Human PD-1 ELISA Kit

For the quantitative determination of human Programmed Death-1 (PD-1) concentrations in biological samples.

Catalogue Number: EL10059
96 tests

FOR LABORATORY RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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TABLE OF CONTENTS

INTENDED USE

This human PD-1 ELISA kit is to be used for the *in vitro* quantitative determination of human Programmed Death-Ligand 1 (PD-1) concentrations in cell culture supernatant and other biological fluids. This kit is intended for LABORATORY RESEARCH USE ONLY.

INTRODUCTION

Programmed Cell Death-1 (PD-1) is a receptor that is elevated on activated T cells, B cells and macrophages. PD-1 is a checkpoint co-inhibitor of T cell-associated immune response. When T cell receptor (TCR) engages with the foreign/cancer antigen, PD-1 is joined with its ligand, programmed death ligand-1 (PD-L1) on the antigen presenting cells simultaneously and triggers an inhibitory signal to suppress IL-2 production and antigen specific CD8+ T cell proliferation. In addition, PD-1/PDL-1 binding promotes regulatory T cells (Tregs) development and function. PD-L1 is expressed abundantly in immune-privileged organs such as placenta, eyes and testes and plays a role in tolerance. Upregulation of PD-L1 has been found in many cancer types, such as bladder cancer, non-small cell lung cancer (NSCLC), renal cancer, melanoma, ovarian cancer, and breast cancer. Evidences showed that the PD-1/PD-L1 pathway is associated with autoimmunity (1, 2, 4, 6), cancer's immune evasion (8), the antigen-specific CD4+ T cell exhaustion during some chronic infection, and neurodegeneration in Alzheimer's disease.

PD-L2, another ligand of PD-1, is mainly expressed in macrophages and dendritic cells and has a low constitutive basal expression. PD-L2 plays a role in modulation of Th2 type of immune response, while PD-L1 is an important negative regulator to Th1 response which is more relevant to cancer immunity (5).

Soluble PD-1 (sPD-1) can be detected in blood. Increased sPD-1 expression is associated with autoimmunity and with the systemic inflammatory condition in cancer patients (3). Characterization of sPD-1 in aplastic anemia patients revealed that it is an alternative slice variant of PD-1, which lacks the transmembrane domain (9). Evidences suggest that sPD-1 may upregulate T cell immunity by blocking the binding of membrane PD-1 with its ligands. It has been observed that increased sPD-1 is correlated with better prognosis in erlotinib treated advanced EGFR-mutated non-small cell lung cancer patients (7).

The PD-1 ELISA kit is for measurement of PD-1 in cell culture and other biological samples. The kit is for research only. The detected value of PD-1 can only be used as reference for scientific study. The kit should not be used in any diagnostic or therapeutic procedures.

PRINCIPLE OF THE ASSAY

This enzyme-linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific for human PD-1. When standards or samples are added to the appropriate microtiter plate wells, human PD-1 in the standards or samples will be immobilized by the pre-coated antibody during incubation. A biotin-conjugated antibody preparation specific for human PD-1 is added to each well and incubated. The biotin labelled antibody attaches to the wells by binding to human PD-1. After plate washing, other proteins, components and unattached biotin labelled antibody is removed. Then, avidin-horseradish peroxidase (HRP) conjugate is added to each well. Avidin has a very high affinity for biotin, thus, it links the tracer (HRP) sturdily to the biotin labelled antibody. The wells are thoroughly washed to remove all unbound avidin-HRP conjugate and a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only wells that contain human PD-1 will exhibit a change in colour. The extent of colour change is proportional to the quantity of human PD-1 presented in the standards/samples. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wave length of 450 nm ± 2 nm.

According to the testing system, the provided standard is diluted (2-fold) with the appropriate diluent and assayed at the same time as the samples. This allows the operator to produce a curve of Optical Density (O.D.) versus human PD-1 concentration (pg/mL) in standards. The concentration of human PD-1 in the samples is then determined by comparing the O.D. of the samples to the standard curve and multiplying with sample dilution factor.

REAGENTS PROVIDED

All reagents provided are stored at 2-8°C. Refer to the expiration date on the label.

	96 tests

1. **HUMAN PD-1 MICROTITER PLATE** (Part EL59-1) ***96 wells***
   Pre-coated with anti-human PD-1 monoclonal antibody

2. **BIOTIN CONJUGATE** (Part EL59-2) ***6 mL***
   Anti-human PD-1 monoclonal antibody conjugated to biotin

3. **AVIDIN-HRP CONJUGATE** (Part EL59-3) ***12 mL***
   Avidin conjugated to horseradish peroxidase

4. **HUMAN PD-1 STANDARD** (Part EL59-4) ***2 vials***
   Recombinant human PD-1 (2000pg/vial) in a buffered protein base with preservative, lyophilized.

5. **CALIBRATOR DILUENT I** (Part EL59-5) ***25 mL***
   Buffered bovine serum with preservative

6. **CALIBRATOR DILUENT II** (Part EL59-6 ***25 mL***
   Cell culture medium with bovine serum and preservative

7. **WASH BUFFER (20X)** (Part 30005) ***60 mL***
   20-fold concentrated solution of buffered surfactant.

8. **TMB SUBSTRATE** (Part 30010) ***11 mL***
   TMB solution

9. **STOP SOLUTION** (Part 30008) ***14 mL***
   2N Sulphuric Acid (H_2SO_4). Caution: Caustic Material!

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips (10-100μL and 50-200μL) for running the assay.
2. Pipettes: 1 mL, 5 mL, and 10 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Eppendorf tubes and racks.
5. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
6. Microtiter plate reader (450 nm ± 2nm)
7. Automatic microtiter plate washer or squirt bottle.
8. Sodium hypochlorite solution, 5.25% (household liquid bleach).
9. Deionized or distilled water.
10. Plastic plate cover.
11. Disposable gloves.
12. Absorbent paper.

PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. All biological samples should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn and good laboratory practices should be followed during the assay procedure.
9. All biological samples should be disposed of in a manner that will inactivate viruses.
   Solid Wastes: Autoclave for 60 minutes at 121°C.
   Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate viruses before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form which must be dissolved by warming to 37°C prior to use.

SAMPLE PREPARATION

STORAGE AND DILUTION:

Samples should be assayed immediately, or be stored at -20°C or lower temperature for long term storage. Bring samples to room temperature prior to the assay. Use CALIBRATOR DILEUNT or a buffer similar to the samples to dilute standards and samples.

PREPARATION OF REAGENTS

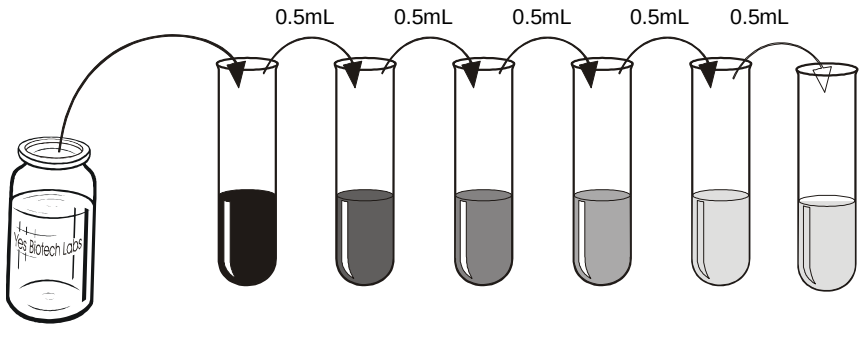
Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as below.

1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water.

2. **Human PD-1 Standard:** Two vials of PD-1 standard are included in the kit. The standard is a human cell expressed recombinant PD-1 protein.

   a) Reconstitute the **Human PD-1 Standard** with 2mL of appropriate Calibrator Diluent. This reconstitution produces a stock solution of 1000pg/mL. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstitution. Aliquot the stock and store frozen at -20°C (short term) or -70°C (long term) if repeated use is expected.

   b) Use the above stock solution to produce a 2-fold serial dilution within the range of this assay (15.6pg/mL to 1000pg/mL) as illustrated. Between each test tube transfer, be sure to mix contents thoroughly. The undiluted human PD-1 standard stock will serve as the high standard (1000pg/mL) and the Calibrator Diluent will serve as the zero-standard (0pg/mL).



1000pg/mL 500pg/mL 250pg/mL 125pg/mL 62.5pg/mL 31.25pg/mL 15.6pg/mL

ASSAY PROCEDURE

1. Prepare Wash Buffer (1X), human PD-1 Standards and Samples before starting assay procedure (Refer to Sample Preparation and Preparation of Reagents). *It is recommended that the table and diagram provided be used as a reference for adding Standards or Samples to the Microtiter Plate.*

Wells	Contents	**Wells**	Contents
1A, 1B	Standard 1 - **0pg/mL** (S1)	**2A, 2B**	Standard 5 – **125pg/mL** (S5)
1C, 1D	Standard 2 – **15.6pg/mL** (S2)	**2C, 2D**	Standard 6 – **250pg/mL** (S6)
1E, 1F	Standard 3 – **31.25pg/mL** (S3)	**2E, 2F**	Standard 7 – **500pg/mL** (S7)
1G, 1H	Standard 4 – **62.5pg/mL** (S4)	**2G,2H**	Standard 8 – **1000pg/mL** (S8)
3A….12H			

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S1	S5	1	5	9	13	17	21	25	29	33	37
B	S1	S5	1	5	9	13	17	21	25	29	33	37
C	S2	S6	2	6	10	14	18	22	26	30	34	38
D	S2	S6	2	6	10	14	18	22	26	30	34	38
E	S3	S7	3	7	11	15	19	23	27	31	35	39
F	S3	S7	3	7	11	15	19	23	27	31	35	39
G	S4	S8	4	8	12	16	20	24	28	32	36	40
H	S4	S8	4	8	12	16	20	24	28	32	36	40

2. Add 100µL of Standards or Samples to the appropriate wells of anti-human PD-1 antibody pre-coated microtiter plate. Cover and incubate for 1 hour at room temperature.

3. Without discarding the standards and samples, add 50µL of Anti-human PD-1 Biotin Conjugate to each well and mix briefly on ELISA plate shaker.

4. Cover and incubate for 1 hour at room temperature.

5. Wash the Microtiter Plate using one of the specified methods indicated below:

   Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note*: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

   Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and

set fill volume at 350μL/well/wash (range: 350-400 μL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

6. Repeat wash procedure as described in Step 5.

7. Dispense 100μL of Avidin-HRP conjugate to each well. Cover and incubate for 1 hour at room temperature.

8. Repeat wash procedure as described in Step 5.

9. Add 100μL TMB Substrate to each well. Cover and incubate for 15 minutes at room temperature.

10. Add 100μL Stop Solution to each well. Mix well.

11. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 30 minutes.

CALCULATION RESULT

The standard curve is used to determine the amount of human PD-1 in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding human PD-1 concentration (pg/mL) on the horizontal (X) axis.

1. First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0pg/mL) before result interpretation. Construct the standard curve using graph paper or statistical software.

2. To determine the amount of human PD-1 in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding human PD-1 concentration. If samples generate values higher than the highest standard, dilute the samples with the appropriate Diluent and repeat the assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

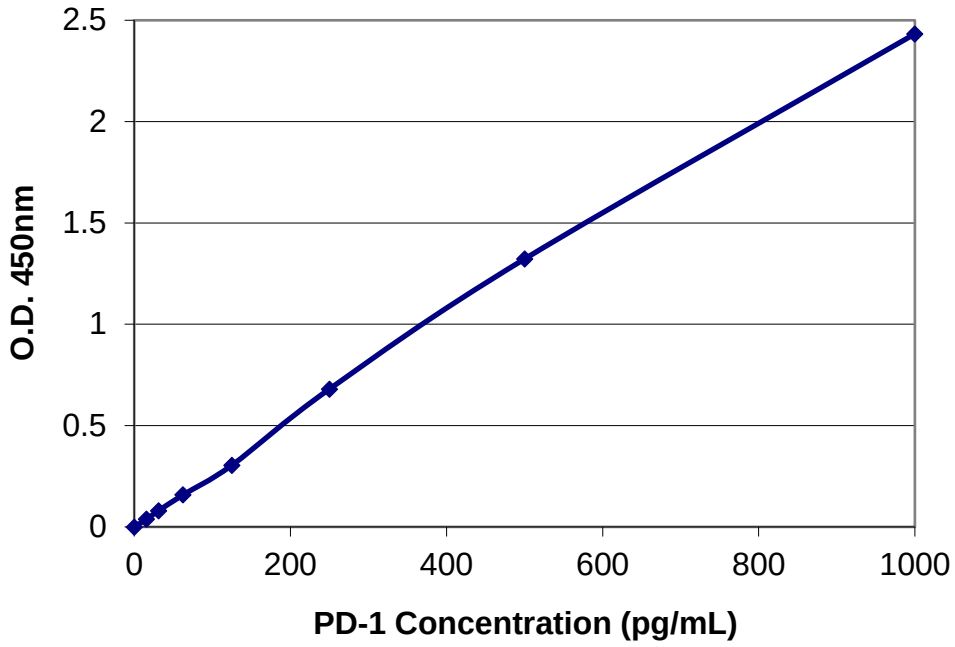
Results of a typical standard run of a human PD-1 ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or

temperature, and kit age can cause variation in result. The following examples are for the purpose of illustration only, and should not be used to calculate user results.

EXAMPLE I

The following data and standard curve were obtained using CALIBRATOR DILUENT I.

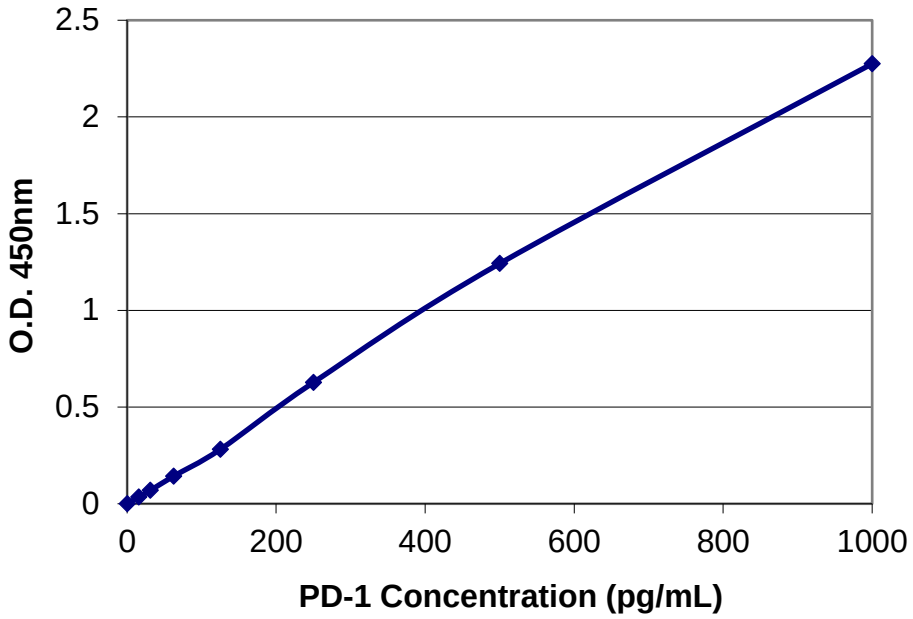
Standard (pg/mL)	**Mean O.D. (450 nm)**	**%CV**	**Zero Standard Subtracted** (Std.)-(S1)
0	0.062	4.12	0.000
15.6	0.100	0.70	0.038
31.25	0.142	3.98	0.080
62.5	0.220	1.29	0.158
125	0.366	0.39	0.304
250	0.744	1.33	0.682
500	1.384	1.28	1.322
1000	2.495	0.03	2.433



EXAMPLE II

The following data and standard curve were obtained using CALIBRATOR DILUENT II.

Standard (pg/mL)	Mean O.D. (450 nm)	%CV	Zero Standard Subtracted (Std.)-(S1)
0	0.065	1.06	0.000
15.6	0.100	7.07	0.035
31.25	0.134	1.06	0.069
62.5	0.208	1.70	0.143
125	0.346	2.86	0.281
250	0.692	2.04	0.627
500	1.307	0.97	1.242
1000	2.343	1.48	2.275



RFORMANCE CHARACTERISTICS

1. INTRA-ASSAY PRECISION

To determine within-run precision, three different samples of PD-1 at low, medium and high level, were assayed in 16 replicates in 1 assay.

Sample	1	2	3
N	16	16	16
Mean (pg/ml)	28.63	121.68	526.54
Coefficient of Variation (%)	4.55	2.25	1.42

2. INTER-ASSAY PRECISION

To determine between-run precision, three different samples of PD-I at low, medium and high level, were measured in 8 different assays.

Sample	1	2	3
N	8	8	8
Mean (pg/mL)	32.51	119.60	503.55
Coefficient of Variation (%)	4.7	4.4	3.0

3. RECOVERY

Human serum samples were spiked with different concentrations of PD-1. The recovery rates at low, medium and high level are listed below.

PD-1 added to serum	Range of Recovery Rate%	Average of Recovery Rate %
31.2 pg/mL	105-111%	107%
125 pg/mL	95-99%	96%
500 pg/mL	103-107%	104%

4. SENSITIVITY

The minimum detectable dose of human PD-1 was determined by adding two standard deviations to the mean optical density value of 16 zero standard replicates and calculating the corresponding concentration from the standard curve. The minimum detectable dose of human PD-1 calculated from calibrate diluent I diluted standard curve was <0.44pg/mL.

5. CROSS-REACTIVITY

The kit was tested with 31 different native and recombinant human proteins. No cross-reactivity were found.

6. SAMPLE TEST

100 normal human serum and plasma samples were tested. sPD-1 concentration in serum samples ranged from 4pg/mL to 530pg/mL. The average concentration of sPD-1 in 25 serum samples were 68pg/mL. sPD-1 concentrations in plasma samples ranged from 4pg/mL to 1444pg/mL. The average concentration of sPD-1 in 75 plasma samples were 194pg/ML.

REFERENCES

1. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunological Review. 2010;236:219–42
2. Kong EK, Prokunina-Olsson L, Wong WH, Lau CS, Chan TM, Alarcon-Riquelme M, et al. A new haplotype of *PDCD1* is associated with rheumatoid arthritis in Hong Kong Chinese. Arthritis and Rheumatology. 2005;52:1058–62.
3. Kruger S, legenstein ML, Rosgen V, Haas M, Modest DP, Westphalen CB, Ormanns S, Kirchner T, Heinemann V, Holdenrieder S, Boech S. Serum levels of soluble programmed death protein 1 (sPD-1) and soluble programmed death ligand 1 (sPD-L1) in advanced pancreatic cancer. Oncoimmunology 2017 31:6(5): e1310358
4. Newby PR, Roberts-Davies EL, Brand OJ, Heward JM, Franklyn JA, Gough SC, et al. Tag SNP screening of the *PDCD1* gene for association with Graves' disease. Clinical Endocrinology. 2007;67:125–8
5. Rozali EN, Stanleyson VH, Bruce WR, Richard AL and Lesterhuis. Programmed death ligand 2 in cancer-induced immune suppression. Clinical and developmental immunology. 2012 Article ID 656340
6. Suarez-Gestal M, Ferreiros-Vidal I, Ortiz JA, Gomez-Reino JJ, Gonzalez A. Analysis of the functional relevance of a putative regulatory SNP of PDCD1, PD-1.3, associated with systemic lupus erythematosus. Genes and Immunity 2008;9:309–15
7. Sorensen SF, Demuth C., Weber B, Sorensen BS, Meldgaard P. Increase in soluble PD-1 is associated with prolonged survival in patients with advanced EGFR-mutated non-small cell lung cancer treated with erlotinib. Lung Cancer. 2016: 100:77-84
8. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. Journal of clinical oncology. 2016: 33(17):1975-82
9. Wu H, Miao M, Zhang G, Hu Y, Ming Z, Zhang X. Soluble PD-1 is associated with aberrant regulation of T cells activation in aplastic anema. Immunological Investigations. 2009:38(5):408-21