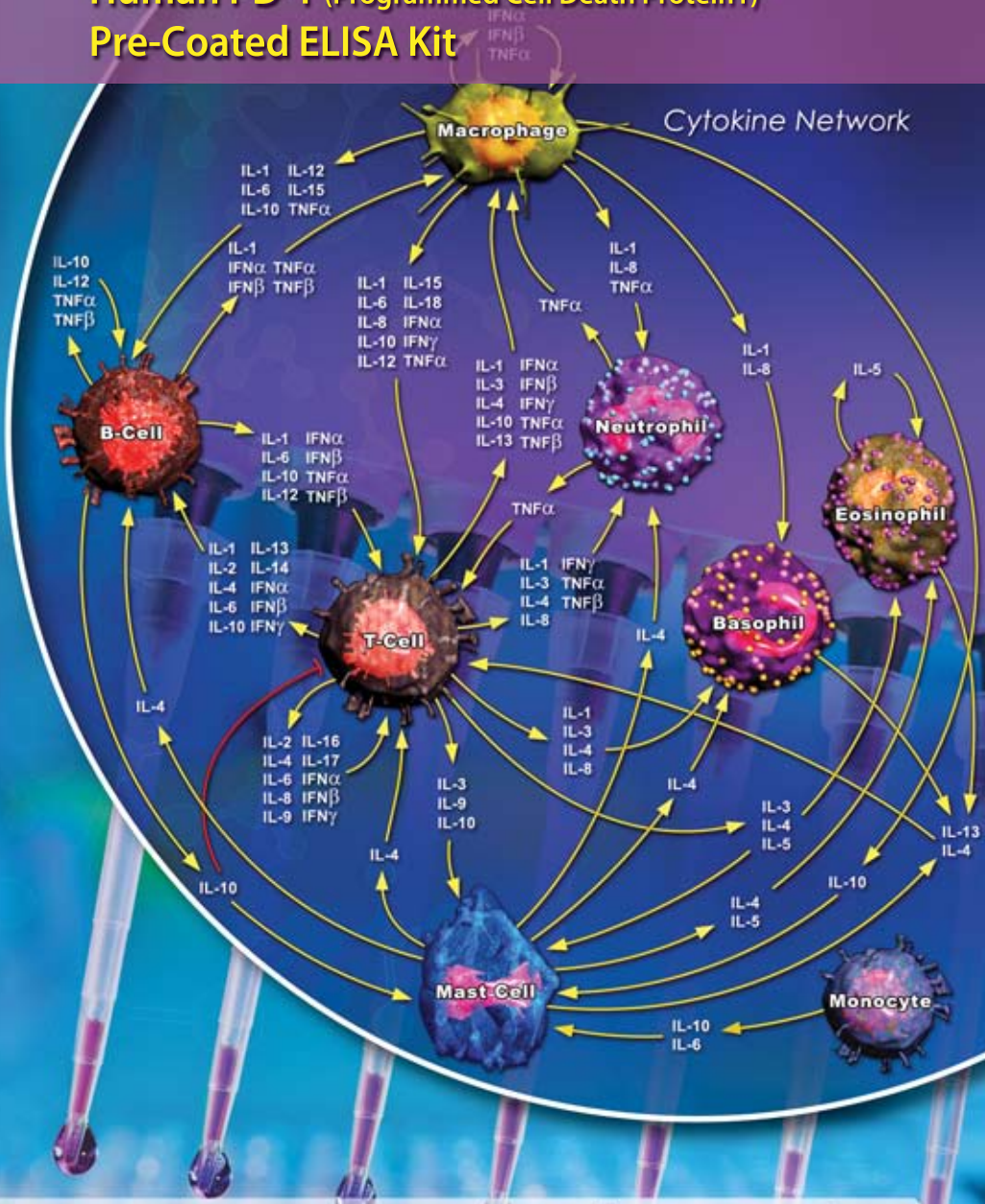
Human PD-1 (Programmed Cell Death Protein1) Pre-Coated ELISA Kit



USER MANUAL

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Human PD-1

(Programmed Cell Death Protein1)

Pre-Coated ELISA Kit

Catalog No: 90-2111
1 × 96 well Format (96 tests)
Detection Range: 0.156 – 10 ng/ml
Sensitivity: < 0.094 ng/ml

This immunoassay kit allows for the in vitro quantitative determination of Human PD-1 concentrations in serum, plasma and other biological fluids.

This kit is for Research Use Only. Not for use in diagnostic/therapeutics procedures.

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I. BACKGROUND

As a member of the CD28/B7 family and co-inhibitory molecules, PD-1 (programmed death 1) and its ligands (PD-Ls), PD-L1 and PD-L2, have been demonstrated to play an important role in autoimmune disease, organ transplant rejection, micro-organism infection, and tumor immune escape. PD-1 is not only a major regulator of apoptosis, which can impact antiviral T cells in chronic infections, but it also has significant effects on cytokine production, such as IFN-γ, TNF-α, and IL-2. PD-1 is expressed more in $CD4^+$ T cells than in $CD8^+$ T cells. Its expression was also detected on lymphocytes.

PD-1 negatively regulates T-cell receptor signaling and decrease proliferation and cytokine production in T cells. Recently, PD-1 has been demonstrated to be a major component in the process of T-cell exhaustion although complex mechanisms are involved in inducing exhaustion. It has been shown that TIM-3, a family member of T-cell immunoglobulin and mucin domain proteins, along with other molecules, are co-expressed with PD-1 on exhausted T cells. The role of PD-1 in human autoimmune diseases, such as T1AD, has been studied using animal models. PD-1 deficiency accelerates the onset and frequency of T1AD in NOD (non-obese diabetic) mice. PD-1 deficiency in antigen-specific $CD4^+$ T cells in NOD mice resulted in increased T-cell numbers in the spleen, pancreatic lymph nodes and pancreas, and induced T1AD.

II. OVERVIEW

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti- Human PD-1 antibody was pre-coated into 96-well plates. Biotin conjugated anti-human PD-1 detection antibody was used. Standards, test samples and biotin conjugated detection antibody were added to the wells subsequently. Wash buffer was used to wash any non-specific binding. HRP conjugated Streptavidin was used as secondary antibody. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the Human PD-1 amount of samples captured in the plate. Optical Density (O.D) can be read at absorbance 450nm in a microplate reader. Concentration of Human PD-1 can be calculated using the standard curve.

III. ADVANTAGES

Multiple samples can be analyzed in a low volume, high-throughput format.

Full analysis can be complete in 4 hours.

IV. STORAGE

Kit can be stored in 4°C, if you are using within a week.

If you are using within 6 months, lyophilized standard can be stored in -20°C and other components at 4°C.

Kit Components

Item	Specifications	Storage
96 well Strip ELISA Plate	8 X 12 well	4°C/-20°C
Lyophilized Standard	2 vials	4°C /-20°C
Sample and Standard Dilution Buffer	20 ml	4°C
Biotinylated Detection Antibody for PD-1	120 µl	4°C/-20°C
Antibody Dilution Buffer	10 ml	4°C
HRP Conjugated Streptavidin (SABC)	120 µl	4°C in dark
SABC Dilution Buffer	10 ml	4°C
TMB Substrate	10 ml	4°C in dark
Stop Solution	10 ml	4°C
25X Wash Buffer	30 ml	4°C
Plate Sealer	5 pieces	
Product Manual	1	

Material Required, (Not Supplied)

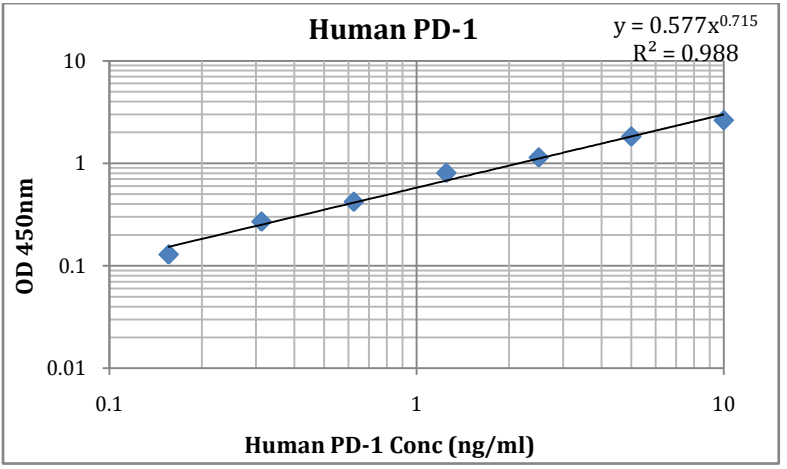
Microplate Reader
37°C Incubator
Plate Reader
Multi Chanel Pipette and disposable tips
Eppendorf Tubes
Deionized Water

V. PRECAUTIONS FOR USE

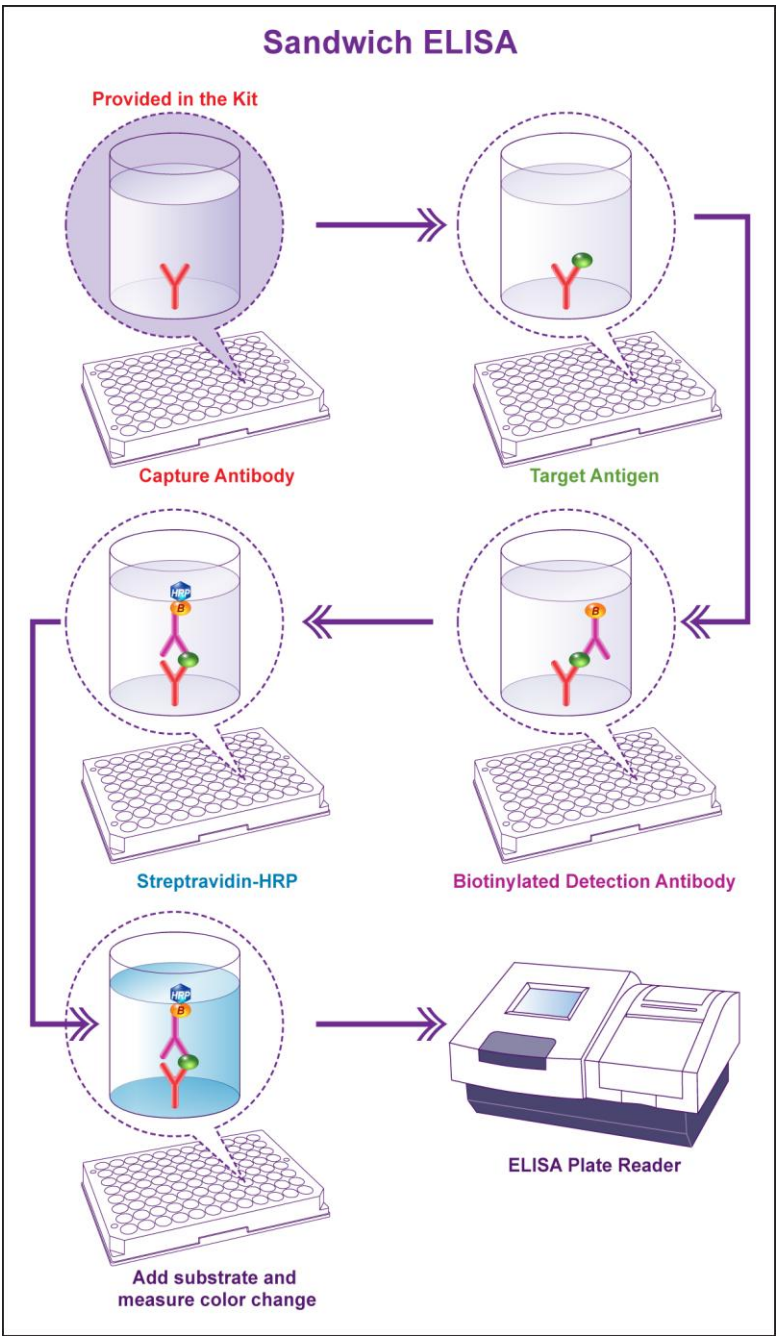
1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let Micro plate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.

VI. STANDARD CURVE

Human PD-1 Standard Curve is shown below.



X	pg/ml	10	5	2.5	1.25	0.625	0.313	0.156	0
Y	O.D.450	2.667	1.855	1.169	0.833	0.448	0.296	0.155	0.026


Sandwich ELISA
Provided in the Kit
Capture Antibody
Target Antigen
Biotinylated Detection Antibody
Streptravidin-HRP
ELISA Plate Reader
Add substrate and
measure color change

VII. REAGENT PREPARATION AND STORAGE

Included buffers and reagents are optimized for use with this kit. Substitution with other reagents is not recommended and may not give optimal results.

1. **Reconstitute the lyophilized Standard:** Standard should be prepared no more than 2 hours before the experiment. Use one tube for each experiment.
   - **a.** Quick spin down one vial of lyophilized standard. ***(DO NOT dilute standard directly on the plate)***. Add 1ml of sample/standard dilution buffer into one of the standard tube. Incubate at room temp. for 10 min. Mix thoroughly by vortex. Stock Standard concentration is 10 ng/ml.
   - **b.** Label 6 eppendorf tubes with 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, 0.156 ng/ml respectively. Add 0.3 ml of sample/standard dilution buffer into each tube. Add 0.3 ml of stock standard (10 ng/ml) into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube mix thoroughly, and so on.

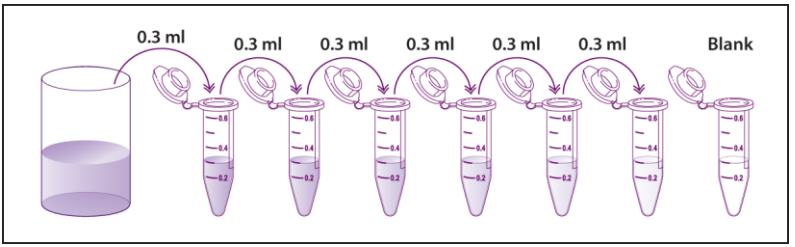


Fig-1: Dilution tubes

Note: *Standard Solutions are best used within 2 hrs. Standard solution should be stored at 4°C for up to 12 hrs. or store at -20°C for up to 48 hrs. Avoid repeated freeze-thaw.*

2. **Sample Preparation and storage:** Test samples should be collected, analyze immediately (within 2 hrs.) or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.
   - **a.** **Cell culture supernatants**: Centrifuge to remove precipitate, analyze immediately or aliquot and store at -20°C.
   - **b.** **Serum**: Coagulate the serum at room temp about 1 hr. Centrifuge approximately 1000 × g for 15 min. Analyze serum immediately or aliquot and store at -20°C.

c. **Plasma:** Collect plasma with heparin or EDTA as the anti-coagulant. Centrifuge for 15 min at 2-8°C at 1500 × g within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at 2-8°C at 10,000 × g. Analyze immediately or aliquot and store frozen at -20°C.

d. **Tissue Homogenates**: For general information, hemolytic blood may affect the results, you should rinse the tissues with ice cold PBS (0.01M, pH 7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces. This will be homogenized in PBS in a cold glass homogenizer. *(Volume depends on the weight of the tissue, 1gram of tissue requires 9 ml of ice cold PBS with protease inhibitor)*. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze- thaw cycle. Homogenates are then centrifuged for 5 min. at 5000 × g to get the supernatant.

Note: *Samples to be used within 5 days may be store at 4°C, otherwise sample should be stored at -20°C (< 1 month) or -80°C (< 2 months) to avoid loss of bioactivity and contamination.* *Hemolyzed samples are not suitable for use in this Assay.*

e. End user should estimate the concentration of the target protein in the test samples first, then select proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit. Dilute the samples with the provided dilution buffer. Several trials may be necessary in practice. The test sample should be well mixed with the dilution buffer. Standard curve and sample should be made before the experiment.

High target protein concentration 100-1000 ng/ml: Dilute 1:100 (add 1 µl of sample into 99 µl of sample/standard dilution buffer).

Medium target protein concentration 10-100 ng/ml: Dilute 1:10 (add 10 µl of sample into 90 µl of sample/standard dilution buffer).

Low target protein concentration 0.156-10 ng/ml: Dilute 1:2 (add 50 µl of sample into 50 µl of sample/standard dilution buffer).

Very low target protein concentration < 0.156 ng/ml: Do not dilute, use 100 µl of sample.

3. **Preparation of Biotin detection antibody working solution:** Prepare within one hour before the experiment. Calculate total volume working solution required. (0.1 ml/well × number of wells. Add 100-200 µl extra).

   Dilute Biotin detection antibody with antibody dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 µl of Biotin conjugated detection antibody into 99 µl of antibody dilution buffer).

4. **Preparation of HRP-Streptavidin Conjugate (SABC) working solution:** Prepare within 30 min before the experiment. Calculate total volume working solution required. (0.1 ml/well × number of wells. Add 100- 200 µl extra).

   Dilute SABC with SABC dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 µl of SABC into 99 µl of SABC dilution buffer).

5. **Preparation of 1 X Wash buffer:** Prepare 1 X Wash buffer by diluting 25X Wash buffer in sterile water. Diluted Wash buffer may be stored at 4°C, however we recommend preparing fresh 1X wash buffer for each experiment.

   For example: *10 ml of 25X Wash buffer in 240 ml of sterile water.*

VIII. ASSAY PROCEDURE

Before starting the experiment, equilibrate the SABC working solution and TMB substrate for at least 30 min at room temp. When diluting samples and reagents, they should be mixed completely and evenly. It is recommended to plot a standard curve for each test.

** If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.*

1. Set standard, test sample and blank (control zero) wells on the pre-coated plate and then record their position. It is recommended to measure each standard and sample in duplicate.

   Note: *Wash plate twice before adding standard, sample and blank into the well.*

2. Add 0.1 ml of standard (10 ng/ml, 5 ng/ml, 2.5 ng/ml. 1.25 ng/ml, 0.622 ng/ml, 0.311 ng/ml, 0.156 pg/ml, control zero dilution buffer) into standard well.
3. Add 0.1 ml of diluted samples into test sample wells.
4. Seal plate with a cover and incubate at 37°C for 90 min.
5. Remove the cover and discard samples and standard solution by tapping plate on an absorbent paper.

   Note: *DO NOT let the wells completely dry any time. DO NOT wash plate.*
6. Add 0.1 ml of Biotin-detection antibody working solution into the above wells (Standards, control zero and samples).
7. Seal plate with cover and incubate at 37°C for 60 min.
8. Remove the cover, and wash plate 3 times with 1X wash buffer.
9. Add 0.1 ml of SABC working solution into each well. Cover the plate and incubate at 37°C for 30 min.
10. Remove the cover and wash plate 5 times with 1X wash buffer. Each time let the wash buffer stay in the well for 1-2 min.
11. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. *(**Note**: This incubation time is for reference use only. The optimal time should be determined by end user)*. The shades of blue can be seen in the first 3-4 wells, only on most concentrated standards. Other wells show no obvious color.
12. Add 50 µl of stop solution into each well and mix thoroughly. Color will change into yellow immediately.
13. Read O.D. absorbance at 450 nm in a micro-plate reader immediately after adding the stop solution.
14. Calculation: Relative O.D. 450 = O.D. for each well – O.D. 450 control zero well. The Standard curve can be plotted as the relative O.D. 450 of each standard solution in Y axis vs. the respective concentration of the standard in X axis. Concentration of the samples can be incorporated from the standard curve. If the samples were diluted, multiply the dilution factor to the concentration.

Table-1

	Standard 1	Standard 2	3	4	5	6	7	8	9	10	11	12
A	**10 ng/ml**	**10 ng/ml**										
B	**5 ng/ml**	**5 ng/ml**										
C	**2.5 ng/ml**	**2.5 ng/ml**										
D	**1.25 ng/ml**	**1.25 ng/ml**										
E	**0.622 ng/ml**	**0.622 ng/ml**										
F	**0.311 ng/ml**	**0.311 ng/ml**										
G	**0.156 ng/ml**	**0.156 ng/ml**										
H	**0**	**0**										

IX. REFERENCES

1. Programmed Death 1 (PD-1) is involved in the development of proliferative diabetic retinopathy by mediating activation-induced apoptosis.
   PMID: 26321864

2. Expression of PD-1 Molecule on Regulatory T Lymphocytes in Patients with Insulin-Dependent Diabetes Mellitus.
   PMID: 26393578

3. PD-1 expression defines two distinct T-cell sub-populations in follicular lymphoma that differentially impact patient survival.
   PMID: 25700246

4. Low programmed cell death-1 (PD-1) expression in peripheral CD4(+) T cells in Japanese patients with autoimmune type 1 diabetes.
   PMID: 25682896

X. TROUBLE SHOOTING

Problem	Probable Cause	Suggestion
No signal	Forgot to add all components.	Prepare check list and add the components in the correct order.
Low signal	Not enough lysates per well.	Check the protein concentration. Add more lysates.
High background	Washing is not sufficient.	Wash plates thoroughly after incubation with Streptavidin-HRP secondary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