

Quantikine™ ELISA

Human PD-1 Immunoassay

Catalog Number DPD10

For the quantitative determination of human Programmed Death-1 (PD-1) concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Programmed Death-1 (PD-1), also known as Programmed cell death protein and CD279, is an extensively studied immune checkpoint inhibitory receptor. Given PD-1's role in peripheral tolerance, it is not surprising that increased PD-1 expression is a mechanism for immune escape, which is permissive for cancer growth and metastasis (1,2). PD-1 is encoded by the *PDCD1* gene (3). The PD-1 glycoprotein is a monomeric 50-55kDa type 1 transmembrane protein that belongs to the immunoglobulin (Ig) superfamily (4). PD-1 expression and induction have been well studied (3,5,6). It is expressed in CD4⁺ and CD8⁺ T cells as well as B cells, macrophages, some dendritic cell subsets and NK cells. PD-1 is induced by T cell receptor (TCR) signaling as well as interleukin 2 (IL-2), IL-7 and type 1 interferons. A variety of reagents are used to experimentally induce PD-1 expression, including, phorbol 12-myristate 13 acetate, ionomycin, concanavalin A, CD3/CD28 antibodies, or most notably lymphocytic choriomeningitis virus *in vivo* (6).

PD-1 expression is also regulated post-translationally (2). The PD-1 extracellular domain is glycosylated at asparagine (N) residues N49 and N72 while in the endoplasmic reticulum. PD-1 subsequently transits to the golgi apparatus where it is fucosylated at the same sites by the core fucosylase FUT8. This post translational modification represents a novel therapeutic target as T cells have a more robust anticancer response due to reduced surface expression of de-fucosylated PD-1. PD-1 is polyubiquitinated at lysine (K) residue K48 by the E3 ligase F-Box 38 (FBX028) (2,7) which results in PD-1 degradation via the proteasome.

PD-1 serves as the receptor for programmed death ligand 1 (PD-L1/CD274/B7-H1) and PD-L2 (CD273/B7-DC) (8,9). The interaction of the extracellular domains of PD-1 and PD-L1 causes a conformational change that results in the phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) by Src family kinases (4). Src homology 2 containing tyrosine phosphatase 2 (SHP-2) and SHP-1 are recruited in order to attenuate T cell activating signals via dephosphorylation of downstream signaling cascades.

The Quantikine™ Human Programmed Death-1 (PD-1) Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human PD-1 in cell culture supernates, cell lysates, serum, plasma, and urine. It contains HEK293-expressed recombinant human PD-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human PD-1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human PD-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human PD-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PD-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human PD-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PD-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human PD-1 Microplate	899256	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human PD-1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human PD-1 Standard	899258	2 vials of recombinant human PD-1 in a buffered protein base with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	Use a new standard for each assay. Discard after use.
Human PD-1 Conjugate	899257	21 mL of a monoclonal antibody specific for human PD-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5Y	895201	21 mL of a concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples
- Human PD-1 Controls (optional; R&D Systems®, Catalog # QC289)
- Lysis Buffer 17 (optional; R&D Systems, Catalog # 895943)

PRECAUTIONS

PD-1 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Urine and cell culture supernates may require dilution due to high endogenous levels.

Multiple dilutions are recommended for unknown samples.

For cell lysate samples, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 3-50 $\mu\text{g}/\text{well}$.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

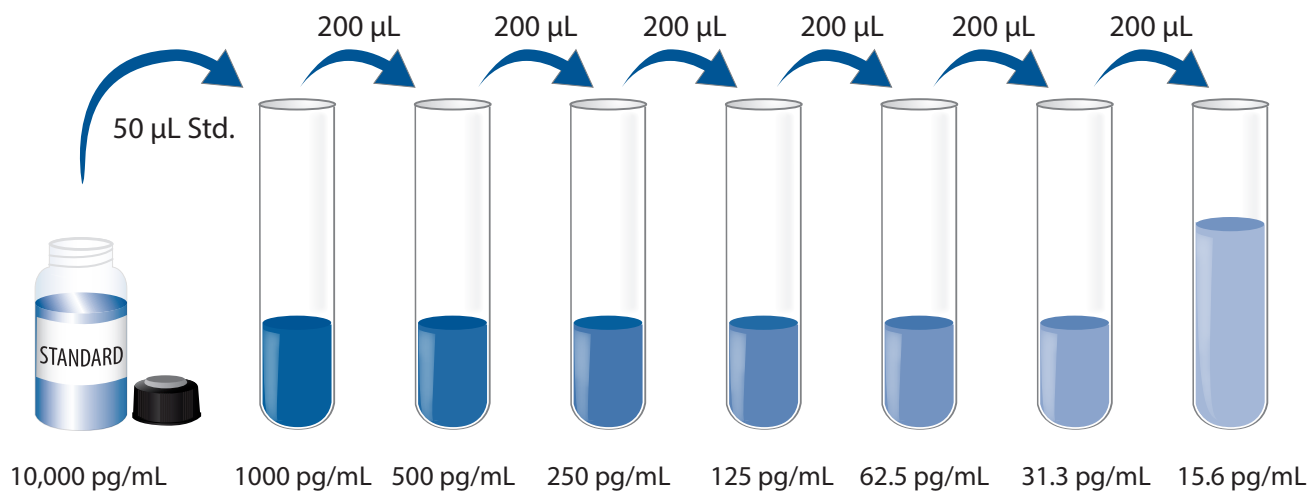
Note: PD-1 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Human PD-1 Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human PD-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. *Vortex and rocker can be used to mix the standard or controls.*

Pipette 450 μ L of Calibrator Diluent RD5Y into the 1000 pg/mL tube. Pipette 200 μ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5Y serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *PD-1 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μ L of Assay Diluent RD1-21 to each well.
4. Add 50 μ L of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μ L of Human PD-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μ L of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 μ L of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Sample may require dilution.

CALCULATION OF RESULTS

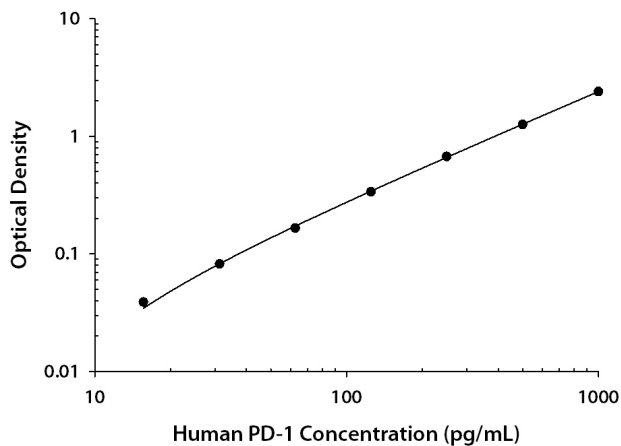
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human PD-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.015 0.018	0.017	—
15.6	0.055 0.057	0.056	0.039
31.3	0.098 0.100	0.099	0.082
62.5	0.182 0.184	0.183	0.166
125	0.354 0.356	0.355	0.338
250	0.688 0.693	0.691	0.674
500	1.267 1.291	1.279	1.262
1000	2.411 2.426	2.419	2.402

PRECISION

Intra-Assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	135	319	655	131	304	626
Standard deviation	3.49	5.60	10.8	7.36	14.6	25.8
CV (%)	2.6	1.8	1.6	5.6	4.8	4.1

RECOVERY

The recovery of human PD-1 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	100	97-103%
Lysis buffer (n=1)	96	94-97%
Serum (n=4)	88	77-98%
EDTA plasma (n=4)	86	81-93%
Heparin plasma (n=4)	85	75-93%
Urine (n=4)	89	83-97%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human PD-1 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Lysates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine* (n=4)
1:2	Average % of Expected	98	92	104	104	102	103
	Range (%)	97-99	89-95	100-106	100-107	100-104	102-105
1:4	Average % of Expected	96	98	108	108	108	105
	Range (%)	91-99	95-101	102-112	104-113	105-110	103-108
1:8	Average % of Expected	96	96	110	109	108	107
	Range (%)	93-99	93-105	106-114	104-114	105-112	106-109
1:16	Average % of Expected	93	99	108	109	109	105
	Range (%)	90-96	93-106	102-113	107-113	105-116	102-107

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-four assays were evaluated and the minimum detectable dose (MDD) of human PD-1 ranged from 0.505-3.27 pg/mL. The mean MDD was 1.59 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified HEK293-expressed recombinant human PD-1 manufactured at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human PD-1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=30)	175	20.6-347	71.1
EDTA plasma (n=30)	168	19.6-346	72.5
Heparin plasma (n=30)	171	20.8-347	70.9
Urine (n=10)	456	196-1070	310

Cell Culture:

Human peripheral blood mononuclear cells (PBMCs) were cultured at 3×10^6 cells/mL in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin and were either left untreated or treated with 10 μ g/mL PHA-L and 20 ng/mL of recombinant human (rh) IL-2 for 5 days. After 5 days, cells were then treated with 10 ng/mL PMA and 500 ng/mL Ionomycin for an additional 24 hours before harvesting conditioned media.

CD4⁺ T cells were isolated from PBMCs using MagCelect™ Human CD4⁺ T Cell Isolation Kit (R&D Systems®, Catalog # MAGH102). CD4⁺ T cells were cultured at 5×10^5 /mL in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin and were either left untreated (Unstimulated) or treated with 5 μ g/mL PHA-L and 20 ng/mL rhIL-2 for 5 days (Th2). Cells were then treated with 10 ng/mL PMA and 500 ng/mL Ionomycin overnight before harvesting conditioned media.

For Cloudz™ stimulation, CD4⁺ T cells were cultured at 5×10^5 /mL using ExCellerate™ Human T Cell Expansion Media, Xeno-Free (R&D Systems, Catalog # CCM030) for the duration. T cells were treated with 25 μ L of Cloudz CD3/28 particles/mL of culture media for 5 days. Cell conditioned media was collected by centrifugation and stored at ≤ -20 °C until assayed. After centrifugation, the remaining cells and Cloudz particles were resuspended with 1X Release Buffer. After dissolution of Cloudz particles, cells were lysed as indicated below.

Cell Supernates - Aliquots of the cell culture supernates were assayed for levels of human PD-1.

Cell Lysates - Untreated or treated cells listed above were washed with PBS and solubilized in Lysis Buffer 17 (R&D Systems, Catalog # 895943) supplemented with protease inhibitors using 3-5 times the pellet volume and put on ice for 15 minutes. Tubes were centrifuged at 14,000 x g for 5 minutes to remove insoluble material. The remaining whole cell extract was removed, aliquoted into a clean test tube, and stored at ≤ -20 °C. Whole cell extract protein concentration was quantified using a total protein assay. 5 μ g of the cell lysate was removed and assayed for human PD-1.

Condition	Cell culture supernates (pg/mL)	Lysates (pg/mL)
Unstimulated CD4 ⁺ T cells	ND	49.7
Stimulated CD4 ⁺ T cells (Cloudz)	58.6	111
Stimulated CD4 ⁺ T cells, Th2	367	693
Unstimulated PBMC	122	116
Stimulated PBMC	394	425

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human PD-1.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human PD-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

B7
B7-1
B7-2
B7-H1
B7-H2
B7-H3
B7-H4
B7-H6
PD-L2

Other recombinants:

canine PD-1
mouse PD-1
porcine PD-1
rat PD-1

Recombinant cynomolgus monkey PD-1 cross-reacts approximately 2.7% and interferes at concentrations > 5 ng/mL in the assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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

NOTES

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