PD-L1 TN-cyclon™ ELISA kit
User Manual

Kit Components（for a 96-well plate）

Component	States of samples	Quantity	Kit Storage
96-well plate	-	1 plate	Room Temperature
Adhesive Plate Sealers	-	5	Room Temperature
Capture Antibody Reagent	Liquid	1 vial ×20 µL (20 µg)	Frozen (under -70℃) Refrigerated after thaw(2 to 8℃)
Detection Antibody Reagent	Liquid	1 vial ×10 µL (5 µg)	Refrigerated (2 to 8℃)
Standard Stock	Liquid	1 vial ×20 µL (0.2 µg)	Frozen (under -70℃) Refrigerated after thaw(2 to 8℃)
Capture Ab Diluent	Liquid	1 vial ×20 mL	Room Temperature
Wash Buffer (20×)	Liquid	1 vial ×50 mL	Room Temperature
Sample Diluent	Liquid	1 vial ×20 mL	Refrigerated (2 to 8℃)
Blocking Reagent	Liquid	1 vial ×40 mL	Refrigerated (2 to 8℃)
Detection Ab Diluent	Liquid	1 vial ×30 mL	Refrigerated (2 to 8℃)
Enzyme Cycling Reagent 1	Powder	4 vial ×2.8 mg (100 mM solution, equivalent to 40 µL)	Frozen (-20 to -30℃)
Enzyme Cycling Reagent 2	Powder	4 vial ×20.4 mg (100 mM solution, equivalent to 300 µL)	Frozen (-20 to -30℃)
Enzyme Cycling Reagent 3	Powder	1 vial ×4.0 mg (Specific activity 40 U/mg, 1,000 U/mL solution, equivalent to 160 µL)	Frozen (-20 to -30℃)

Enzyme Cycling Reagent 4	Powder	1 vial ×2.3 mg (50 mM solution, equivalent to 120 µL)	Frozen (-20 to -30℃)
Enzyme Cycling Diluent1	Liquid	1 vial ×20 mL	Refrigerated (2 to 8℃)
Enzyme Cycling Diluent2 (Thio-NAD Diluent)	Liquid	1 vial ×1 mL	Refrigerated (2 to 8℃)
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WARNING

The components are not subject to the Industrial Safety and Health Act. However, when handling, please ensure to wear protective equipment such as goggles and gloves, and take adequate precautions to avoid direct contact with skin or eyes.
For Enzyme Cycling Reagent 4*, please refer to the SDS from the following URL.

※ If crystals have formed in the concentrate, warm at 37℃ water bath and mix it gently until crystals have completely dissolved.

※ The ingredient of Enzyme Cycling Reagent 4 is 17β-Methoxy-5β-androstan-3α-ol 3-phosphate（A3P）. Please download the SDS from the following URL.
https://www.biophenoma.com/pdl1kit

《Expiration date》：2 months from the date of manufacture.

Other Required Supplies

- Distilled Water
- 100 µL – 1000 µL single channel pipette and tips
- 10 µL – 200 µL single channel pipette and tips
- 1 µL – 10 µL single channel pipette and tips
- 50 µL – 200 µL multi-channel pipette and tips
- Multi-channel pipette reservoirs
- 1.5 mL tubes and 15 mL、50 mL centrifuge tubes
- Paper towels
- Microplate reader with 405 nm wavelength filter and 37℃ incubator
- Vortex mixer
- Benchtop Centrifuge
- Graduated cylinder
- Plate shaker
- Automated washer
- Methanol（G.R.）

Assay principle

PD-L1 is a protein molecule that has immunosuppressive functions by binding to its receptor, PD-1, which either suppresses or halts T-cell activity. While PD-L1 is normally expressed on the surface of antigen-presenting cells, it is also known to be expressed on the surface of tumor cells and non-transformed cells within the tumor microenvironment. Blocking the interaction between PD-1 and PD-L1 with immune checkpoint inhibitors has been shown to inhibit the growth of tumor cells, and the expression level of PD-L1 in tumor tissue, as well as its release into the blood, is a useful candidate biomarker for predicting efficacy.

PD-L1 is also found in serum (as soluble PD-L1) and exosomes. Exosomes, particularly in cancer, play a crucial role in mechanisms such as horizontal transmission, which is linked to cancer metastasis. Furthermore, a small amount of PD-L1 is present in the serum of healthy individuals, making it important to understand how the quantity changes when transitioning from a healthy state to cancer. This knowledge can contribute to the re-evaluation of the significance of immune checkpoints and improve the effectiveness of inhibitors.

However, since exosomes are limited in quantity and PD-L1 levels in the serum of healthy individuals are also low, it is essential to measure ultra-small amounts of PD-L1 when distinguishing between healthy individuals and early cancer patients.

The measurement of PD-L1 with this kit is based on TN-cyclon™ [1),2)], a proprietary technology that combines the sandwich ELISA and enzymatic cycling methods (following Figure). This method enables highly sensitive quantification of human PD-L1 in samples containing serum or exosomes.

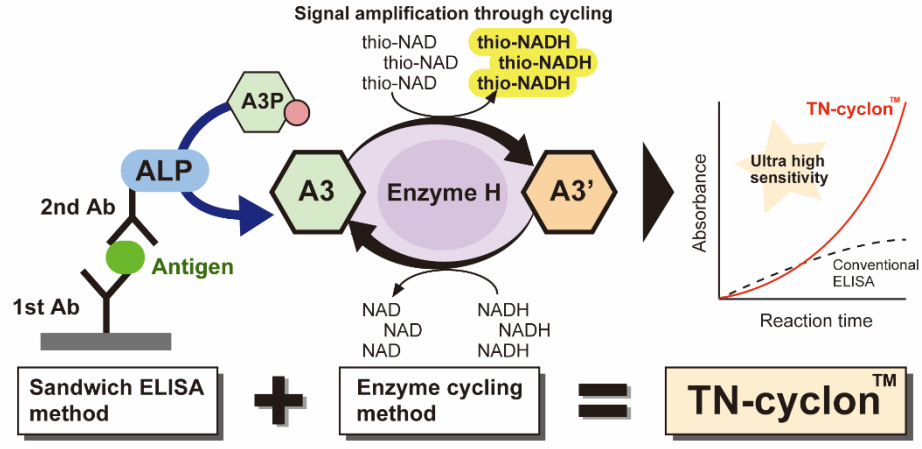


Figure Principle of TN-cyclon™ method.

(A3P (Enzyme Cycling Reagent 4) is our proprietary substrate.)

(The principle (simplified version) is as follows: The antigen is captured in a sandwich ELISA using a primary antibody (capture antibody) and a secondary antibody (detection antibody). The secondary antibody is labeled with alkaline phosphatase (ALP). When a substrate with a phosphate group (A3P) is applied, ALP catalyzes the removal of the phosphate group from A3P, converting it to A3. The resulting A3 is then amplified using the enzyme cycling method. In this cycling process, the key enzyme is 3α-hydroxysteroid dehydrogenase (3α-HSD), with NADH and Thio-NAD added as cofactors. During the cycling reaction, Thio-NADH accumulates, and its absorbance peak at 405 nm is measured. This absorbance change correlates with the original antigen concentration. For more details, please refer to the relevant references.)

《Target》 : Human PD-L1

《Sample types》 : Serum or samples including exosomes

※ This kit is for research use only. Not for diagnostic use. It is important that you read this entire manual carefully before starting your experiment.

Reagent Preparation

・1× Wash Buffer

Dilute the Wash Buffer (20x) 20 times with deionized or distilled water.

ex) For one plate, you will need over 900 mL of 1x Wash Buffer. To prepare 900 mL of 1x Wash Buffer, dilute 45 mL of 25x Wash Buffer with 855 mL of deionized or distilled water.

・Capture antibody solution

Dilute the Capture Antibody Reagent 1,000 times with Capture Antibody Diluent.

※ Gently tap the bottom of Capture Antibody Reagent to mix. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom of the vial.

ex) To prepare 10 mL of Capture Antibody Solution, dilute 10 µL of Capture Antibody Reagent with 9,990 µL of Capture Antibody Diluent.

・Standard Solution

Prepare Standard Solution with Standard Stock (10 µg/mL) and Sample Diluent.

※ Bring Sample Diluent back to room temperature before use.

※ Gently tap the bottom of Standard Stock to mix. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom of the vial.

※ Dilute 2 µL of Standard Stock (10 µg/mL) with 198 µL of Sample Diluent in 1.5 mL tube to make Dil1 (100 ng/mL).

The following are instructions for the preparation of a Standard dilution series.

Sample Diluent	-	98 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL
	-	Dil1	Dil2	Std1	Std2	Std3	Std4	Std5	Std6	-
Transfer Volume	-	2 µL	100 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	-
(pg/mL)	10,000 [**Dil1**]	200 [**Dil2**]	40 [**Std1**]	20 [**Std2**]	10 [**Std3**]	5 [**Std4**]	2.5 [**Std5**]	1.25 [**Std6**]	0.625 [**Std7**]	0 [**Blank**]

※ Dil1 should be used immediately and not stored for future use.

・Detection Antibody Solution

Dilute Detection Antibody Reagent (2,500x) 10 times with Detection Antibody Diluent to prepare Detection Antibody Reagent (250x), then dilute it 250 times again with Detection Antibody Diluent

※ Bring Detection Antibody Diluent back to room temperature before use.

※ 250x Detection Antibody Reagent should be used immediately and not stored for future use.

※ Gently tap the bottom of Detection Antibody Reagent to mix. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom of the vial.

ex）Dilute 5 µL of Detection Antibody Reagent（2,500x）with 45 µL of Detection Antibody Diluent in 1.5 mL tube to make Detection Antibody Reagent (250x), then dilute 40 µL of it with 9,960 µL of Detection Antibody Diluent in 50 mL centrifuge tube.

・Enzyme Cycling Solution

The following are instructions for the preparation of dissolved Enzyme Cycling Reagent.

Enzyme Cycling Reagent (Lid color)	Reagent 1 (Blue)	Reagent 2 (Yellow)	Reagent 3 (Green)	Reagent 4 (Pink)
Solvent to be added	Enzyme Cycling Diluent 1	Enzyme Cycling Diluent 2	Enzyme Cycling Diluent 1	Methanol (G.R.)
Add Volume	100 µL	300 µL	160 µL	160 µL

※ Bring Enzyme Cycling Diluent 1 and 2 back to room temperature before use.

※ The Enzyme Cycling Reagent 1 is clear and colorless to light yellow immediately after dissolution; however, it may turn a deeper yellow or discolor during storage. Do not use discolored solutions; prepare a new solution instead.

※ Store the Enzyme Cycling Reagent 2 after dissolution in a light-shielding sample box.

※ When dissolving the Enzyme Cycling Reagent 1, 2, and 4, mix it using a vortex mixer. If dissolution is difficult, also use pipetting to assist. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom of the vials.

※ Gently tap the bottom of Enzyme Cycling Reagent 3 to mix after dissolution. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom of the vial

※ Store all reagents after dissolution in a refrigerator at 2 to 8°C

＜Sample Preparation＞

【For Serum】:

・Dilute serum 100 times with Sample Diluent.

【For Exosome】:

・Add the exosome suspended in a solution such as Tris-buffered saline (pH 7.5) Sample Diluent. If necessary, add a detergent such as Triton™ X-100 to Sample Diluent to achieve a final concentration of 1%, and then mix with the exosome suspension.

Assay Procedure

***Day* 1**

1．Capture antibody coating

・Add 100 µL of capture antibody solution per well, cover with a plate sealer, and incubate for over 16 hours at 4℃.

***Day* 2**

2．Plate wash

・Aspirate the liquid from each well and wash 3 times by an automated washer. Wash by adding approximately 300 µL of 1x Wash buffer.

※ If you don’t have an automated washer, please use multi-channel pipette or decant the liquid.

・After washing, invert the plate and tap against clean paper towel.

3．Blocking

・Add 300 µL of blocking solution per well, cover with a plate sealer, and incubate for 1 hour at room temperature.

4．Plate wash

・Aspirate the liquid from each well and wash 9 times by an automated washer. Wash by adding approximately 300 µL of 1x Wash buffer.

※ If you don’t have an automated washer, please use multi-channel pipette or decant the liquid.

・After washing, invert the plate and tap against clean paper towel.

5．Antigen and Sample

・Add 100 µL of Standard, Blank, or Sample per well, cover with a plate sealer, and incubate for 1 hour at room temperature. Mix wells on a plate shaker at approximately 400 rpm while incubation.

6. Plate wash

・Aspirate the liquid from each well and wash 9 times by an automated washer. Wash by adding approximately 300 µL of 1x Wash buffer.

※ If you don't have an automated washer, please use multi-channel pipette or decant the liquid.

・After washing, invert the plate and tap against clean paper towel.

7. Detection Antibody

・Add 100 µL of detection antibody solution per well, cover with a plate sealer, and incubate for 1 hour at room temperature. Mix wells on a plate shaker at approximately 400 rpm while incubation.

8. Plate wash

・Aspirate the liquid from each well and wash 9 times by an automated washer. Wash by adding approximately 300 µL of 1x Wash buffer.

※ If you don't have an automated washer, please use multi-channel pipette or decant the liquid.

・After washing, invert the plate and tap against clean paper towel.

9. TN-cyclon

・The following is an instruction for the preparation of an Enzyme Cycling Reagent. Add the dissolved Enzyme Cycling Reagent 1, Reagent 2, Reagent 3, and Reagent 4 sequentially to Enzyme Cycling Diluent 1, as indicated in the table below. Depending on the preparation volume, use a 1.5 mL microtube, a 15 mL conical tube, or a 50 mL conical tube.

・Mix thoroughly to ensure homogeneity, and promptly dispense 100 µL into each well.

	For 100 µL/1 well	ex)For 100 wells
Enzyme Cycling Diluent 1	95.2 µL	9520 µL
Dissolved Enzyme Cycling Reagent 1	1.0 µL	100 µL
Dissolved Enzyme Cycling Reagent 2	2.0 µL	200 µL
Dissolved Enzyme Cycling Reagent 3	1.0 µL	100 µL
Dissolved Enzyme Cycling Reagent 4	0.8 µL	80 µL

※ Do not use discolored Enzyme Cycling Reagent 1.

※ Wrong addition order of Enzyme Cycling Reagents may have a negative impact on absorbance. Please follow the numbers when you prepare.

※ Gently tap the bottom of Enzyme Cycling Reagent mixture to mix. Avoid vigorous mixing with a vortex mixer.

※ We recommend using a multichannel pipette to add the Enzyme Cycling Reagent mixture to the wells. When using a multichannel pipette, transfer the Enzyme Cycling Reagent mixture to a reservoir before dispensing it into the wells.

10. Measuring Absorbance

・Perform the colorimetric reaction using the enzyme cycling method at 37°C, and measure the absorbance at 405 nm at any desired time point after the reaction begins.

Example of measurement

Set the measurement wavelengths to 405 nm (primary wavelength) and 660 nm (secondary wavelength). Measure the absorbance at 5-minute intervals for a total of 13 measurements to obtain data similar to the example shown

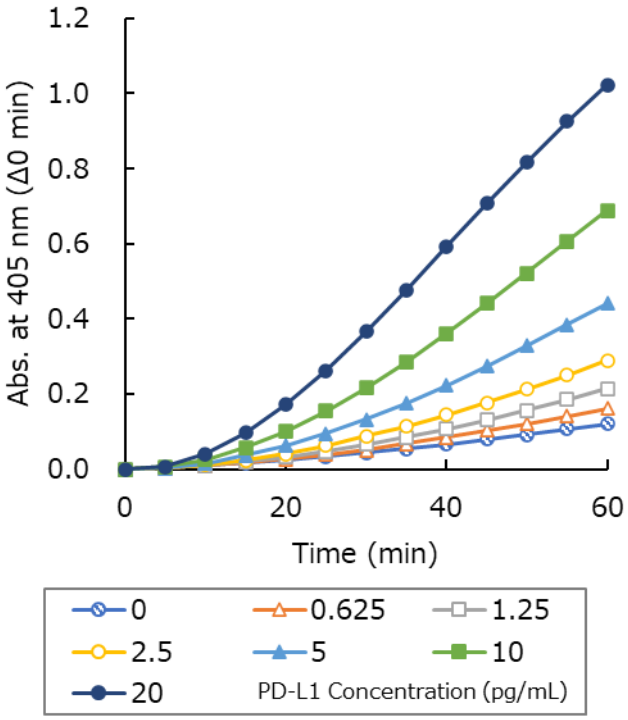
below.

※ In the example, the secondary wavelength of 660 nm was used to calculate the true absorbance of Thio-NADH as［Primary Wave Length］-［Secondary Wave Length］. However, measurements and analyses can also be performed without using a secondary wavelength.

※ If a microplate reader with a temperature control function is not available, use a thermal device such as a hot plate that can maintain a temperature of 37°C.

※ Ensure that the microplate reader with temperature control or the thermal device (e.g., hot plate) is preheated to 37°C before starting the reaction. If the device does not reach 37°C at the start of the reaction, it may affect the measurement data.

※ Before measuring the wells with the microplate reader, confirm that the solution is free of bubbles, as bubbles may interfere with the measurement data.

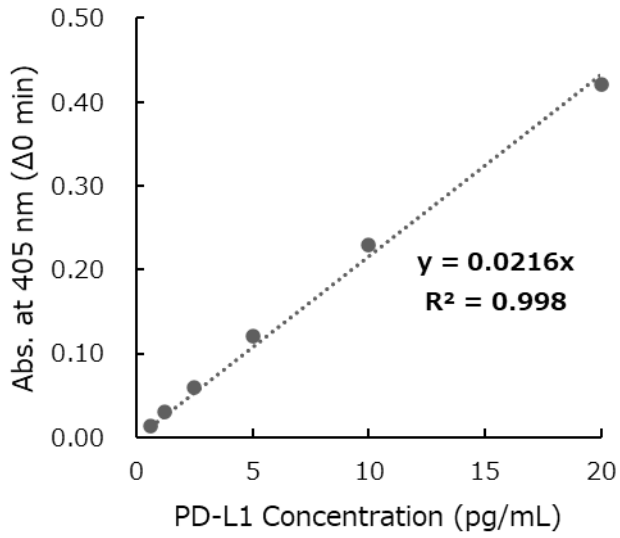


Assay Example

※This example is based on the results of experiments conducted following the protocol in this operation manual.

・ Calibration Curve

The absorbance values at 405 nm (Δ0 min) for the PD-L1 standard concentrations (pg/mL) are shown in the figure below (measurement wavelengths: 405 nm [primary wavelength] and 660 nm [secondary wavelength], measurement time: 60 min). Note that a calibration curve should be generated for each assay to calculate the concentration in the samples.



The absorbance values and CV values for each PD-L1 concentration are shown in the table below.

PD-L1 (pg/mL)	Absorbance(405 nm)			Ave. absorbance	CV(%)
	1	2	3		
0	0.125	0.119	0.122	0.122	4.64
0.625	0.168	0.154	0.164	0.162	3.63
1.25	0.218	0.199	0.226	0.214	5.28
2.5	0.305	0.278	0.287	0.290	3.87
5	0.442	0.430	0.455	0.442	2.31
10	0.692	0.672	0.700	0.688	1.71
20	1.110	0.975	0.985	1.023	6.00

・ Limit of Detection

The limit of detection, calculated using the standard deviation of the blank absorbance (SD: 3SD applied) and the slope of the calibration curve, was below 0.2 pg/mL (0.154 pg/mL in the example above).

・ Spike and Recovery test（serum）

The recovery rate of PD-L1 at a known concentration (100 pg/mL) added to serum samples was calculated as shown in the table below. (Serum samples were diluted 100-fold before use; measurements were performed in triplicate, n=3).

Sample	Measured Concentration (pg/mL)	Recovery Rate (%)
Serum (x100 diluent)	93.6	93.6

References

1) Tsurusawa N, Iha K, Sato A, Tsai HY, Sonoda H, Watabe S, Yoshimura T, Wu DC, Lin MW, Ito E. Ultrasensitive Detection of GRP78 in Exosomes and Observation of Migration and Proliferation of Cancer Cells by Application of GRP78-Containing Exosomes. Cancers. 2022 Aug 11;14(16):3887. doi: 10.3390/cancers14163887.

2) Iha K, Tsurusawa N, Tsai HY, Lin MW, Sonoda H, Watabe S, Yoshimura T, Ito E. Ultrasensitive ELISA detection of proteins in separated lumen and membrane fractions of cancer cell exosomes. Anal Biochem. 2022 Oct 1;654:114831. doi: 10.1016/j.ab.2022.114831.