each sample, so a total of 200 µL supernatant was needed to generate duplicate data. Samples run with the Simple Plex PD-L1 assay cartridge on Ella were diluted 1:1 by mixing 30 µL of sample with 30 µL of Sample Diluent. 50 µL of diluted sample was then applied to one well in the Simple Plex 72x1 PD-L1 cartridge, which generates triplicate results.

The ELISA assay didn't detect endogenous levels of PD-L1 in any of the untreated PBMC samples, while the Simple Plex assay detected endogenous levels of PD-L1 in three of the four untreated PBMC samples (**Table 2**). Both the ELISA and Simple Plex assays detected increases in PD-L1 protein levels after treatment with either IFNγ, IFNγ + LPS, or PMA (**Figure 3**). There was a 2-fold increase in PD-L1 expression in most samples except for one set of PBMC cells treated with IFNγ and LPS that increased PD-L1 expression by 5-fold. And in the HDLM-2 samples, both the ELISA and Simple Plex assays showed a 3.3- and 2.9-fold increase in PD-L1 protein levels, respectively.

Conclusion

Ella's Simple Plex assay is ideal for detecting critical targets like PD-L1 from precious human samples and cancer cell lines. The Simple Plex PD-L1 assay can measure endogenous PD-L1 at low picogram per mL levels in low volumes with single-digit CVs — so you can trust the results of your assays. When comparing validated traditional ELISA assays with Simple Plex assays, we saw very good correlation between the two techniques. In the group of samples where PD-L1 was detected, 86.7% of them had less than a 20% difference, and both techniques measured similar increases in PD-L1 after treatment with PMA, IFNγ, or IFNγ + LPS demonstrating the high degree of data equivalency between the two techniques.

The big difference was that our results consistently showed the Simple Plex PD-L1 assay was more sensitive. The ELISA assay only detected endogenous PD-L1 in half of the untreated glioma cell supernatant samples we ran, and detected nothing in the untreated PBMC

samples. On the other hand, the Simple Plex PD-L1 assay detected endogenous PD-L1 in all untreated glioma cell supernatants tested, and in all but one of the untreated PBMC samples. Our experiments also showed the lower limit of quantitation for the Simple Plex PD-L1 assay was lower than the commercial ELISA assay, coming in at 2.35 pg/mL. CVs for both assays were in the single-digit range at <9.6% for the traditional ELISA and <6.8% for the Simple Plex assay. And it has built-in triplicates for each sample, so it also uses 3X less sample than if you ran duplicates with ELISA.

The Simple Plex PD-L1 format gives you the same specificity of an ELISA with far better sensitivity and dynamic range. Plus you can make decisions a lot faster because your data is available in an hour.

References

1. Costimulatory molecule B7-H1 in primary and metastatic clear cell renal cell carcinoma, RH Thompson, MD Gillett, JC Cheville, CM Lohse, H Dong, WS Weber, L Chen, H Zincke, ML Blute, BC Leibovich, ED Kwon, *Cancer*, 2005; 104 (10): 2084-91.

2. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways, BT Fife, JA Bluestone, *Immunol Rev*, 2008; 224:166-82.

3. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD28+ T cells, S Spranger, RM Spaapen, Y Zha, J Williams, Y Meng, TT Ha, TF Gajewski, *Sci Transl Med*, 2013; 5 (200):200ra116.

4. Improved reproducibility and quality of the GvHD biomarker assay: application of multiplex microfluidic channel system, P Anandi, X Tian, F Chinian, CR Cantilena, N Dunavin, N Hensel, D Draper, E Koklanaris, S Maxwell, J Superata, P Muranski, M Battiwalla, S Paczesny, AJ Barrett, S Ito, *Bone Marrow Transplant*, 2016; doi: 10.1038/bmt.2016.226.

proteinsimple a bio-techne brand

Toll-free: (888) 607-9692
Tel: (408) 510-5500
info@proteinsimple.com
proteinsimple.com

94-3002-00, Rev A