KRIBIOLISA® PD-1/PD-L1 Functional Blocking / Potency ELISA

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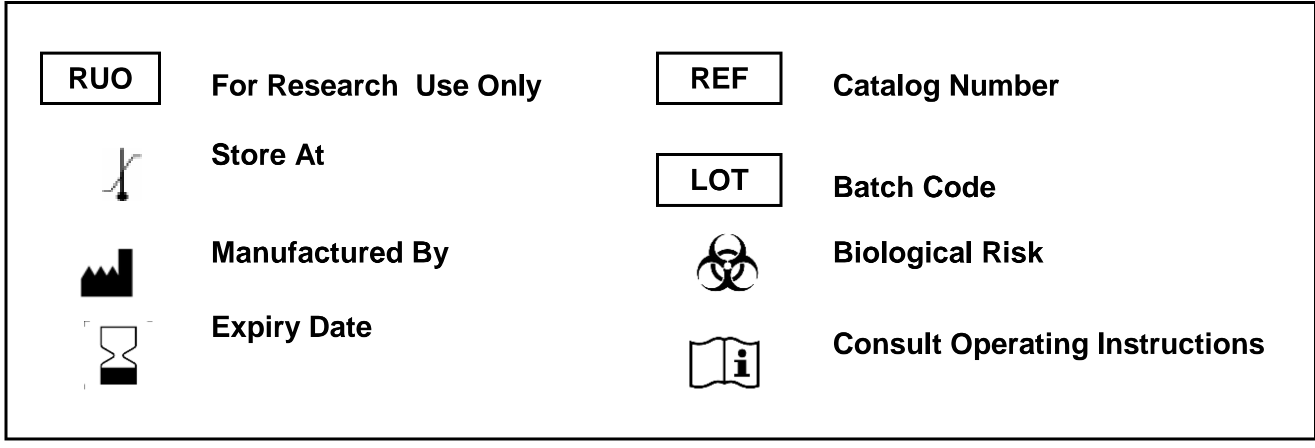
Ver 1.0

RUO

Enzyme Immunoassay for the Qualitative Determination of PD-1/PD-L1 Functional Blocking / Potency in Human serum, plasma and other biological samples



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Introduction:

The KRIBIOLISA® ELISA kits are used for assessing the specific biomarker in samples analytes which may be serum, plasma and cell culture supernatant as validated with the kit. The kit employs a competitive ELISA technique.

Intended Use:

The KRIBIOLISA® PD-1/PD-L1 Functional Blocking / Potency ELISA kit is used as an analytical tool for qualitative determination of PD-1/PD-L1 Functional Blocking / Potency in Human serum, plasma and other biological samples.

Principle:

This kit was based on Competitive-ELISA detection method. The microtiter plate provided in this kit has been pre-coated with PD-L1 protein. Human PD-1 Protein is used as an analytical Assay Control. During the reaction, Inhibitor in the sample/ positive control Inhibit PD1 and PDL1 binding. Excess unbound sample or positive control are washed from the plate, and Biotinylated PD-1 Antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. Then TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The inhibition ratio of Inhibitor in the samples is then determined by comparing the OD of the samples to the standard.

Materials Provided:

1. PD-L1 protein coated Microtitre plate (8 x 12 wells) - 1 no.
2. Assay Control (Lyophilized, 10 ng/vial) – 10 vials
3. Positive Control - 1 ml
4. Biotinylated PD-1 Antibody (concentrated) - 120 ul
5. Streptavidin:HRP Conjugate (concentrated) - 120 ul
6. Sample Diluent - 20 ml
7. Biotin Antibody Dilution Buffer - 10 ml
8. HRP Conjugate Dilution Buffer - 10 ml
9. (20X) Wash Buffer - 25 ml
10. TMB Substrate - 12 ml
11. Stop Solution - 12 ml
12. Instruction manual

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Clean tubes and Eppendorf tubes.
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator.
8. Timer.

Handling/Storage:

1. All reagents should be stored as indicated on the component label.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Sample Preparation and Storage:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
2. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
3. **Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 15-min at 2000-3000 rpm. Remove the supernatant carefully. If precipitation appears, recentrifuge.
4. **Urine-** Collect urine in a sterile container, centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
5. **Cell Culture Supernatant-** Collect sample in a sterile container. Centrifuge for 20-mins at 2000-3000 rpm. Remove the supernatant carefully. When examining the components within the cell, dilute cell suspension with PBS (pH 7.2-7.4), if cell concentration is greater than 1 million/ml. Damage the cells by repeated freeze-thaw cycles to release intracellular components. Centrifuge for 20-min at 2000-3000 rpm. If precipitation appears, centrifuge again.
6. **Tissue Samples-** Rinse tissues in PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (pH7.4) with a glass homogenizer on ice. Thaw at 2-8°C or freeze at -20°C. Centrifuge at 2000-3000 RPM for approximately 20 minutes and collect the supernatant carefully.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Sample Dilution

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided sample diluent, and several trials may be necessary. The test sample must be well mixed with the sample diluent. And also standard curves and sample should be making in pre-experiment. If samples with very high concentrations, dilute samples with PBS first and then dilute the samples with the Sample Diluent.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make **Wash Buffer (1X) 500 ml;** dilute **25 ml of (20X) Wash Buffer in 475 ml of DI water**.
4. **Biotinylated PD-1 Antibody Working Solution**: Prepare it within 30 minutes before experiment.Calculate required total volume of the working solution: 0.05 ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Biotinylated PD-1 Antibody (concentrated) with Biotin Antibody Dilution Buffer at 1:100 and mix them thoroughly (i.e. Add 1 ul Biotinylated PD-1 Antibody into 99 ul Biotin Antibody Dilution Buffer).
5. **Streptavidin:HRP Conjugate Working Solution**: Prepare it within 30 minutes before experiment. Calculate required total volume of the working solution: 0.1ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Streptavidin:HRP Conjugate with Streptavidin:HRP Conjugate Dilution Buffer at 1:100 and mix them thoroughly (i.e. Add 1 ul of Streptavidin:HRP Conjugate into 99 ul of Streptavidin:HRP Conjugate Dilution Buffer).
6. **Assay Control Working solution:** Add 500ul Sample Dilution Buffer into Assay Control tube, keep the tube at room temperature for 1-2 minutes and mix them thoroughly, to get the concentration of (20 ng/ml).

Note: Store the dissolved Assay Control (20ng/ml) at 2-8°C and use it within 12h.

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of PD-1/PD-L1 inhibitor. High Dose Hook Effect is due to excess of antibody for very high concentrations of PD-1/PD-L1 inhibitor present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent.
3. PD-1/PD-L1 inhibitor concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
4. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of PD-1/PD-L1 inhibitor.
5. It is recommended that all Controls and Samples be assayed in duplicates or triplicates.
6. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
7. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
8. The plates should be read within 30 minutes after adding the Stop Solution.
9. Make a work list in order to identify the location of Controls and Samples.

Assay Procedure:

1. Bring all reagents to room temperature prior to use. It is strongly recommended that Samples should be run in duplicates.

2. Add **50 ul Assay Control to** respective to each wells except blank wells.

3. Add **50 ul Positive Control** to respective wells.

4. Add **100 ul Sample Diluent** to Blank wells and 50 ul to Assay Control well.

5. Add **50 ul Diluted Sample** to Sample wells.

6. Cover the plate with the sealer and incubate for **90 minutes** at **37°C**.

7. Aspirate and wash plate 2 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

8. Pipette **100 ul Biotinylated PD-1 Antibody Working Solution** to all wells.

9. Cover the plate with a sealer and incubate for **60 minutes** at **37°C**.

10. Aspirate and wash plate 3 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

11. Add **100 ul of Streptavidin:HRP Conjugate** to all wells.

12. Cover the plate with the sealer and incubate for 30 minutes at 37°C.

13. Aspirate and wash plate 5 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

14. Pipette **100 ul TMB Substrate** into each well.

15. Incubate the plate at **37°C** for **10 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

16. Pipette **100 ul of Stop Solution** to all wells. The wells should turn from blue to yellow in color.

17. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Test Group	Assay Control (10ng/ml PD-1 Protein)	Positive Control	Sample	Blank
Number	A	B	C	D
OD450	1.8 – 2.4	0.3 – 0.6	0.1 – 2.4	0.1 – 0.25

Data calculation: Inhibition rate (%)=OD450[(A-C)/A]×100

Safety Precautions:

- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Source materials maybe derived from Human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.

- Since the kit contains potentially hazardous materials, the following precautions should be observed.
- Do not smoke, eat or drink while handling kit material.
- Always use protective gloves.
- Never pipette material by mouth.
- Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	Interpolated Concentration
1A 2A	Blank Blank			
1B 2B	Positive Control Positive Control			
1C 2C	Negative Control Negative Control			
1D 2D	Sample Sample			
1E 2E	Sample Sample			
1F 2F	Sample Sample			
1G 2G	Sample Sample			
1H 2H	Sample Sample			
3A 4A	Sample Sample			
3B 4B	Sample Sample			

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SYMBOLS KEY

Symbol	Meaning
MTP	Coated Microtiter Plate (8 x 12 wells)
POS CNTRL	Positive Control
ASY CNTRL	Assay Control
BIOTIN AB	Biotinylated Antibody
HRP CONJ	Conjugate Horseradish Peroxidase
BIOTIN DIL	Biotin Antibody Dilution Buffer
HRP DIL	HRP Conjugate Dilution Buffer
SAMP DIL 1	Sample Diluent 1
SAMP DIL 2	Sample Diluent 2
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
i	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature