TN-cyclon™ ELISA Development Kit

RUO (Research Use Only)

User Manual

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Kit Components（for a 96-well plate）

See Table 1 for a list of kit components, Figures 1 and 2 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 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/devkit

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

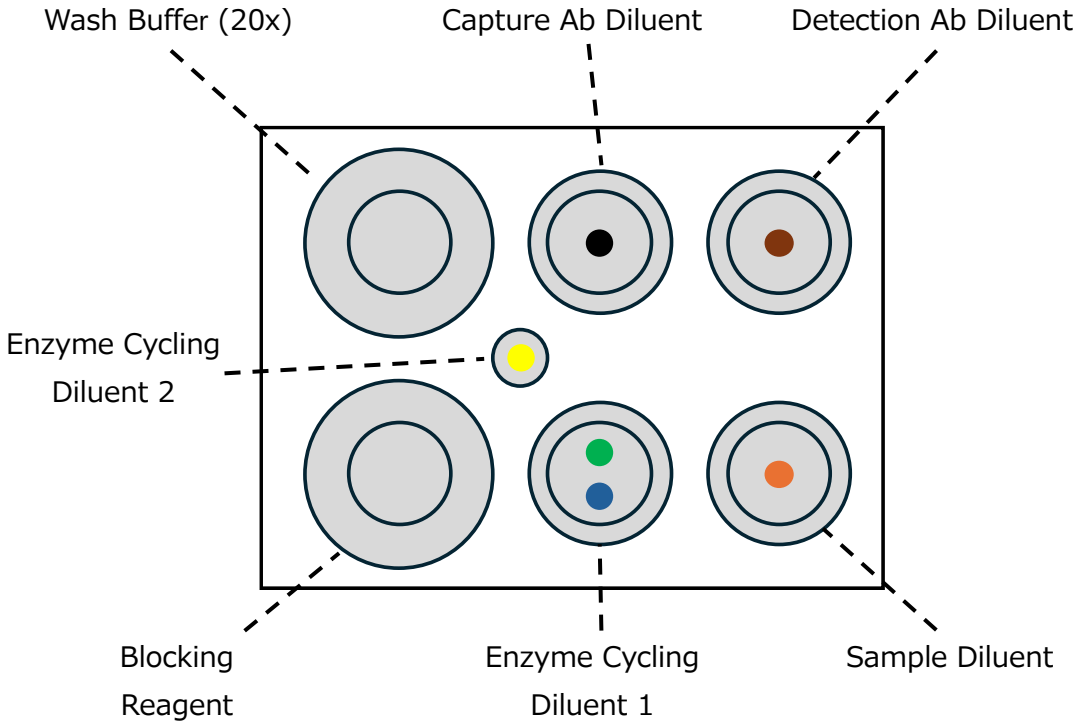

Fig. 1 Reagent Box Layout (2–8°C)

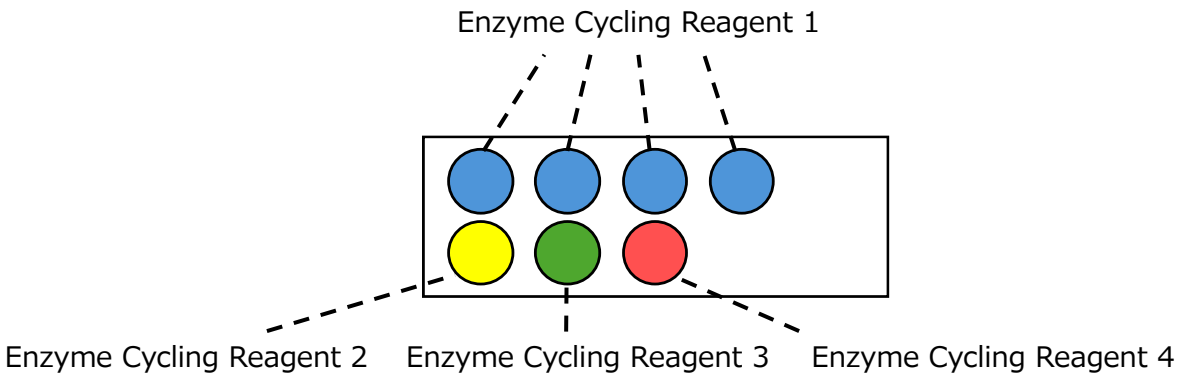

Fig. 2 Reagent Box Layout (−30 to −20°C)

Other Required Supplies

- Capture antibody
- Detection antibody
- Standard antigen
- 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)

Assay Principle

TN-cyclon™ is our unique protein detection technique that combines sandwich ELISA with enzyme cycling method[1), 2)]. 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.

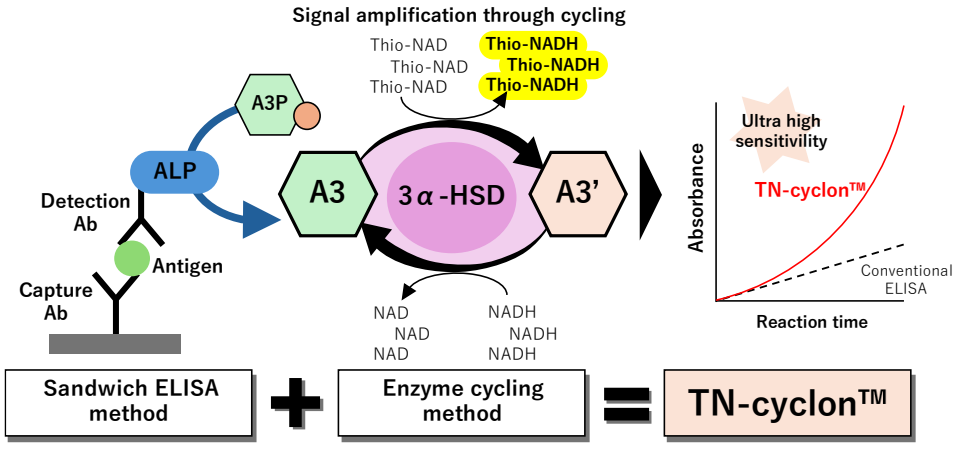

Fig. 3 Principle of TN-cyclon™

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

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

※ 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

Prepare your capture antibody at the appropriate concentration using Capture Ab Diluent. Use a 1.5 mL tube, 15 mL centrifuge tube, or 50 mL centrifuge tube, depending on the volume to be prepared.

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

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

※ You can use your own solution for the capture antibody diluent, in which case 50 mM carbonate-bicarbonate buffer (pH 9.6) is recommended.

・Standard solution

Prepare the optional dilution series by mixing your standard antigen with the Sample Diluent. Use 1.5 mL tubes, 15 mL centrifuge tubes, or 50 mL centrifuge tubes depending on the volume of preparation.

※ Bring Sample Diluent back to room temperature before use.

※ Gently tap the bottom of standard solution vials to mix. Briefly centrifuge the vials to ensure that all reagent is collected at the bottom.

※ You can use your own solution for the sample diluent, in which case TBS or other Tris-based buffers (pH 7.5) are recommended.

・Detection antibody solution

Prepare your detection antibody at the appropriate concentration using Detection Ab Diluent. Use a 1.5 mL tube, 15 mL centrifuge tube, or 50 mL centrifuge tube, depending on the volume to be prepared.

※ Please use ALP-labelled antibody for detection.

※ Alkaline Phosphatase Labeling Kit – SH (LK13; Dojindo Laboratories) is recommended to label ALP for detection antibody.

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

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

※ You can use your own solution for the detection antibody diluent, in which case TBS or other Tris-based buffers (pH 7.5) are recommended.

・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 dissolution.

※ 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.

Assay Procedure

※ This protocol requires the optimal conditions for your capture antibody, standard antigen, and detection antibody.
※ This protocol is based on the condition of using a 96-well plate (or a 96-well plate in a strip format) and coating with 100 µL of capture antibody solution (our recommended condition). When changing the well volume or the amount of capture antibody solution, adjust the amount of each solution accordingly.

1. Capture antibody coating

・Add 100 µL of capture antibody solution per well.
※ Any plate for ELISA can be used for capture antibody coating, but we recommend the use of plates optimized for antibody binding (e.g. Thermo Fisher Scientific, MaxiSorp™).
・Cover the wells with a plate seal and incubate under suitable condition for the antibody you are using.

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.
※ Any solution for blocking can be used, in which case a Tris-based solution such as TBS is recommended as the base buffer. A buffer pH of 7.5 is recommended.

・Cover the wells with a plate seal, and incubate for 1 hour at room temperature.

※ Adjust the conditions suitable for your samples.

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 under suitable conditions for the standard or samples you are using.

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 under the suitable condition for the detection antibody you are using.

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 wells
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. 4.

(Fig. 4 is an example of recombinant PD-L1 measurement by TN-cyclon™.)

※ 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.

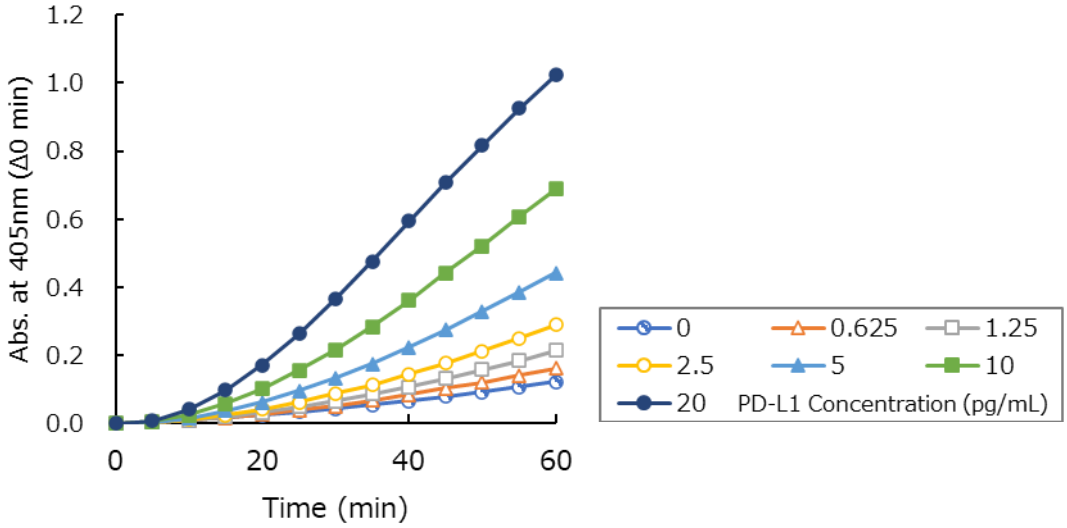

Fig. 4 Measurement of PD-L1

Assay Example: Measurement of recombinant PD-L1

※This example is based on experiments conducted by our company according to the protocol in this manual. Actual results may vary depending on the antibodies or antigens used, and the same values are not guaranteed.

・ 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. 5.

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

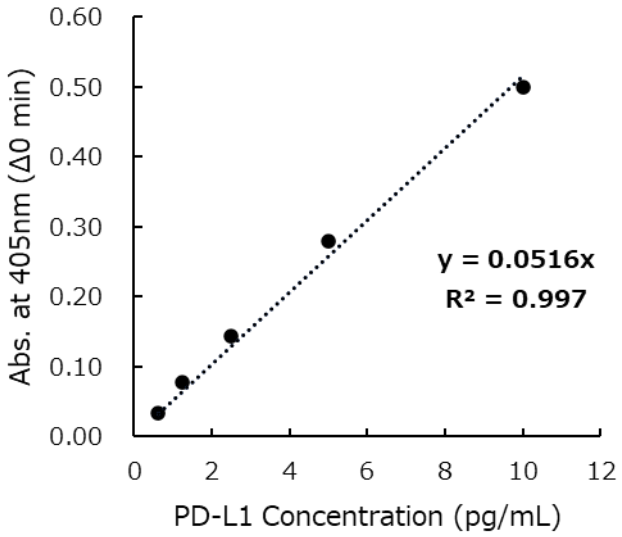

Fig. 5 Calibration curve

・ 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) 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.
2) 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.

For inquiries, contact :
BioPhenoMA Inc.
info@biophenoma.com