PD-L1 TN-cyclon™ ELISA kit

RUO (Research Use Only)

User Manual

TOC

Kit Components（for a 96-well plate）

See Table 1 for a list of kit components, and Figures 1, 2 and 3 for the arrangement of each reagent in the kit box.

Table 1

Components	Reagent Forms	Quantity	Storage	Shipping Temperature
96-well plate	-	1 plate (12 strips of 8 wells)	Room Temperature (15 - 30℃)	Refrigerated (2 - 8℃)
Adhesive Plate Seals	-	5	Room Temperature (15 - 30℃)	Refrigerated (2 - 8℃)
Capture Ab Reagent (1,000x)	Liquid	20 µL x 1 (20 µg)	Frozen (≤ -70 °C) Refrigerated after thaw (2 - 8℃)	Frozen (≤ -70 °C)
Detection Ab Reagent (2,500x)	Liquid	10 µL x 1 (5 µg)	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Standard Stock (10 µg/mL)	Liquid	20 µL x 1 (0.2 µg)	Frozen (≤ -70 °C) Refrigerated after thaw (2 - 8℃)	Frozen (≤ -70 °C)
Capture Ab Diluent	Liquid	20 mL x 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Wash Buffer (20x)	Liquid	50 mL × 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Sample Diluent	Liquid	20 mL x 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Blocking Reagent	Liquid	40 mL × 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Detection Ab Diluent	Liquid	30 mL × 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Enzyme Cycling Reagent 1	Powder	2.8 mg x 4 (100 mM solution, equivalent to 40 µL)	Frozen (-30 - -20℃)	Frozen (-30 - -20℃)
Enzyme Cycling Reagent 2	Powder	20.4 mg x 1 (100 mM solution, equivalent to 300 µL)	Frozen (-30 - -20℃)	Frozen (-30 - -20℃)
Enzyme Cycling Reagent 3	Powder	160 U x 1 (1,000 U/mL solution, equivalent to 160 µL)	Frozen (-30 - -20℃)	Frozen (-30 - -20℃)
Enzyme Cycling Reagent 4	Powder	2.3 mg x 1 (50 mM solution, equivalent to 120 µL)	Frozen (-30 - -20℃)	Frozen (-30 - -20℃)
Enzyme Cycling Diluent 1	Liquid	20 mL x 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
Enzyme Cycling Diluent 2	Liquid	1 mL x 1	Refrigerated (2 - 8℃)	Refrigerated (2 - 8℃)
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WARNING The components of this kit are not regulated under Japan's Industrial Safety and Health Act. This information is provided for reference only.
When handling these materials, please wear appropriate protective equipment such as safety glasses and gloves, and take sufficient precautions to avoid contact with the human body.

※ If crystals have formed in the buffers, warm them at 37℃ in a water bath and gently mix until the 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/en/pdl1kit

《Expiration Date》 Please check the label information printed on the kit's outer box.

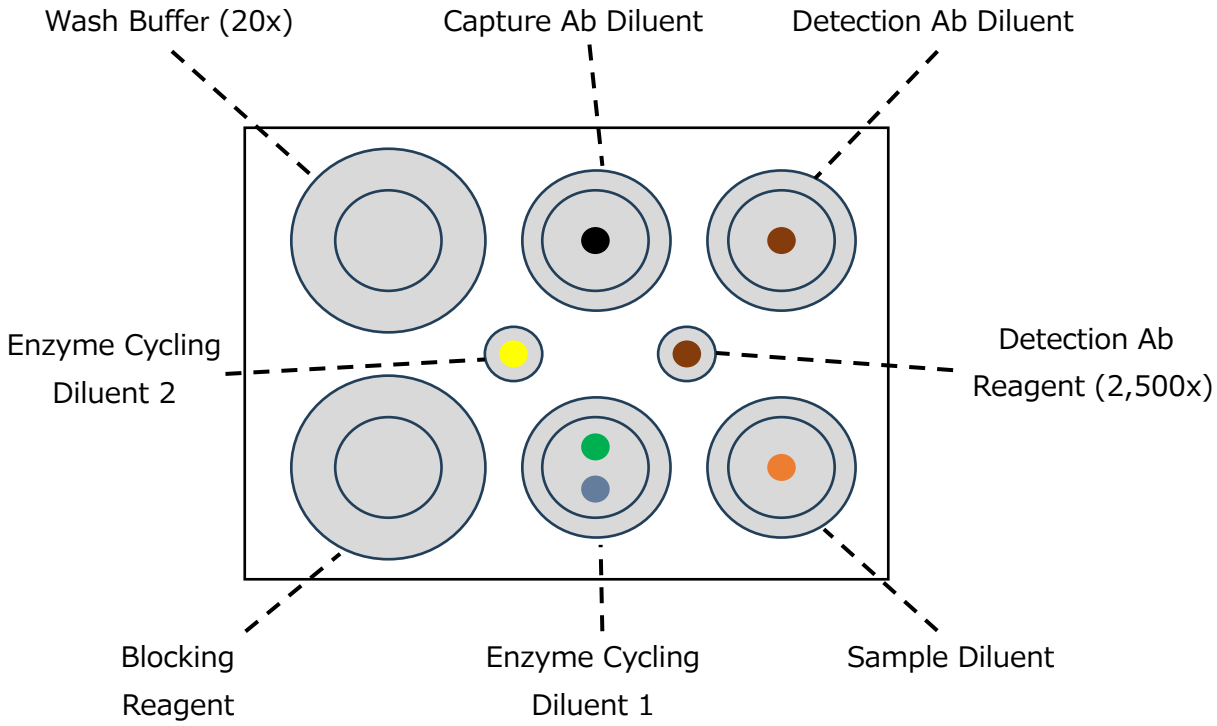

Fig. 1　Reagent Box Layout (2–8°C)

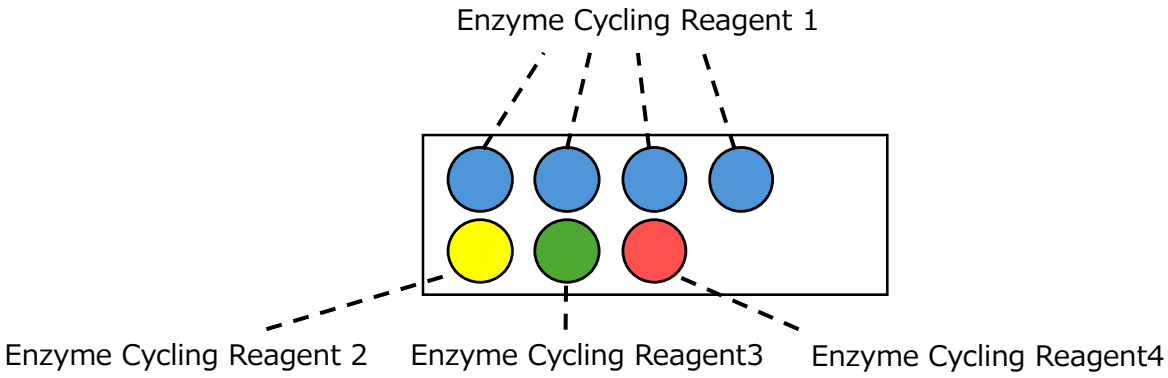

Fig. 2　Reagent Box Layout (−30 to −20°C)

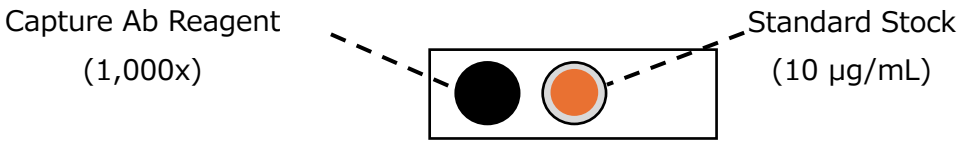

Fig. 3　Reagent Box Layout (≤ −70 °C)

Other Required Supplies

- Ultrapure 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
- Reagent reservoirs
- 1.5 mL tubes and 15 mL, 50 mL centrifuge tubes
- Paper towels
- Microplate reader with absorbance measurement and temperature control function
  ※ 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.
- Vortex mixer
- Benchtop centrifuge
- Graduated cylinder (for 1 L)
- Plate shaker
- Automated washer
- Methanol (Guaranteed Reagent)

Background

PD-L1 is a protein with immunosuppressive function that inhibits or suppresses T-cell activation through binding to its receptor, PD-1. PD-L1 is normally expressed on the surface of antigen-presenting cells; however, it is also known to be expressed on the surface of tumor cells as well as non-transformed cells present in the tumor microenvironment. In cancer research, blockade of the interaction between PD-L1 and PD-1 by immune checkpoint inhibitors has been shown to inhibit tumor cell proliferation. Therefore, the expression level of PD-L1 in tumor tissues and the concentration of PD-L1 released into the bloodstream (soluble PD-L1) are considered promising predictive biomarkers of therapeutic efficacy.

PD-L1 is present not only on the cell surface and in the bloodstream but also in exosomes. In cancer, exosomes are utilized for the horizontal transfer of various factors and are known to play an important role in the mechanisms of cancer metastasis[1)]. Accordingly, the amount of PD-L1 contained in exosomes has attracted increasing attention in recent years[2)]. However, because exosomes are inherently present in limited quantities, the amount of PD-L1 contained in exosomes derived from cancer patients is expected to be extremely low. The concentration of PD-L1 in the blood of healthy individuals is presumed to be even lower; therefore, ultra-sensitive measurement of PD-L1 is required for distinguishing early-stage cancer patients from healthy individuals.

Measurement of PD-L1 using this kit is performed based on TN-cyclon ™[3), 4)], a proprietary technology that combines the sandwich ELISA method with an enzyme cycling method (see “Assay Principle” for details). This method enables highly sensitive quantification of human PD-L1 in serum or in samples containing exosomes.

Assay Principle

TN-cyclon™ is our unique protein detection technique that combines sandwich ELISA with enzyme cycling method[3), 4)]. This innovative approach allows for the measurement of proteins with higher sensitivity than conventional sandwich ELISA methods.

The principle (simplified version) of TN-cyclon™ is as follows: The antigen is captured in a sandwich ELISA using a capture antibody and a detection antibody. The detection antibody is labeled with alkaline phosphatase (ALP). When a 17β-methoxy-5β-androstan-3α-ol 3-phosphate (substrate A3P) is applied, ALP catalyzes the dephosphorylation of 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.

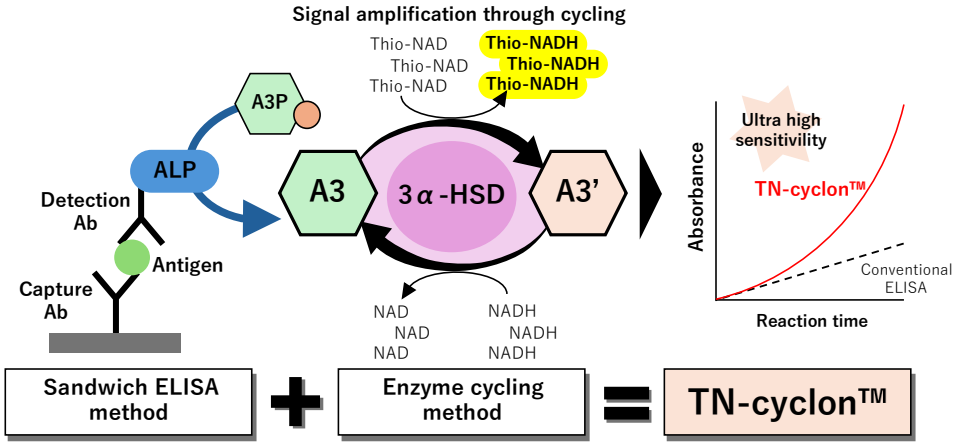

Fig. 4 Principle of TN-cyclon™

(TN-cyclon™ is a signal amplifying technique that combines sandwich ELISA with enzyme cycling method)

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

《Target》: Human

《Specimen》: Serum or exosome-containing samples

《Analyte》: PD-L1

※ 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

・1x Wash Buffer

Dilute the Wash Buffer (20x) 20 times with ultrapure water.

Example:

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 Wash Buffer (20x) with 855 mL of ultrapure water.

※ Bring Wash Buffer (20x) back to room temperature before use.

※ Do not use phosphate-based buffers such as phosphate-buffered saline (PBS) because ALP-labeled antibodies are used in this protocol. Phosphate-based buffers may affect the measurement results. We recommend using Tris-hydroxymethylaminomethane (hereinafter referred to as “Tris”) based buffers such as Tris-buffered saline (TBS). A buffer pH of 7.5 is recommended.

・ Capture antibody solution

Dilute the Capture Ab Reagent (1,000×) 1,000-fold with Capture Ab Diluent.

Use a 1.5 mL tube, 15 mL centrifuge tube, or 50 mL centrifuge tube according to the required preparation volume.

※ Bring the Capture Ab Diluent to room temperature before use.

※ Gently mix the capture antibody by tapping or similar methods. Avoid vigorous mixing using a vortex mixer.

※ After mixing, spin down briefly using a benchtop centrifuge before use.

Example:

Mix 10 µL of Capture Ab Reagent (1,000×) with 9,990 µL of Capture Ab Diluent in a 15 mL or 50 mL centrifuge tube.

・Standard solution

Prepare a serial dilution by mixing the Standard Stock (10 µg/mL) with Sample Diluent.

Use 1.5 mL tubes, 15 mL centrifuge tubes, or 50 mL centrifuges tube according to the required preparation volume.

※ Bring the Sample Diluent to room temperature before use.

※ Gently mix the standard soltuions by tapping or similar methods. Avoid vigorous mixing using a vortex mixer.

※ After mixing, spin down briefly using a benchtop centrifuge before use.

Example:

Mix 2 µL of Standard Stock (10 µg/mL) with 198 µL of Sample Diluent in a 1.5 mL tube to prepare a 100-fold dilution, resulting in a 100 ng/mL solution (designated as Dil1).

Next, prepare the solutions using 1.5 mL tubes as shown in the table below.

Sample Diluent	-	998 µL	640 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL
Solution to add	-	Dil1	Dil2	Std1	Std2	Std3	Std4	Std5	Std6	-
Volume to add	-	2 µL	160 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	-
Standard concentration (pg/mL)	100,000 [**Dil1**]	200 [**Dil2**]	40 [**Std1**]	20 [**Std2**]	10 [**Std3**]	5 [**Std4**]	2.5 [**Std5**]	1.25 [**Std6**]	0.625 [**Std7**]	0 [**Blank**]

Use Std1 to Std7 and Blank as standard solutions for generating the calibration curve.

※ Prepare Dil1 immediately before use.

・Detection antibody solution

Dilute the Detection Ab Reagent (2,500×) 10-fold with Detection Ab Diluent to prepare a 250× detection antibody concentrate.

Next, dilute the 250× detection antibody concentrate 1:250 with Detection Ab Diluent.

Use a 1.5 mL tube, 15 mL centrifuge tube, or 50 mL centrifuge tube according to the required preparation volume.

※ Bring the Detection Ab Diluent to room temperature before use.

※ Prepare the 250× detection antibody concentrate immediately before use.

※ Gently mix the detection antibody by tapping or similar methods. Avoid vigorous mixing using a vortex mixer.

※ After mixing, spin down briefly using a benchtop centrifuge before use.

Example:

Mix 5 µL of Detection Ab Reagent (2,500×) with 45 µL of Detection Ab Diluent in a 1.5 mL tube to prepare 50 µL of a 250× detection antibody concentrate.

Then, mix 40 µL of the 250× detection antibody concentrate with 9,960 µL of Detection Ab Diluent in a 50 mL centrifuge tube.

・Enzyme Cycling Solution

The following Table 2 is an instruction for the preparation of dissolved Enzyme Cycling Reagents.

The dissolved Enzyme Cycling Reagents will be referred to as “Dissolved Enzyme Cycling Reagent 1”, “Dissolved Enzyme Cycling Reagent 2”, “Dissolved Enzyme Cycling Reagent 3”, and “Dissolved Enzyme Cycling Reagent 4”, respectively.

Table 2

Enzyme Cycling Reagent (Lid color)	Reagent 1 (Blue)	Reagent 2 (Yellow)	Reagent 3 (Green)	Reagent 4 (Pink)
Solvent to be added (Lid sticker color)	Enzyme Cycling Diluent 1 (Blue・Green)	Enzyme Cycling Diluent 2 (Yellow)	Enzyme Cycling Diluent 1 (Blue・Green)	Methanol (Guaranteed Reagent)
Add Volume	40 µL	300 µL	160 µL	120 µL

※ Use the Enzyme Cycling Reagents immediately after reconstitution.
※ Bring Enzyme Cycling Diluent 1 and 2 back to room temperature before use.
※ The Enzyme Cycling Reagent 1 is clear and either colorless or 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.
※ Gently tap the bottom of Enzyme Cycling Reagent 3 vial to mix after dissolution. Briefly centrifuge the vial to ensure that all reagent is collected at the bottom.
※ Store all reagents after dissolution in a refrigerator at 2–8°C.

<Sample Preparation>
[For Serum]:
・Dilute serum 100-fold with Sample Diluent.

[For Exosomes]:
・Add exosomes suspended in Tris-buffered saline (pH 7.5) or a similar buffer to Sample Diluent.
・If necessary, add a surfactant such as Triton™ X-100 to the Sample Diluent to 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 the wells with a plate seal and incubate at 4°C for at least 16 hours.

***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 Reagent per well.

※ Bring Blocking Reagent back to room temperature before use.

・Cover the wells with a plate seal, 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．Add standard or sample

・Add 100 µL of standard, blank, or sample per well.

・Cover the wells with a plate seal and incubate for 1 hour at room temperature with shaking at approximately 400 rpm using a plate shaker.

※ If using a dial-type plate shaker, adjust the shaking speed to approximately 400 rpm.

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．Add detection antibody

・Add 100 µL of detection antibody solution per well.

・Cover the wells with a plate seal and incubate at room temperature for 1 hour with shaking at approximately 400 rpm using a plate shaker.

※ If a dial-type plate shaker is used, set the shaking speed to a level equivalent to approximately 400 rpm.

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．Add Enzyme Cycling Reagent mixture

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

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

Table 3

Diluent/Reagent name (Lid sticker or lid color)	For 100 µL/1 well	Example: For 100 well
Enzyme Cycling Diluent 1 (Blue・Green)	95.2 µL	9,520 µL
Dissolved Enzyme Cycling Reagent 1 (Blue)	1.0 µL	100 µL
Dissolved Enzyme Cycling Reagent 2 (Yellow)	2.0 µL	200 µL
Dissolved Enzyme Cycling Reagent 3 (Green)	1.0 µL	100 µL
Dissolved Enzyme Cycling Reagent 4 (Pink)	0.8 µL	80 µL

※ Do not use discolored dissolved Enzyme Cycling Reagent 1.

※ Wrong addition order of Enzyme Cycling Reagents may have a negative impact on absorbance. Please follow the order number 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 an appropriate time point after the reaction begins.

Example of measurement

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

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

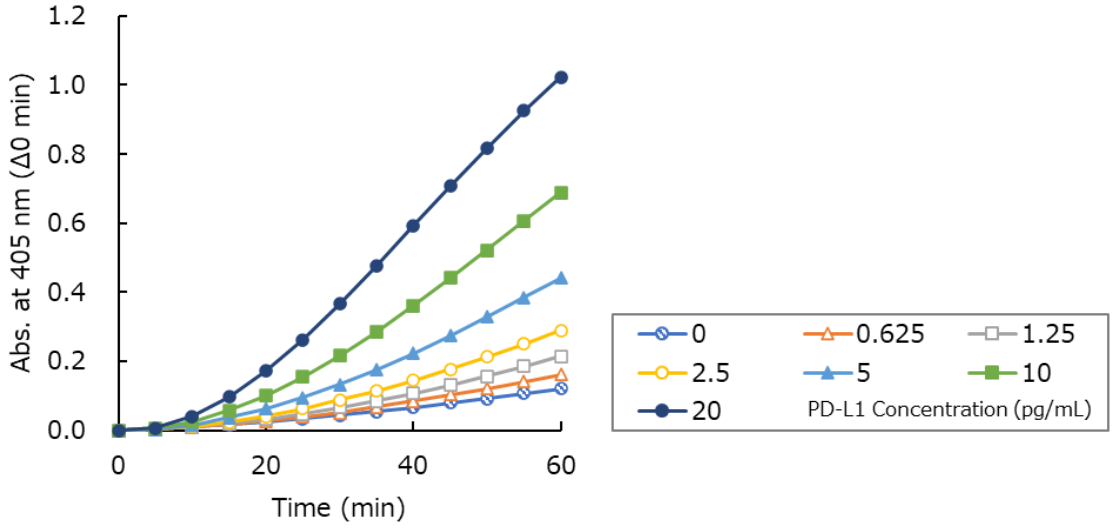

Fig. 5 Measurement of PD-L1

Assay Example

※ This example is based on experiments conducted by our company according to the protocol in this manual.

・ Calibration Curve

The absorbance values (405 nm (Δ0 min)) and coefficients of variation (CV) for the standard PD-L1 concentrations (pg/mL) are shown in Table 4 (measurement wavelength: 405 nm [primary wavelength], 660 nm [reference wavelength]; measurement time: 55 min). The calibration curve generated from the absorbance values listed in Table 4 is shown in Fig. 6.

Table 4

PD-L1 (pg/mL)	Absorbance (405 nm)			Average A_{405}	CV (%)
	1	2	3		
0	0.111	0.105	0.106	0.107	2.45
0.625	0.150	0.133	0.143	0.142	4.91
1.25	0.187	0.172	0.197	0.185	5.54
2.5	0.264	0.238	0.250	0.251	4.24
5	0.386	0.374	0.398	0.386	2.54
10	0.611	0.590	0.620	0.607	2.07
20	1.005	0.878	0.892	0.925	6.15

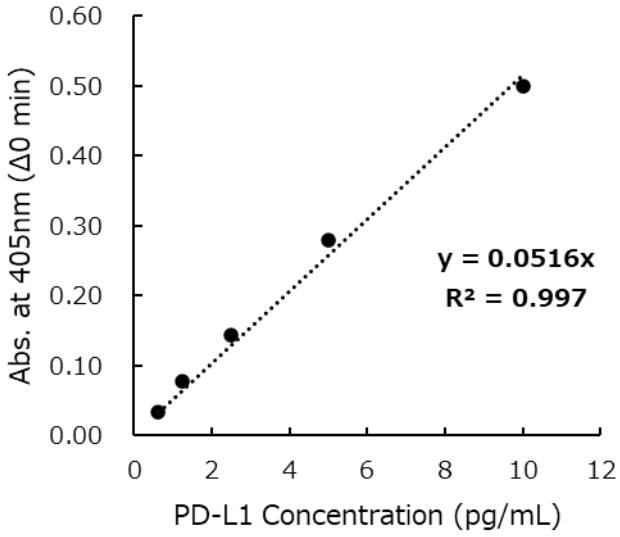

Fig. 6 Calibration curve

・ Limit of detection

The limit of detection, calculated using the standard deviation (SD) of the blank absorbance (3SD applied) and the slope of the calibration curve, was below 0.2 pg/mL (0.153 pg/mL in the example above).

・ Spike and recovery test

The recovery rate of PD-L1 at a known concentration (100 pg/mL) added to serum samples was calculated as shown in Table 5 (serum samples were diluted 100-fold with Sample Diluent before use; measurements were performed in triplicate, n=3).

Table 5

Sample (n=3)	Measured concentration (pg/mL)	Recovery rate (%)
Serum (100-fold dilution)	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) Okita K, Arita H,Sudo K, Yoshimura T, Ito E. Establishment of an Assay with Ultrahigh Sensitivity for Detecting sEV-Derived PD-L1 as a Serum Biomarker for Lung Cancer-A Pilot Study Using TN-cyclon™. Curr Issues Mol Biol. 2025 Jul 18:47(7):564. doi: 10.3390/cimb47070564.
3) Watabe S, Kodama H, Kaneda M, Morikawa M, Nakaishi K, Yoshimura T, Iwai A, Miura T, Ito E. Ultrasensitive enzyme-linked immunosorbent assay (ELISA) of proteins by combination with the thio-NAD cycling method. Biophysics. 2014 Sep 5:10:49-54. doi: 10.2142/biophysics.10.49.
4) Kobayashi Y, Kyosei Y, Ogawa R, Okita K, Yoshimura T, Ito E. Ultrasensitive protein-level detection for respiratory infectious viruses. Front Immunol. 2024 Dec 2:15:1445771. doi: 10.3389/fimmu.2024.1445771.

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