Product Information & ELISA Manual

Human PD-L1 ELISA Kit (Colorimetric)
NBP3-43469

Enzyme-linked Immunosorbent Assay
for quantitative detection.

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Novus kits are guaranteed for 6 months from date of receipt.

For research use only. Not for diagnostic or therapeutic procedures.

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1. Intended Use

The Human PD-L1 ELISA Kit (Colorimetric) is to be used for the *in vitro* quantitative determination of human PD- L1 in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

PD-L1, (also known as CD274 or B7-H1), is an immuno-coinhibitory member of the B7 family and functions as an immune checkpoint via its interaction with its receptors PD-1 (CD279) and CD80 (also known as B7.1) (1). PD-L1 is expressed by hematopoietic cells such as T, B and dendritic cells and by parenchymal cells in response to cytokine (i.e., IFN-γ) induction. Ligation of PD-1 by PD-L1 (B7-H1; CD274) or PD-L2 (B7-DC; CD273) inhibits TCR-mediated T cell proliferation and production of IL-1, IL-4, IL-10 and IFN-γ and also inhibits BCR mediated signaling (2). PD-L1 binding to CD80 inhibits T cells proliferation. PD-1 deficient mice have a defect in peripheral tolerance and spontaneously develop autoimmune diseases (3). A novel approach to fight cancer is to target immune checkpoint proteins with antibody inhibitors, such as anti-PD-1 or anti-PD-L1 that function as a tumor suppressing factor via the modulation of immune cell-tumor cell interactions (4).

The soluble form of PD-L1 (sPD-L1) is expressed upon immune cells activation and can be secreted in the serum/plasma. Soluble checkpoints, such as sPD-1 or sPD-L1, are involved in positive or negative immune regulation and changes in their serum/plasma levels affect the development, prognosis and treatment of cancer (5). Increase of sPD-L1 or sPD-1 levels have been detected in patients with different types of cancer (6). This PD-L1 (human) ELISA Kit is developed to detect specifically human soluble PD-L1 in different biological fluids.

3. General References

1) The diverse functions of the PD1 inhibitory pathway: A.K. Sharpe & K.E. Pauken; Nat. Rev. Immunol. **18**, 153 (2018)

(2) CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms: R.V. Parry, et al.; Mol. Cell. Biol. **25**, 9543 (2005)

(3) Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice: H. Nishimura, et al.; Science **291**, 319 (2001)

(4) PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome: H.O. Alsaab, et al.; Front. Pharmacol. **8**, 561 (2017)

(5) Hiding in Plain Sight: Soluble Immunomodulatory Receptors: L.N. Dahal, et al.; Trends Immunol. **39**, 771 (2018)

(6) Soluble PD-L1 as a biomarker in malignant melanoma treated with checkpoint blockade: J. Zhou, et al.; Cancer Immunol. Res. **5**, 480 (2017)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human PD-L1 in cell culture supernatants, serum and plasma (EDTA, heparin or citrate). A monoclonal antibody specific for PD-L1 has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, PD-L1 is recognized by the addition of a biotinylated monoclonal antibody specific for PD-L1 (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450nm after acidification and is directly proportional to the concentration of PD-L1 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human PD-L1 Standard (lyophilized)	(100 ng)	(STD)
• 1 vial Detection Antibody	(60 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with PD-L1 Antibody	(6 x 16-well strips)	
• 2 plate covers (plastic film)		
• 2 silica gel minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:200 in ELISA Buffer 1X (50 µl DET + 10 ml ELISA Buffer 1X).

  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human PD-L1 Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - This reconstitution produces a stock solution of 1 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
  - Suggested standard points are:
    150, 75, 37.5, 18.75, 9.375, 4.6875, 2.3437 and 0 pg/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of PD-L1 (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X
1.5 ng/ml	150µl of PD-L1 (10 ng/ml)	850 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
150 pg/ml	100 µl of PD-L1 (1.5 ng/ml)	900 µl of ELISA Buffer 1X
75 pg/ml	300 µl of PD-L1 (150 pg/ml)	300 µl of ELISA Buffer 1X
37.5 pg/ml	300 µl of PD-L1 (75 pg/ml)	300 µl of ELISA Buffer 1X
18.75 pg/ml	300 µl of PD-L1 (37.5 pg/ml)	300 µl of ELISA Buffer 1X
9.375 pg/ml	300 µl of PD-L1 (18.75 pg/ml)	300 µl of ELISA Buffer 1X
4.6875 pg/ml	300 µl of PD-L1 (9.375 pg/ml)	300 µl of ELISA Buffer 1X
2.3437 pg/ml	300 µl of PD-L1 (4.6875 pg/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Serum: Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma: Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma and Cell Culture Supernatant have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/4 dilution of serum or plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with PD-L1 antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma, serum or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **2 hours at Room Temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 hour at Room Temperature.**
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at Room Temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at Room Temperature in the dark for 20 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard and sample and subtract the average blank value (obtained with the 0 pg/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding PD-L1 concentration (pg/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the PD-L1 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation.
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human PD-L1 in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

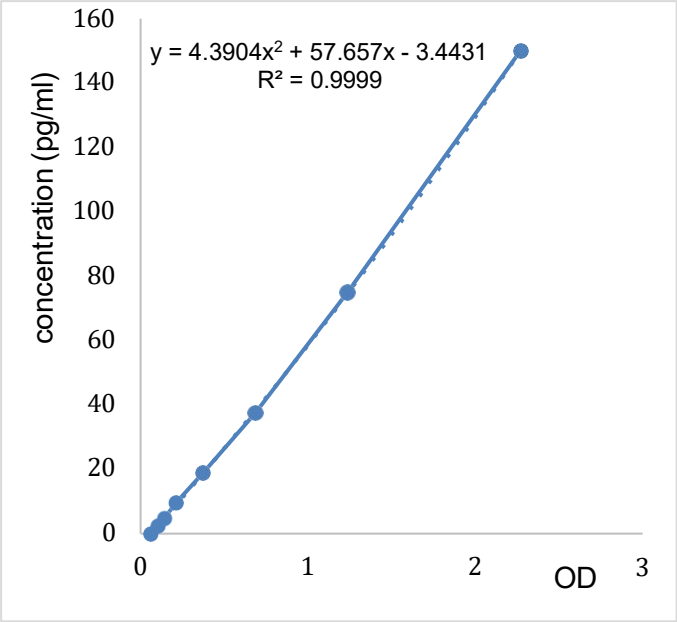

Figure: Standard curve

Standard PD-L1 (pg/ml)	Optical Density (mean)
150	2.27
75	1.237
37.5	0.6865
18.75	0.373
9.375	0.2075
4.6875	0.1415
2.3437	0.1025
0	0.061

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of human PD-L1 that can be detected by this assay is **0.8 pg/ml**.

NOTE: *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 2.343 pg/ml – 150 pg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human PD-L1 [CD274].

D. Intra-assay precision:

Four samples of known concentrations of human PD-L1 were assayed in replicates 6 times to test precision within an assay.

Samples	Means (pg/ml)	SD	CV (%)	n
A1	149.85	5.84	3.90	6
A2	174.28	8.32	4.77	6
A3	147.74	4.36	2.95	6
A4	109.87	8.19	7.45	6

E. Inter-assay precision:

Four samples of known concentrations of human PD-L1 were assayed in 5 separate assays to test precision between assays.

Samples	Means (pg/ml)	SD	CV (%)	n
B1	105.01	4.03	3.84	5
B2	181.27	5.43	2.99	5
B3	223.56	5.58	2.50	5
B4	103.90	4.73	4.55	5

F. Recovery:

When samples are spiked with known concentrations of human PD-L1, the recovery averages range from 90% to 103%.

G. Linearity:

Different samples containing human PD-L1 were diluted several fold (1/2 to 1/8 for sera and plasmas) and the measured recoveries ranged from 90% to 113%.

H. Expected values:

Human PD-L1 levels range in serum and plasma from **<40pg/ml to >200pg/ml.**

12. Technical Hints and Limitations

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes