SunLong Biotech Co.,LTD
Tel: 0086-571-56623320 Fax: 0086-571-56623318
E-mail: sales@sunlongbiotech.com
www.sunlongbiotech.com

Sunlong Medical™ Human PD-1/CD279 Sandwich ELISA Kit

Catalog Number: EL0996Hu
Size: 96 Test

Storage: 2-8℃/-20℃
Validity Period: 2-8℃: 6 months; -20℃: 12 months

Sensitivity: 4.5 pg/mL
Assay range: 31.25-2000 pg/mL

For the quantitative detection of human PD-1/CD279 concentrations in serum, plasma, cell culture supernatant, urine

This package insert must be read entirely before using this product. For proper performance, follow the protocol provided with each individual kit.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

ASSAY PROCEDURE SUMMARY 1

Introduction

Description 2

Principle of the Assay 2

Limitations of the Procedure 2

General Information

Materials Provided 3

Storage 3

Other Supplies Required 3

Precaution 3

Technical Hints 4

Assay Protocol

Sample Collection and Storage 4

Reagent Preparation 4

Assay Procedure 5

Analysis

Calculation of Results 6

Typical Data 6

Sensitivity 9

Precision 9

Recovery 10

Linearity 10

Calibration 10

Sample Values 10

Specificity 11

ASSAY PROCEDURE SUMMARY

1. Take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

↓

2. Preset the layout of the microplate, including control group, standard group and sample group, add 100 µL of each standard and sample to the appropriate wells.(Make sure sample addition is uninterrupted and completed within 5 to 10 minutes, It is recommended to assay all standards, controls, and samples in duplicate).

↓

3. Seal plate with cover seal, pressing it firmly onto top of microwells. Incubate the plate for 2 hours at 37°C.

↓

4. Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times with 1X Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

↓

5. Add 100 µL of 1X Detection antibody, HRP-conjugated solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate for 40 minutes at 37°C.

↓

6. Repeat wash step in 8.4.

↓

7. Signal development: Add 100 µL of TMB substrate solution to each well, protected from light. Incubate for 15 to 20 minutes. Substrate Solution should remain colorless until added to the plate.

↓

8. Quenching color development: Add 100 µL of Stop Solution to each well in the same order as addition of the TMB substrate. Mix by tapping the side of the plate gently. NB: Avoid skin and eye contact with the Stop solution.

↓

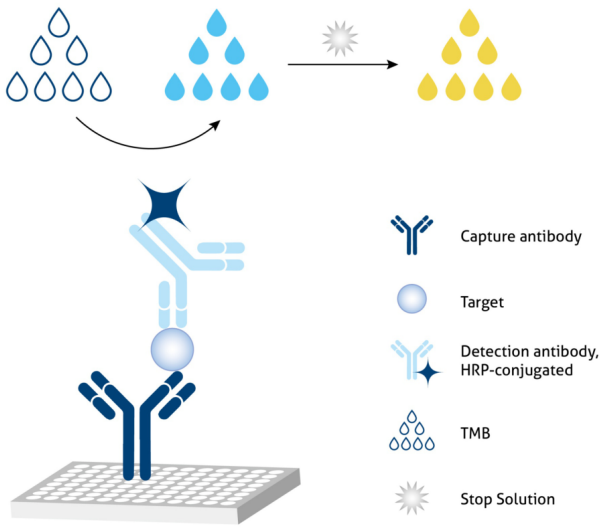
9. Read results: Immediately after adding Stop solution read the absorbance on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

DESCRIPTION

Programmed cell death 1 (PD-1, also known as CD279) is an immunoinhibitory receptor that belongs to the CD28/CTLA-4 subfamily of the Ig superfamily. It is a 288 amino acid (aa) type I transmembrane protein composed of one Ig superfamily domain, a stalk, a transmembrane domain, and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 can be expressed on activated T cells, B cells, natural killer T cells, monocytes, and dendritic cells (DCs). Engagement of PD-1 by its ligands PD-L1 or PD-L2 transduces a signal that inhibits T-cell proliferation, cytokine production, and cytolytic function. It is critical for the regulation of T cell function during tolerance, autoimmunity and infection. Blockade of PD-1 can overcome immune resistance and also has been shown to have antitumor activity.

PRINCIPLE OF THE ASSAY

Sandwich ELISA structure (Detection antibody labeled with HRP) A capture antibody is pre-coated onto the bottom of wells which binds to analyte of interest. A detection antibody labeled with HRP also binds to the analyte. TMB acts as the HRP substrate and the solution color will change from colorless to blue. A stop solution containing sulfuric acid turns solution yellow. The color intensity is proportional to the quantity of bound protein which is measurable at 450 nm with the correction wavelength set at 630 nm.



LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use expired kit or reagents.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- If concentration of assayed factor in samples is higher than the highest standard, dilute the samples with the appropriate Sample Diluent and reanalyze.
- Any variation in testing personnel, sample preparation, standard dilution, pipetting technique, washing techniques, incubation time, temperature, kit age and equipment can cause variation in results.
- This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED (96 Test)

Unopened kit should be stored at 2 - 8℃.

- **Microplate:** antibody coated 96-well microplate (8 well × 12 strips) (1 plate)
- **Protein standard:** 4000 pg/bottle; lyophilized (2 bottles)
- **Detection antibody, HRP-conjugated (100×):** 120 µL/vial* (1 vial)
- **Sample Diluent PT 3:** 30 mL/bottle. For human serum, plasma and urine. (1 bottle)
- **Sample Diluent PT 4B1:** 30 mL/bottle. For cell culture supernatants. (1 bottle)
- **Sample Diluent PT 3-ef:** 30 mL/bottle. For cell lysates. (1 bottle)
- **Detection Diluent:** 30 mL/bottle (1 bottle)
- **Wash Buffer Concentrate (20×):** 30 mL/bottle (1 bottle)
- **Extraction Reagent:** 30 mL/bottle (1 bottle)
- **Tetramethylbenzidine Substrate (TMB):** 12 mL/bottle (1 bottle)
- **Stop Solution:** 12 mL/bottle (1 bottle)
- Plate Cover Seals (4 pieces)

STORAGE

Store kit reagents between 2 and 8℃. Immediately after use remaining reagents should be returned to cold storage (2 to 8℃). Expiration date of the kit and reagents is stated on the labels.

Expiration date of the kit components can only be guaranteed if the components are stored properly.

	Condition
Unopened kit	Store at 2 - 8℃ for 6 months or -20℃ for 12 months. (See expiration date on the label.)
Opened kit	All reagents stored at 2 - 8℃ for 7 days. Please use a new standard for each assay.

OTHER SUPPLIES REQUIRED

- A microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 630 nm.
- Calibrated, adjustable precision pipettes and disposable plastic tips. A manifold multi-channel pipette is recommended for large assays.
- Plate washer: automated or manual.
- Absorbent paper towels.
- Glass or plastic tubes to prepare standard and sample dilutions.
- Beakers and graduated cylinders.
- Log-log or semi-log graph paper or computer and software for ELISA data analysis. A four-parameter logistic (4-PL) curve-fit is recommended.

PRECAUTION

- Avoid any skin and eye contact with Stop Solution and TMB. In case of contact, wash thoroughly with water.
- Do not use the kit after the expiration date.
- Do not mix or substitute reagents or materials from other kit lots or other sources.
- Be sure to wear protective equipment such as gloves, masks and goggles during the experiment.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer to improve assay precision

TECHNICAL HINTS

- Bring all reagents to room temperature before use.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period before washing step and/or rotating the plate between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.
- The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.
- It is recommended that all samples and standards be assayed in duplicate.

SAMPLE COLLECTION AND STORAGE

Serum

Allow blood samples to clot for 30 minutes, followed by centrifugation for 15 minutes at 1000xg. Clear serum can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

Plasma

Use EDTA, heparin, or citrate as an anticoagulant for plasma collection. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. The plasma can be assayed immediately or aliquoted and stored at -20℃. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatant

Remove particulates by centrifugation for 5 minutes at 500xg and assay immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles.

Urine

Collect urine samples and centrifuge for 20 minutes at 1000xg. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20℃. Avoid repeated freeze-thaw cycles.

Cell Lysate

1) Collect cells and wash by centrifuging at 500 x g for 5 minutes before resuspension in pre-cooled PBS buffer. Perform this step three times. 2) Count cells and then discard the supernatant. 3) Add protease inhibitor cocktail to the Extraction Reagent to a final concentration immediately prior to performing cell lysis. 4) Add 1 mL of Extraction reagent (containing protease inhibitor cocktail) Per 1 x 107 cells, Incubate cell suspension on ice for 30 minutes, use ultrasound to treat the samples. 5) Centrifuge cell lysate at 10,000 x g for 5 minutes at 4℃. 6) Measure the concentration of total protein in cell lysate using BCA assay. Where possible, keep samples on ice to avoid protein degradation.

REAGENT PREPARATION

Bring all reagents and samples to room temperature before use. If crystals have formed in the Wash Buffer Concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.

Wash Buffer (1X)

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 30 mL of Wash Buffer Concentrate(20X) to 570 mL deionized or distilled water to prepare 1X Wash Buffer.

Detection Antibody, HRP-conjugated(1X)

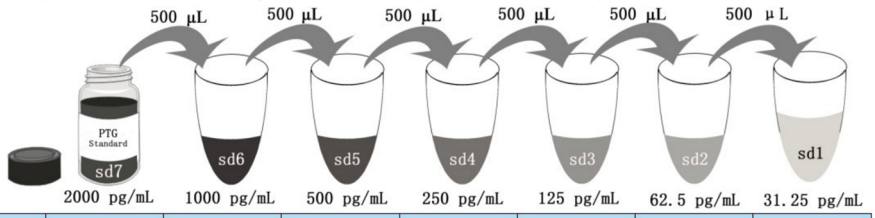
Dilute 100X Detection Antibody, HRP-conjugated 1:100 using Detection Diluent prior to assay. Suggested 1:100 dilution: 10 µL 100X Detection Antibody, HRP-conjugated + 990 µL Detection Diluent (Centrifuge the 100X Detection Antibody solution, HRP-conjugated for a few seconds prior to use)

Sample Dilution

Different samples should be diluted with corresponding Sample Diluent, samples may require further dilution if the readout values are higher than the highest standard OD reading. Variations in sample collection, processing and storage may affect the results of the measurement. Recommended Dilution for different sample types: 1:2 or 1:4 is recommended for human serum, plasma, cell culture supernatant and urine; 1:4 or 1:8 is recommended for cell lysate.

Standard Serial Dilution

For human serum, plasma and urine, add 2mL Sample Diluent PT 3 in protein standard. For cell culture supernatant, add 2mL Sample Diluent PT 4B1 in protein standard. For cell lysate, add 2mL Sample Diluent PT 3-ef in protein standard.



Add # µL of Standard diluted in the previous step	—	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
# µL of Sample Diluent PT 3 or PT 4B1 or PT 3-ef	**2000 µL**	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
	"sd7"	"sd6"	"sd5"	"sd4"	"sd3"	"sd2"	"sd1"

ASSAY PROCEDURE

Bring all reagents to room temperature before use (Detection antibody, HRP-conjugated can be used immediately). To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

1. Take out the required number of microplate strips and return excess strips to the foil pouch containing the drying reagent pack and reseal; store at 4°C immediately. Microplate strips should be used in one week.

2. Preset the layout of the microplate, including control group, standard group and sample group, add 100 µL of each standard and sample to the appropriate wells.(Make sure sample addition is uninterrupted and completed within 5 to 10 minutes, It is recommended to assay all standards, controls, and samples in duplicate).

3. Seal plate with cover seal, pressing it firmly onto top of microwells. Incubate the plate for 2 hours at 37°C.

4. Wash: 1) Gently remove the cover seal. Discard the liquid from wells by aspirating or decanting. Remove any residual solution by tapping the plate a few times on fresh paper towels. 2) Wash 4 times with 1X Wash Buffer, using at least 350-400 µL per well. Following the last wash, firmly tap plates on fresh towels 10 times to remove residual Wash Buffer. Avoid getting any towel fibers in the wells or wells drying out completely.

5. Add 100 µL of 1X Detection antibody, HRP-conjugated solution (refer to Reagent Preparation 7.2) to each well. Seal plate with cover seal and incubate for 40 minutes at 37°C.

6. Repeat wash step in 8.4.

7. Signal development: Add 100 µL of TMB substrate solution to each well, protected from light. Incubate for 15 to 20 minutes. Substrate Solution should remain colorless until added to the plate.

8. Quenching color development: Add 100 µL of Stop Solution to each well in the same order as addition of the TMB substrate. Mix by tapping the side of the plate gently. NB: Avoid skin and eye contact with the Stop solution.

9. Read results: Immediately after adding Stop solution read the absorbance on a microplate reader at a wavelength of 450 nm. If possible, perform a double wavelength readout (450 nm and 630 nm).

Step	Reagent	Volume	Incuba-tion	Wash	Notes
1	Standard and Samples	100 µL	120 min	4 times	Cover Wells incubate at 37°C
2	Diluent Detection antibody, HRP-conjugated Solution	100 µL	40 min	4 times	Cover Wells incubate at 37°C
3	TMB Substrate	100 µL	15-20 min	Do not wash	Incubate in the dark at 37°C
4	Stop Solution	100 µL	0 min	Do not wash	-
5	Read plate at 450 nm and 630 nm immediately after adding Stop solution. DO NOT exceed 5 minutes.				

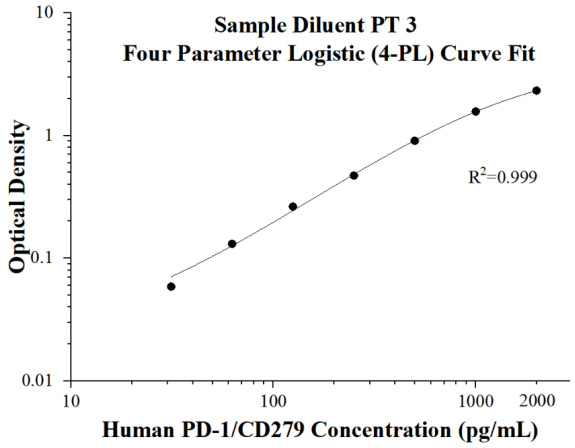
CALCULATION OF RESULTS

Data analysis: Calculate the average of the duplicate readings (OD value) for each standard and sample, and subtract the average of the zero standard absorbance. Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, use four-parameter logistic curvefit (4-PL) analysis to do this. If the samples have been diluted, the OD readout from the standard curve must be multiplied by the dilution factor used.

TYPICAL DATA

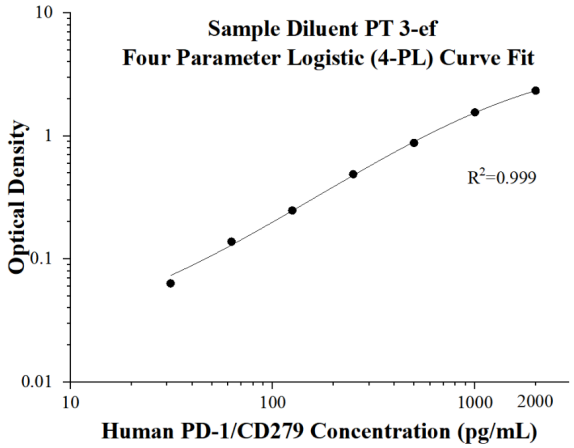
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Standard curve 1



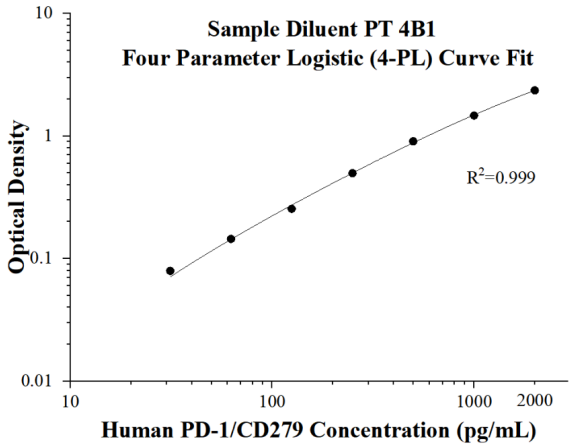
(pg/mL)	O.D.	Average	Corrected
0	0.0655 0.065	0.06525	-
31.25	0.1275 0.1204	0.12395	0.0587
62.5	0.1996 0.1928	0.1962	0.13095
125	0.3299 0.3283	0.3291	0.26385
250	0.5404 0.5326	0.5365	0.47125
500	0.9607 0.9839	0.9723	0.90705
1000	1.6478 1.6279	1.63785	1.5726
2000	2.3961 2.3825	2.3893	2.32405

Standard curve 2



(pg/mL)	O.D.	Average	Corrected
0	0.0431 0.0429	0.043	-
31.25	0.1055 0.1072	0.10635	0.06335
62.5	0.1786 0.1841	0.18135	0.13835
125	0.295 0.2871	0.29105	0.24805
250	0.5391 0.5255	0.5323	0.4893
500	0.9441 0.8977	0.9209	0.8779
1000	1.6173 1.5803	1.5988	1.5558
2000	2.3623 2.3933	2.3778	2.3348

Standard curve 3



(pg/mL)	O.D.	Average	Corrected
0	0.0691 0.0838	0.07645	-
31.25	0.1458 0.1659	0.15585	0.0794
62.5	0.2178 0.2247	0.22125	0.1448
125	0.3377 0.3246	0.33115	0.2547
250	0.6033 0.5444	0.57385	0.4974
500	0.9994 0.9638	0.9816	0.90515
1000	1.5961 1.5005	1.5483	1.47185
2000	2.4483 2.4263	2.4373	2.36085

SENSITIVITY

The minimum detectable dose of human PD-1/CD279 is 4.5 pg/mL. This was determined by adding two standard deviations to the concentration corresponding to the mean O.D. of 20 zero standard replicates.

PRECISION

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested 8 times on one plate to assess intra-assay precision. Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in 16 separate assays to assess inter-assay precision.

Intra-assay Precision

Sample	n	Mean (pg/mL)	SD	CV%
1	8	948.7	63.9	6.7
2	8	253.2	11.2	4.4
3	8	134.9	6.3	4.7

Inter-assay Precision

Sample	n	Mean (pg/mL)	SD	CV%
1	16	952.0	55.4	5.8
2	16	257.2	13.7	5.3
3	16	140.7	10.6	7.5

RECOVERY

The recovery of human PD-1/CD279 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type		Average% of Expected	Range (%)
Human plasma	1:4	94	88-98
Cell culture supernatant	1:2	112	110-114
	1:4	109	107-112
Urine	1:8	95	91-100
	1:16	94	88-98
Cell lysate	1:16	99	84-113
	1:32	103	101-106

LINEARITY

To assess the linearity of the assay, samples were diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. (The cell lysate was initially diluted 1:2.)

		Human plasma (Sample Diluent PT 3)	Cell culture supernatant (Sample Diluent PT 4B1)	Urine (Sample Diluent PT 3)	Cell lysate (Sample Diluent PT 3-ef)
1:2	Average% of Expected	100	100	100	100
	Range (%)	-	-	-	-
1:4	Average% of Expected	89	94	108	99
	Range (%)	84-95	83-105	101-115	98-100
1:8	Average% of Expected	89	85	113	109
	Range (%)	85-93	75-96	99-127	108-110
1:16	Average% of Expected	90	-	109	127
	Range (%)	89-91	-	108-109	122-131

CALIBRATION

This immunoassay is calibrated against a highly purified recombinant **human Human PD-1/CD279 Sandwich ELISA Kit** produced at Sunlong Medical™.

SAMPLE VALUES

Human plasma/Urine - human plasma and urine were evaluated for the presence of human PD-1/CD279 in this assay.

Cell culture supernatant - Human peripheral blood mononuclear cells (PBMC) (1 x 106 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 5µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human PD-1/CD279. ND*=Non-detectable Cell lysate

Sample Type	Mean (pg/mL)	Range (pg/mL)
Human plasma (n=16)	196.5	51.5-1,362.8
Urine (n=8)	320.4	96.5-739.7

Condition	Day 5 (pg/mL)
Unstimulated	ND
Stimulated	277.9

	Human PD-1/CD279 (pg/mL)	Total protein (mg/mL)
MOLT-4 cell lysate	2,150.3	1.2

SPECIFICITY

This kit specifically recognizes native and recombinant human PD-1/CD279.

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												