

PD-1 ENZYME IMMUNOASSAY TEST KIT

Catalog Number: BC-1301



Enzyme Immunoassay for the Quantitative Determination of PD-1 Concentration in Human Serum and Plasma

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

INTRODUCTION

Programmed cell death protein 1, (PD-1, CD279, PDCD1), is a cell surface receptor that is critical for the regulation of T cell inflammatory activity and maintains peripheral tolerance in the immune system (1). With an extracellular IgV domain, a transmembrane domain, and an intracellular tail with two phosphorylation sites (SHP-1 and SHP-2), PD-1 is able to interact with ligands PD-L1 and PD-L2 to down-regulate the immune system and suppress T cell activity (2). Upon antigen recognition, an activated T cell expresses PD-1 on its surface and produces interferons, which induces expression of PD-L1, thereby promoting apoptosis or cell death. (3) However when hijacked by tumor cells, aberrant expression of PD-1 ligands inhibits immune-modulatory T cell activation, leading to disease progression (4).

Elevated levels of PD-1 in serum and plasma have been associated with rheumatoid arthritis and skin sclerosis (5,6). Also, PD-1 is predominantly expressed by tumor infiltrating T cells(7). Further research implies that using monoclonal antibodies to target the PD-1 immunologic checkpoint has contributed to breakthrough progress in understanding and treating cancers such as melanoma and other various non-small cell lung cancer (4).

PRINCIPLE OF THE ASSAY

The PD-1 ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody pair directed against a distinct antigenic determinant on the PD-1 molecule. One mouse monoclonal anti-PD-1 antibody is used for solid phase immobilization (on the microtiter wells). Another mouse monoclonal anti-PD-1 antibody conjugated to horseradish peroxidase (HRP) is in the enzyme conjugate solution. The test samples are allowed to react sequentially with the two antibodies, resulting in the PD-1 molecules to be sandwiched between the solid phase and enzyme-linked antibodies. After two separate 60- minute incubation steps at room temperature, the wells are rinsed with Wash Buffer to remove unbound labeled antibodies. TMB Reagent is added and incubated

for 30 minutes under dark conditions, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution, changing the color to yellow. The concentration of PD-1 is directly proportional to the color intensity of the test samples. Absorbance is measured spectrophotometrically at 450 nm.

REAGENTS AND MATERIALS PROVIDED

1. Antibody-Coated Wells (1 plate, 96 wells)
Microtiter wells coated with mouse monoclonal anti-PD-1
2. 50 ng/ml PD-1 Standard (0.5 mL /vial)
50 ng/mL PD-1 in phosphate buffer-BSA solution with preservatives
3. Standard and Sample Diluent (30 mL/bottle, 1 bottle)
Contains phosphate buffer-BSA solution with preservatives
4. Enzyme Conjugate Reagent (12 mL/vial, 1 vial)
Contains mouse monoclonal anti-PD-1 conjugated to horseradish peroxidase
5. 20X Wash Buffer (50 mL/bottle, 1 bottle)
Phosphate buffer with detergents
6. TMB Reagent (11 mL/bottle, 1 bottle)
Contains one-step TMB solution
7. Stop Solution (11 mL/bottle, 1 bottle)
Contains diluted hydrochloric acid (1N HCl)

STORAGE CONDITIONS

1. Store the unopened kit at 2-8°C upon receipt and when it is not in use, until the expiration shown on the kit label. Refer to the package label for the expiration date.
2. Keep microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

REAGENT PREPARATION

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. For each test run, prepare a fresh standard set.
3. **Dilute 50 ng/mL to 10 ng/mL with Standard/Sample diluent. Prepare two-fold serial dilutions of the 10 ng/mL Standard with Standard/Sample Diluent:**
 - a. 10 ng/mL: 0.12 mL of 50 ng/mL + 0.48 mL of Standard/Sample Diluent
 - b. 5 ng/mL: 0.25 mL of 10 ng/mL + 0.25 mL of Standard/Sample Diluent
 - c. 2.5 ng/mL: 0.25 mL of 5 ng/mL + 0.25 mL of Standard/Sample Diluent
 - d. 1.25 ng/mL: 0.25 mL of 2.5 ng/mL + 0.25 mL of Standard/Sample Diluent
 - e. 0.625 ng/mL: 0.25 mL of 1.25 ng/mL + 0.25 mL of Standard/Sample Diluent
 - f. 0.313 ng/mL: 0.25 mL of 0.625 ng/mL + 0.25 mL of Standard/Sample Diluent

- h. 0.156 ng/mL: 0.25 mL of 0.313 ng/mL + 0.25 mL of Standard Diluent
- i. 0 ng/mL: 0.25 mL of Standard Diluent
- Patient samples need to be diluted 4-fold prior to use. Prepare a series of small tubes (i.e., 1.5 mL microcentrifuge tubes) and mix 60 µL of serum with 180 µL Standard/Sample Diluent.**
 - Working Wash Buffer:** Preparation of 1X Wash Buffer from 20X Stock. Add 50 mL of 20X Wash Buffer Stock to 950 mL of DI H₂O. The Working Wash Buffer is stable at 2-8°C for 30 days. NOTE: Any crystals that may be present due to high salt concentration must be redissolved at room temperature before making the dilution.

ASSAY PROCEDURE

- Prepare Standards. See Reagent Preparation.**
- Dilute samples 1:4 dilution. See Reagent Preparation.**
- Secure the desired number of coated wells in the holder.
- Dispense 100 µL of PD-1 standards, and **DILUTED** specimens into the appropriate wells.
- Incubate for 60 minutes at room temperature (18-25 °C).
- Remove incubation mixture by flicking plate contents into a waste container. Rinse and flick the microtiter wells 5 times with 300 µL Working Wash Buffer. Strike the wells onto absorbent paper or paper towels to remove all residual water droplets.
- Dispense 100 µL of PD-1 Working Enzyme Conjugate Reagent into each well.
- Incubate for 60 minutes at room temperature (18-25 °C).
- Remove incubation mixture by flicking plate contents into a waste container. Rinse and flick the microtiter wells 5 times with 300 µL Working Wash Buffer. Strike the wells onto absorbent paper or paper towels to remove all residual water droplets.
- Dispense 100 µL TMB solution into each well.
- Incubate for 30 minutes at room temperature (18-25 °C).
- Stop the reaction by adding 100 µL of Stop Solution into each well.
- Gently mix for 30 seconds. **It is important to make sure that all the blue color changes to yellow color completely.**
- Read absorbance at 450 nm with a microtiter well reader within 15 minutes.

CALCULATION OF RESULTS

- Calculate the mean absorbance value (OD₄₅₀) for each set of reference standards, controls and samples.
- Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/mL on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
- The corresponding concentration of PD-1 (ng/mL) can be determined from the standard curve using the mean absorbance value for each sample. Depending on experience and/or the availability of computer capability, other methods of

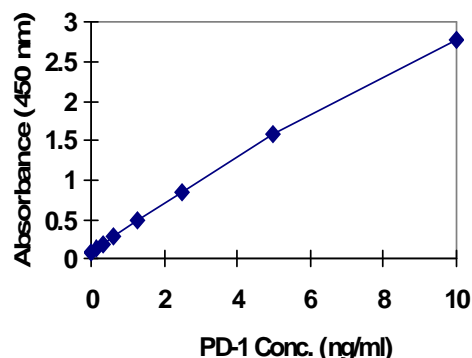
data reduction may be employed.

- The obtained values of the samples should be multiplied by the dilution factor of 4 to obtain PD-1 results in ng/mL.

EXAMPLE OF STANDARD CURVE

Results of a typical standard run with absorbency readings at 450 nm shown on the Y axis against PD-1 concentrations shown on the X axis. **NOTE:** This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must generate its own data and standard curve in each experiment.

PD-1 (ng/ml)	Absorbance (450 nm)
0	0.089
0.156	0.138
0.313	0.190
0.625	0.284
1.25	0.481
2.5	0.851
5	1.581
10	2.776



PERFORMANCE CHARACTERISTICS

- Sensitivity**
The minimum detectable concentration of the PD-1 ELISA assay as measured by 2SD from the mean of a zero standard is estimated to be 0.15 ng/mL.
- Precision**
 - Intra-Assay Precision**
Within-run precision was determined by replicate determinations of three different samples in one assay. Within-assay variability is shown below:

Sample	1	2	3
# Replicates	24	24	24
Mean PD-1 (ng/mL)	8.4	13.2	22.9
S.D.	0.6	1.0	0.9
C.V. (%)	7.0%	7.2%	3.8%

b. *Inter-Assay Precision*

Between-run precision was determined by replicate measurements of three different samples over a series of individually calibrated assays. Between-assay variability is shown below.

Sample	1	2	3
# Replicates	20	20	20
Mean PD-1 (ng/mL)	9.1	13.5	25.3
S.D.	0.8	1.1	0.9
C.V. (%)	9.2%	7.9	3.7

3. **Recovery and Linearity Studies**

a. *Recovery*

Samples were spiked with known PD-1 levels and assayed in duplicate. The mean recovery was 105.3%

PAIR NUMBER	EXPECTED [PD-1] (ng/mL)	OBSERVED [PD-1] (ng/mL)	%RECOVERY
1	8	8.8	110.0%
2	12.5	13.1	104.8%
3	25	25.3	101.2%

b. *Linearity*

Three samples were serially diluted to determine linearity. The mean recovery was 99.6%

#	Dilution	Expected Conc. (ng/ml)	Observed Conc. (ng/ml)	%Expected
1.	1:2	8.8	10.1	114.8%
	1:4		8.8	N/A
	1:8		7.8	88.6%
<i>Mean = 101.7%</i>				
2.	1:2	13.1	14.5	110.6%
	1:4		13.1	N/A
	1:8		11.6	88.5%
<i>Mean = 99.6%</i>				
3.	1:2	25.3	26.5	104.7%
	1:4		25.3	N/A
	1:8		23.0	90.0%
<i>Mean = 97.4%</i>				

REFERENCES

1. Fife, B. T., & Pauken, K. E. (2011). The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Annals of the New York Academy of Sciences*, 1217(1), 45-59.
2. Riella, L. V., Paterson, A. M., Sharpe, A. H., & Chandraker, A. (2012). Role of the PD-1 Pathway in the Immune Response. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 12(10), 2575-2587.
3. Zak, Krzysztof & Kitel, Radoslaw & Przetocka, Sara & Golik, Przemyslaw & Guzik, Katarzyna & Musielak, Bogdan & Dömling, Alexander & Dubin, Grzegorz & Holak, Tad. (2015). Structure of the Complex of Human Programmed Death 1, PD-1, and Its Ligand PD-L1. *Structure*. 23. . 10.1016/j.str.2015.09.010.
4. Zoran Gatalica, Carrie Snyder, Todd Maney, Anatole Ghazalpour, Daniel Holterman, Nianqing Xiao, Peggy Overberg, Inga Rose, Gargi D. Basu, Semir Vranic, Henry T. Lynch, Daniel D. Von Hoff and Omid Hamid. Programmed Cell Death 1 (PD-1) and Its Ligand (PD-L1) in Common Cancers and Their Correlation with Molecular Cancer Type. *Cancer Epidemiol Biomarkers Prev*. December 1 2014 (23) (12) 2965-2970.
5. Greisen, S., Rasmussen, T., Stengaard-Pedersen, K., Hetland, M., Hørslev-Petersen, K., Hvid, M., & Deleuran, B. (2013). Increased soluble programmed death-1 (sPD-1) is associated with disease activity and radiographic progression in early rheumatoid arthritis. *Scandinavian Journal of Rheumatology*, 43(2), 101-108.
6. Yanaba, K., Hayashi, M., Yoshihara, Y., & Nakagawa, H. (2016). Serum levels of soluble programmed death-1 and programmed death ligand-1 in systemic sclerosis: Association with extent of skin sclerosis. *The Journal of Dermatology*, 43(8), 954-957.
7. Ahmadzadeh, M., Johnson, L. A., Heemskerk, B., Wunderlich, J. R., Dudley, M. E., White, D. E., & Rosenberg, S. A. (2009). Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*, 114(8), 1537-1544.

TECHNICAL CONSULTATION

BioCheck, Inc.
 323 Vintage Park Dr.
 Foster City, CA 94404
 Tel: (650) 573-1968 Email: info@biocheckinc.com

FOR RESEARCH USE ONLY
 Not for use in diagnostic procedures