EP101-EN.01

PD-1 [Biotinylated] : PD-L1 Inhibitor Screening ELISA Assay Pair

Pack Size: 96 tests

Catalog Number： EP-101

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedures

INTENDED USE

This kit is developed for screening for inhibitors of human PD-1 binding to human PD-L1.

It is intended for research use only (RUO).

PRINCIPLE OF THE ASSAY

Immune checkpoint pathway is a focal point of today's cancer research. PD-1 is one of the best characterized checkpoint proteins. The binding between PD-1 and its ligand PD-L1 suppresses T-cell activation and allows cancer cells to escape from body's immune surveillance. Therefore, the pharmaceutical inhibition of PD-1 or its ligand has been considered a promising strategy by many oncologists.

This inhibitor screening ELISA pair is designed to facilitate the identification and characterization of new PD-1 pathway inhibitors. This assay employs a simple colorimetric ELISA platform, which measures the binding between immobilized human PD-L1 and in-house developed biotinylated PD-1 protein. This product is uniquely suitable for rapid high-throughput screening of putative PD-1 and PD-L1 inhibitors. Briefly, we provide you with a human PD-1-Biotin protein, a human PD-L1 protein, an anti-PD-1 neutralizing antibody (as method verified Std.), and Streptavidin-HRP reagent. Your experiment will include 4 simple steps:

1) Coat the plate with human PD-L1.

2) Add your molecule of interest to the tests.

3) Add human PD-1-Biotin to bind the coated human PD-L1.

4) Add Streptavidin-HRP followed by TMB or other colorimetric HRP substrate.

Finally, the ability of your compound to inhibit PD-1: PD-L1 binding will be determined by comparing OD readings among different experimental groups.

MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (96 tests)	Format	Storage	
				Unopened	Opened

EP101-C01	High-bind Plate	1 plate	Solid	2-8℃	2-8℃
EP101-C02	Human PD-L1	25 μg	Powder	2-8℃	-70℃
EP101-C03	Anti-PD-1 Neutralizing Antibody	20 μg	Powder	2-8℃	-70℃
EP101-C04	Human PD-1-Biotin	5 μg	Powder	2-8℃	-70℃
EP101-C05	Streptavidin-HRP	10 μg	Powder	2-8℃, avoid light	-70℃, avoid light
EP101-C06	Coating Buffer	12 mL	Liquid	2-8℃	2-8℃
EP101-C07	10xWashing Buffer	50 mL	Liquid	2-8℃	2-8℃
EP101-C08	Blocking Buffer	50 mL	Liquid	2-8℃	2-8℃
EP101-C09	Substrate Solution	12 mL	Liquid	2-8℃, avoid light	2-8℃, avoid light
EP101-C10	Stop Solution	7 mL	Liquid	2-8℃	2-8℃

REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

Single or dual wavelength microplate reader with 450 nm filter；

Centrifuge；

37 ºC Incubator；

Single channel or multichannel pipettes with 10 μL, 200 μL and 1000 μL precision；

10 μL, 200 μL and 1000 μL pipette tips；

Test Tubes；

Graduated cylinder；

Deionized or distilled water for dilution；

1 x PBS

STORAGE AND VALIDITY INSTRUCTIONS

The unopened kit is stable for 12 months from the date of manufacture if stored at 2°C to 8°C.

The opened kit should be stored per TABLE 1. The shelf life is 30 days from the date of opening.

Note: a. Do not use reagents past their expiration date.

b. Find the expiration date on the outside packaging.

REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (20-25℃) before use.

2. Reconstitute the provided lyophilized materials to stock solutions with PBS as recommended in Tab.2, Solubilize for 15 to 30 min at room temperature with occasional gentle mixing. Avoid vigorous shaking or vortexing. The reconstituted stock solutions should be stored at -70℃. **Avoid freeze-thaw cycles**.

***Note**: Streptavidin-HRP stock solution should be protected from light.*

TABLE 2. RECONSTITUTION METHODS FOR 96 TESTS

Catalog	Components	Amount	Stock Solution Con.	Reconstitution Buffer and Vol.
EP101-C02	Human PD-L1	25 μg	250 μg/mL	100 μL 1xPBS
EP101-C03	Anti-PD-1 Neutralizing Antibody	20 μg	250 μg/mL	80 μL 1xPBS
EP101-C04	Human PD-1-Biotin	5 μg	100 μg/mL	50 μL 1xPBS
EP101-C05	Streptavidin-HRP	10 μg	50 μg/mL	200 μL 1xPBS

RECOMMENDED PROTOCOL

1. Working fluid preparation

1.1 Preparation of **1×Washing Buffer**:

Dilute 50 mL 10×Washing Buffer with ultrapure water/deionized water to 500 mL.

1.2 Preparation of **Dilution Buffer**:

Dilute **Blocking Buffer (EP101-C08)** at 1:3 with **1×Washing Buffer**. For example: 10 mL **Blocking Buffer** (EP101-C08) + 30 mL **1×Washing Buffer**.

2. Coating

1) Dilute **Human PD-L1** stock solution (250 μg/mL) to 2 μg/mL with **Coating Buffer** to make **Human PD-L1** working solution.

2) Please leave a couple of wells uncoated for **No-Coating Control (Tab. 3)**.

3) Add 100 μL of **Human PD-L1** working solution (2 μg/mL) to each well, seal the plate with microplate sealing film and incubate overnight (or 15 hours) at 4℃.

3. Washing

Remove the remaining solution by aspiration, add 300 µL of **1×Washing Buffer** to each well, gently tap the plate for 1 min, remove any remaining **1×Washing Buffer** by aspirating or decanting, invert the plate and blot it against paper towels. **Repeat the wash step above for three times**.

Note: *For best results, the complete removal of the* ***Human PD-L1*** *solution is essential. The use of a manifold dispenser or an auto-washer may be necessary.*

4. Blocking

Add 300 µL **Blocking Buffer** to each well, seal the plate with microplate sealing film and incubate at 37℃ for 1.5 h.

5. Washing

Repeat step 3. At meantime, you can start to prepare your samples.

6. Add Samples

1）Make series dilution of the samples as appropriate.

2）If you intend to use the provided **Anti-PD-1 Neutralizing Antibody** as a standard (Std.), you may dilute the antibody as recommended in **Fig 2.**. And plate layout as recommended in **Fig 3**.

3）Add 50 µL of sample solution to each well according to our recommendation (**Fig 1.**) or your own plate setup.

Fig 1. PLATE LAYOUT FOR INHIBITORS/ACTIVATORS ASSAY

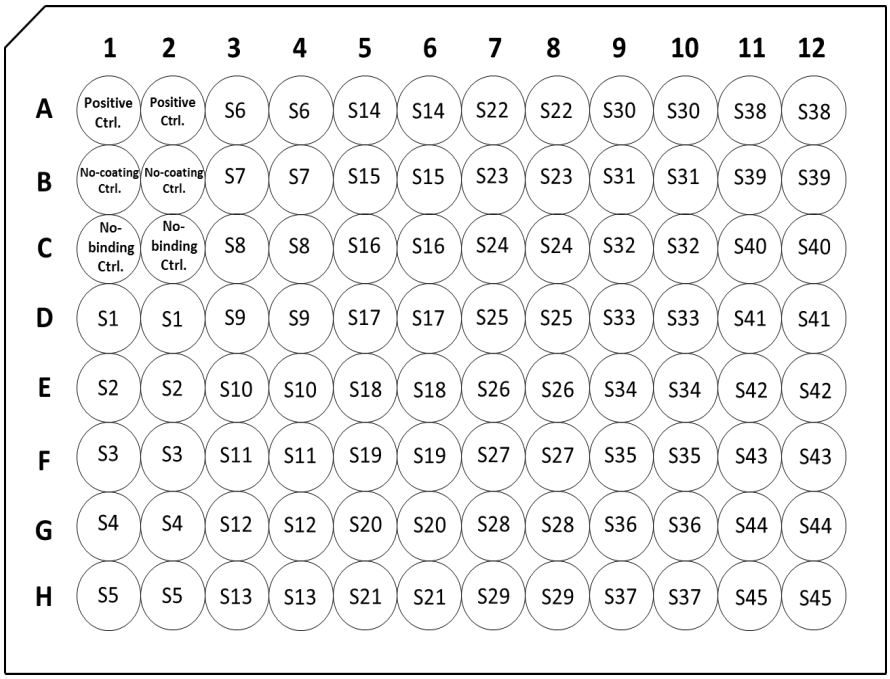
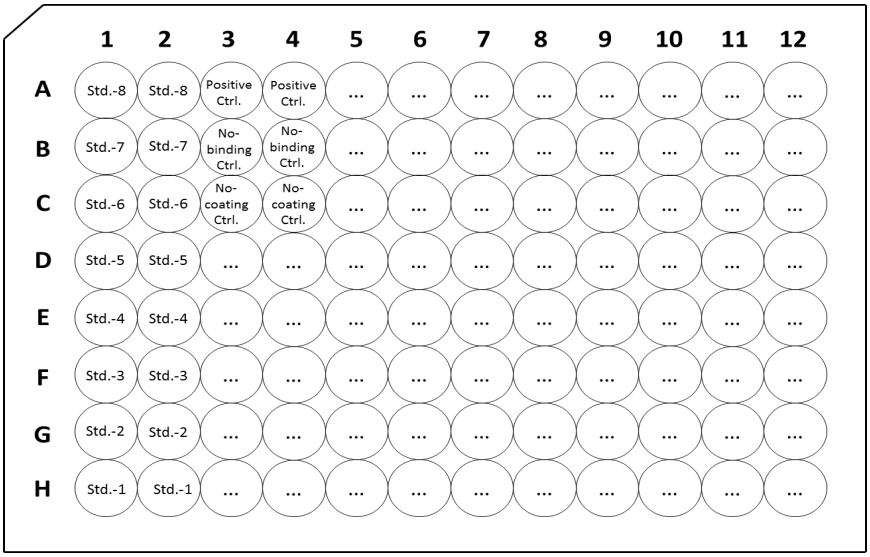


Fig 2. PREPARATION OF 1:1 SERIAL DILUTIONS OF THE ANTI-PD-1 NEUTRALIZING ANTIBODY

Tubes/ Solution Code	Anti-PD1-Neutralizing-Antibody stock solution	Std.-1	Std.-2	Std.-3	Std.-4	Std.-5	Std.-6	Std.-7	Std.-8
Operating	20 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL
Solution Con.	250 µg/mL	10 µg/mL	5 µg/mL	2.5 µg/mL	1.25 µg/mL	0.625 µg/mL	0.313 µg/mL	0.156 µg/mL	0.078 µg/mL
Dilution Buffer Vol.		480 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL

Fig 3. PLATE LAYOUT FOR STANDARD ASSAY



7. Binding

1) Dilute **Human PD-1-Biotin** stock solution (100 µg/mL) to 0.4 µg/mL with **Dilution Buffer** to make **Human PD-1-Biotin** working solution.

2) For **No-binding ctrl.** wells, please add 50 µL **Dilution Buffer**.

3) For all other wells, please add 50 µL **human PD-1-Biotin** working solution and mix the samples by gently tapping the plate. Seal the plate with microplate sealing film and incubate at 37°C for 1.0 h.

8. Washing

Repeat step 3.

9. Add Streptavidin-HRP

1) Dilute **Streptavidin-HRP** stock solution (50 μg/mL) to 0.4 μg/mL with **Dilution Buffer** to make **Streptavidin-HRP** working solution.
2) For all wells, add 100 μL **Streptavidin-HRP** working solution, seal the plate with microplate sealing film and incubate at 37℃ for 1.0 h, **avoid light**.

10. Washing

Repeat step 3.

11. Substrate Reaction

Add 100 μL **Substrate Solution** to each well. Seal the plate with microplate sealing film and incubate at 37℃ for 20 min. Avoid light.

12. Termination

Add 50 μL **Stop Solution** to each well, and gently shake the plate to allow thorough mixing.

Note: *the color in the wells should change from blue to yellow.*

13. Data Recording

Read the absorbance at 450 nm using UV/Vis microplate spectrophotometer.

Note*: Subtracting the value read at* $OD_{450\ nm}$ *with* $OD_{630\ nm}$ *can be used to reduce the background noise.*

TAB. 3 ASSAY PROTOCOL

Steps Code	Steps	Reagents & Instruments	Reaction Conditions	Samples	No-binding Ctrl.	No-coating Ctrl.	Positive Ctrl.
1	Working fluid preparation	N/A	N/A	N/A	N/A	N/A	N/A
2	Coating	Human PD-L1 Working Solution	4℃ for overnight	100 μL	100 μL	—	100 μL
3	Washing	1×Washing Buffer	Wash for 3 times	300 μL	300 μL	300 μL	300 μL
4	Blocking	Blocking Buffer	37℃ for 1.5 h	300 μL	300 μL	300 μL	300 μL
5	Washing	1×Washing Buffer	Wash for 3 times	300 μL	300 μL	300 μL	300 μL
6	Add Samples	Samples	—	50 μL	—	—	—
		Dilution Buffer		—	50 μL+50 μL	50 μL	50 μL
7	Binding	Human PD-1-Biotin	Mix by gentle tapping,	50 μL	—	50 μL	50 μL

		Working Solution	incubate at 37℃ for 1.0 h				
8	Washing	1×Washing Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
9	Streptavidin-HRP	Streptavidin-HRP Working Solution	37℃ for 1.0 h	100 µL	100 µL	100 µL	100 µL
10	Washing	1×Washing Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
11	Substrate Reaction	Substrate Solution	37℃ for 20 min	100 µL	100 µL	100 µL	100 µL
12	Termination	Stop Solution	Mix by gentle tapping	50 µL	50 µL	50 µL	50 µL
13	Data Recording	UV/Vis spectrophotometer	Measure absorbance at 450 nm, with the correction wavelength set at 630 nm				

Note for TAB. 3:

1) ***Samples:*** *Your samples of interest.*
2) ***No-binding Ctrl.:*** *Reaction without* **Human PD-1-Biotin** *added. The absorbance should be around 0.05(< 0.1) at 450 nm.*
3) ***No-coating Ctrl.:*** *Reaction without* ***Human PD-L1 co****ated on the wells. The absorbance should be around 0.05(< 0.1) at 450 nm.*
4) ***Positive Ctrl.:*** *Determined the max value in 450nm absorbance, when out of inhibitors.*
5) *It is recommended that all samples, controls and standards should be done in duplicates.*

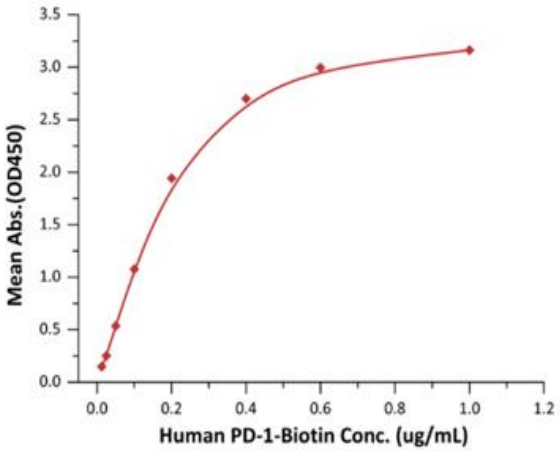
PRECAUSIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. This kit should be used according to the provided instructions.
3. Do not mix reagents from different lots.
4. All reagents should be balanced to room temperature (20℃-25℃) before use.
5. This kit should be stored at 2℃-8℃.
6. Please prepare the working solution of each component according to the needs of the experiment. Except for 1x Washing Buffer, all prepared working solution is for one-time use and cannot be stored.

METHOD V ER IFIC ATION

1. PD-1 [BIOTINYLATED]: PD-L1 BINDING IN THE ABSENCE OF INHIBITORS

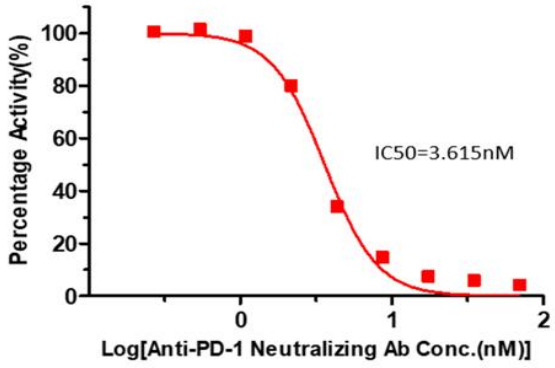
Immobilized **Human PD-L1** protein at 2 µg/mL (100 µL/well) can bind **Human PD-1-Biotin** with a linear range of 0.0125-0.2 µg/mL when detected by **Streptavidin-HRP**. Background was subtracted from data points before curve fitting.



Human PD-1-Biotin(ug/ml)	Mean Abs.(OD450)
1	3.163
0.6	2.996
0.4	2.700
0.2	1.942
0.1	1.076
0.05	0.536
0.025	0.251
0.0125	0.148

2. INHIBITION OF PD-1 [BIOTINYLATED]: PD-L1 BINDING BY ANTI-PD-1 NEUTRALIZING ANTIBODY

Serial dilutions of anti-PD-1 neutralizing antibody (Catalog # EP101-C03) (1:1 serial dilutions, from 10 μg/mL to 0.078 μg/mL) was added into PD-L1 : PD-1-Biotin binding reactions. The assay was performed according to the above described protocol. Background was subtracted from data points prior to log transformation and curve fitting.



Anti-PD-1 Neutralizing Antibody Ab Con.(ug/ml)	Anti-PD-1 Neutralizing Antibody Ab Con.(nM)	Mean Abs.(OD450)	Percentage Activity(%)
0	0.000	2.380	100.00
0.078	0.544	2.361	99.20
0.156	1.088	2.353	98.87
0.313	2.176	1.903	79.96
0.625	4.352	0.811	34.08
1.25	8.704	0.353	14.83
2.5	17.407	0.181	7.61
5	34.814	0.141	5.92
10	69.628	0.102	4.29