KRIBIOLISA™ PD-L1 Pathway Inhibitor (PD-1) Screening ELISA

REF : KBINP1025

Ver.1.1

RUO

Enzyme Immunoassay for the Quantitative Determination of PD-L1 Pathway Inhibitor (PD-1) screening in serum, plasma and biological solutions

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

REF KBINP1025 **Σ 96 tests**

KRISHGEN BioSystems | For US / Europe: toll free +1(888)-970-0827 tel: +1(562)-568-5005
For Asia / India: tel: +91(22)-49198700
Email: sales@krishgen.com

Introduction:

Programmed death-ligand 1 also known as cluster of differentiation 274 or B7 homolog 1 is a Trans membrane protein that in humans is encoded by the CD274 gene. The binding of PD-L1 to the inhibitory checkpoint molecule PD-1 transmits an inhibitory signal based on interaction with phosphatases (SHP-1 or SHP-2) via Immunoreceptor Tyrosine-Based Switch Motif (ITSM).[6] This reduces the proliferation of antigen-specific T-cells in lymph nodes, while simultaneously reducing apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells) – further mediated by a lower regulation of the gene Bcl-2..

Intended Use:

The KRIBIOLISA™ PD-L1 Pathway Inhibitor (PD-1) Screening ELISA kit is used for the quantitative estimation of PD-L1 Pathway Inhibitor (PD-1) screening in serum, plasma and biological solutions including pharmaceutical preparations.

Principle:

The PD-L1 Pathway Inhibitor ELISA is a competitive immunoassay for the determination of PD-L1 Pathway Inhibitor. Plates are coated with PDL1 protein. A varying concentration of unlabeled standard antibody or sample and constant concentration of Human PD1/PDCD1 protein:Biotin Conjugate will bind in sequence to the Human PD-L1/B7-H1 protein coated on the microplate. Upon washing, unbound Human PD1/PDCD1 protein:Biotin Conjugate will be removed. Bound Human PD1/PDCD1 protein:Biotin Complex will bind to Streptavidin:HRP to produce a soluble blue colored product after the addition of TMB Substrate. The enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of bound PD-L1 Pathway Inhibitor present in the standards or samples.

Materials Provided:

Part	Description	Qty
Recombinant Human PD-L1 protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Recombinant Human PD-L1 protein Microtiter Plate	1 x 96 wells
PD1 Antibody Standard	PD1 Antibody standard (lyophilized, concentration - 1 ug/ml) buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	2 vials
Human PD1 protein:Biotin Conjugate	Human PD1 protein:Biotin Conjugate buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
Streptavidin:HRP Conjugate	Streptavidin:HRP Conjugate (concentrated volume - 160ul)	1 vial
Streptavidin:HRP Diluent	Buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane and diluted with 1:100 Human serum	10 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	50 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized TMB Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Handling/Storage:

1. All reagents should be stored at 2ºC to 8ºC for stability.
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.

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

Sample Preparation and Storage:
Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

Biological samples and preparations should be completely dissolved to ensure accuracy of results.

Preparation Before Use:
Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 ul sample diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

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 **(1X) Wash Buffer**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 500 ul of Standard Diluent to obtain 2,000 ng/ml. Keep the vial for 15 mins with gentle agitation and then run the assay procedure. Use the Standard Diluent as the zero standard. Below table shows the calculation for the standard range.

Standard Concentration (ng/ml)	Standard No.	Dilution Particulars
1000 ng/ml	Lyophilized standard	Lyophilized Standard + 500ul of Standard Diluent
2000 ng/ml	Standard No.7	Reconstituted Standard
1000 ng/ml	Standard No.6	250 ul Standard No.7 + 250 ul Standard Diluent
500 ng/ml	Standard No.5	250 ul Standard No.6 + 250 ul Standard Diluent
250 ng/ml	Standard No.4	250 ul Standard No.5 + 250 ul Standard Diluent
125 ng/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent
62.5 ng/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent
31.25 ng/ml	Standard No.1	250 ul Standard No.2 + 250 ul Standard Diluent
0 ng/ml	Standard No.0	250 ul Standard Diluent

Mix each tube thoroughly before the next transfer. Use the standards for experiment within one hour of preparation of standard. Discard standard after use.

5. **Streptavidin:HRP:** The **Streptavidin:HRP** is provided in a concentrated form. Dilute as required prior to running the assay using **Streptavidin:HRP Diluent**. The dilution should be done in the ratio of approximately 1:70. (for example, 71.5 ul of conc. Streptavidin:HRP and 4930 ul of Streptavidin HRP Diluent.

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. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity of the conjugate resulting in under-estimation of the antibodies.
3. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
5. 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.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a work list in order to identify the location of Standards and Samples.
8. Making serial dilution in the wells directly is not permitted.
9. Prepare the Standard within 15 minutes prior to running the assay.
10. Please carefully dilute Standards according to the instruction, and avoid foaming. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated.
11. If crystals have formed in the Wash Solution (20X) concentrate, warm to room temperature and mix gently until the crystals are completely dissolved.
12. Contaminated water or container for reagent preparation will influence the detection results.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at Room Temperature.

2. Pipette out **100 ul of Standards and Samples** to the respective wells.

3. Add **100 ul Human PD1 protein:Biotin Conjugate** to each well.

4. Cover the plate and incubate for 60 mins at 37°C.

5. Aspirate and wash plate 4 times with **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.

6. Add **100 ul** of **diluted Streptavidin:HRP Conjugate** in each well.

7. Incubate the microplate for 60 minutes at 37°C.

8. Aspirate and wash plate 4 times with **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

9. Add **100 ul** of **TMB Substrate** in each well.

10. Incubate the plate at 37°C for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

11. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

12. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown PD-L1 Pathway Inhibitor concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the PD-L1 Pathway Inhibitor Concentration. If samples were diluted, multiply by the appropriate dilution factor.

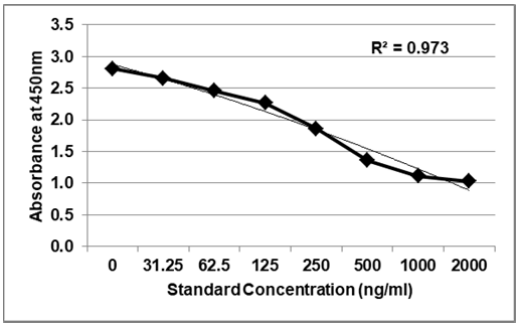
Software which is able to generate a linear regression like cubic spline or 4PL (2nd order) is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 2000 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	2.806	--	--
31.25	2.658	33.1	106.1
62.5	2.458	75.2	120.3
125	2.264	117.7	94.2
250	1.863	232.6	93.1
500	1.361	549.6	109.9
1000	1.111	1087.4	108.7
2000	1.027	1613.3	80.7

Typical Graph



abs = absorbance at 450nm

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics of the Kit:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD.
10 replicates of '0' standards were evaluated and the LOD was found to be less than 30 ng/ml

Specificity:
The recombinant protein PD1 was expressed using a DNA sequence encoding the human PDCD1 (NP_005009.2) (Met1-Gln167) expressed with the Fc region of human IgG1 at the C-terminus. The recombinant protein PDL1 was expressed using a DNA sequence encoding the human CD274 (NP_054862.1) (Met1-Arg238) in mammalian cells,

Linearity:
Standards provided in the kit will be used for measuring the linearity range of PD-L1 Pathway Inhibitor (PD-1) present in matrix. The standard graph range indicated is 0 ng/ml to 2000 ng/ml with a regression coefficient of >0.90

Precision:
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (31.25 ng/ml), medium (250 ng/ml) and high (2000 ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

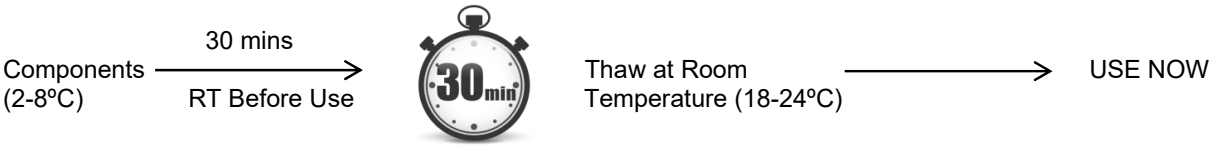
Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<8%	<5%
High	<8%	<5%

Safety Precautions:

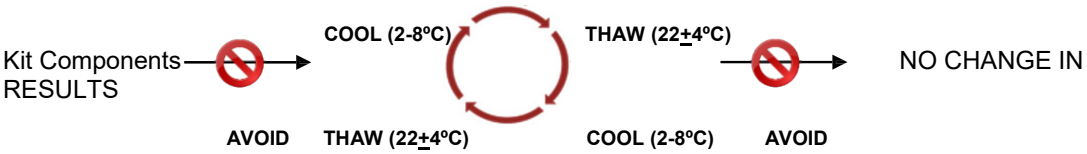
- **This kit is For Research Use Only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- 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.

SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.



2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.



3. Pipette **100 ul Standards / Samples** into each well.

4. Pipette **100 ul Biotinylated Human PD1 protein** into the respective wells.

5. Cover plate and incubate for 60min at 37°C.

6. Aspirate and wash wells 4 times with **Wash Buffer (1X).**

7. Pipette **100 ul diluted Streptavidin:HRP** into each well.

8. Cover plate and incubate for 60min at 37°C.

9. Aspirate and wash wells 4 times with **Wash Buffer (1X).**

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

11. Cover plate and incubate for 30min at 37°C.

12. Pipette **100 ul Stop Solution** into each well.

13. Read absorbance at 450nm with a microplate reader within 30min of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml PD-L1 Pathway Inhibitor
1A 2A	zero standard zero standard			
1B 2B	31.25 ng/ml 31.25 ng/ml			
1C 2C	62.5 ng/ml 62.5 ng/ml			
1D 2D	125 ng/ml 125 ng/ml			
1E 2E	250 ng/ml 250 ng/ml			
1F 2F	500 ng/ml 500 ng/ml			
1G 2G	1000 ng/ml 1000 ng/ml			
1H 2H	2000 ng/ml 2000 ng/ml			
3A 4A	Sample			
3B 4B	Sample			
3C 4C	Sample			

LIMITED WARRANTY

Krishgen Biosystems does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by Krishgen Biosystems, or against damages resulting from such non-Krishgen Biosystems made products or components. Krishgen Biosystems passes on to customer the warranty it received (if any) from the maker thereof of such non-Krishgen made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.

THIS WARRANTY IS EXCLUSIVE. The sole and exclusive obligation of Krishgen Biosystems shall be to repair or replace the defective product in the manner and for the period provided above. Krishgen Biosystems shall not have any other obligation with respect to the products or any part thereof, whether based on contract, tort, strict liability or otherwise. Under no circumstances, whether based on this Limited Warranty or otherwise, shall Krishgen Biosystems be liable for incidental, special, or consequential damages.

This Limited Warranty states the entire obligation of Krishgen Biosystems with respect to the product. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

Krishgen Biosystems. 2024

THANK YOU FOR USING KRISHGEN PRODUCT!

SYMBOLS KEY

MTP	Recombinant Human PD-L1 protein coated Microtiter Plate (12x8 wells)
STD	PD1 Antibody Standard
BIO CONJ	Human PD1 protein:Biotin Conjugate
STRP HRP	Streptavidin:HRP Conjugate (conc)
STRP HRP DIL	(1X) Streptavidin–HRP diluent
1X SAMP DIL	(1X) Sample Diluent
1X STD DIL	(1X) Standard Diluent
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature