KRIBIOLISA™ Pembrolizumab (KEYTRUDA™) ELISA

REF : KBI1084

Ver 1.5

RUO

Enzyme Immunoassay for the Quantitative Determination of Pembrolizumab in human serum and plasma.

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

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REF **KBI1084** **Σ 96 tests**

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KRIBIOLISA™ Pembrolizumab (KEYTRUDA™) ELISA

Introduction:

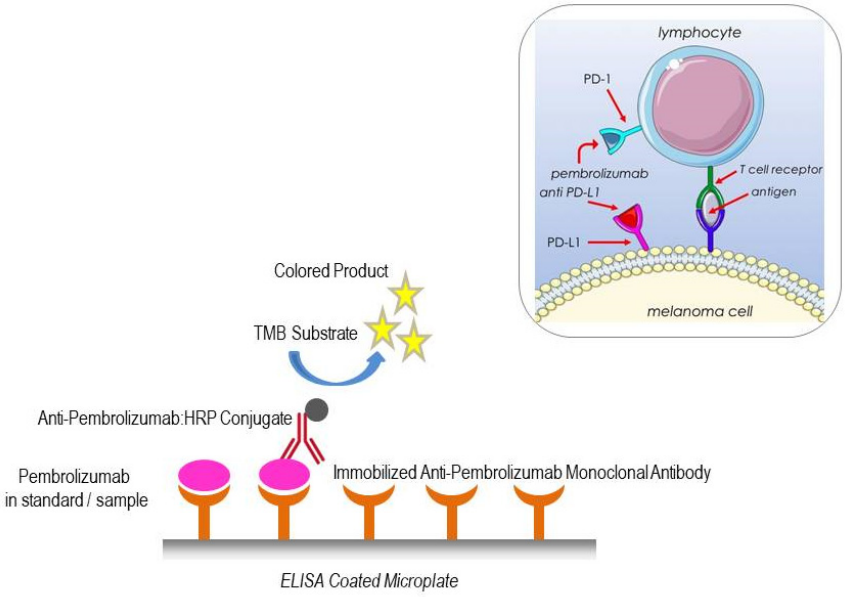
Pembrolizumab, sold under the brand name Keytruda, is a humanized antibody used in cancer immunotherapy that treats melanoma, lung cancer, head and neck cancer, Hodgkin lymphoma, stomach cancer, and cervical cancer. Pembrolizumab was approved for medical use in the United States in 2014. In 2017, the US Food and Drug Administration (FDA) approved it for any unresectable or metastatic solid tumor with certain genetic anomalies (mismatch repair deficiency or microsatellite instability). It is on the World Health Organization's List of Essential Medicines.

Intended Use:

The KRIBIOLISA™ Pembrolizumab ELISA is used as an analytical tool for quantitative determination of Pembrolizumab in human serum and plasma.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Pembrolizumab are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Pembrolizumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-pembrolizumab antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Pembrolizumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



PRINCIPLE OF THE KRIBIOLISA™ PEMBROLIZUMAB ELISA

Materials Provided:

Part	Description	Qty
Anti-Pembrolizumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Pembrolizumab monoclonal antibody.	1 x 96 wells
Pembrolizumab Standard	Recombinant Pembrolizumab in a buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.- (lyophilized, concentrated 1 ug/ml)	2 vials
Anti-Pembrolizumab:HRP Conjugate concentrated	Anti-Pembrolizumab conjugated to Horseradish Peroxidase concentrated (1mg/ml)	1 vial

Part	Description	Qty
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane with 1:1000 dilution of human serum	10 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 Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

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. Standard graph paper or software for data analysis
6. Timer
7. Absorbent Paper

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. Anti-Pembrolizumab: HRP Conjugate concentrated shoud be stored at -20ºC. Upon receipt, make aliquots and store the aliquots at -20ºC.
4. 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.
5. 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:

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.

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 - Serum and Plasma samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul sample diluent) prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires the samples to be kept at -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 **Wash Buffer (1X)**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of Standard Diluent to obtain a concentration of 1ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 640 ul of reconstituted **Standard (1 ug/ml)** with 360 ul of Standard Diluent to generate a **640 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1 ug/ml	Lyophilized Standard	Lyophilized Standard provided in the Kit + 1ml of Standard Diluent
640 ng/ml	Standard No.7	640ul Reconstituted Standard (1 ug/ml) + 360 ul Standard Diluent
320 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent
160 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent
80 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent
40 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent
20 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent
10 ng/ml	Standard No. 1	500 ul Standard No.2 + 500 ul Standard Diluent
0 ng/ml	Standard No.0	Only Standard Diluent

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

5. **Working Anti-Pembrolizumab: HRP Conjugate – Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

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 Pembrolizumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Pembrolizumab present in the sample.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Pembrolizumab.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. 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.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

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 37°C

2. Add **100 ul** of **prepared Standards or diluted Samples** into the respective wells.

3. Cover the plate and incubate for 60 minutes at 37°C

4. 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.

5. Add **100 ul** of **working Anti-Pembrolizumab:HRP Conjugate** into each well.

6. Cover the plate and incubate for 60 minutes at 37°C

7. 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.

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

9. 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.

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

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

Calculation of Results:

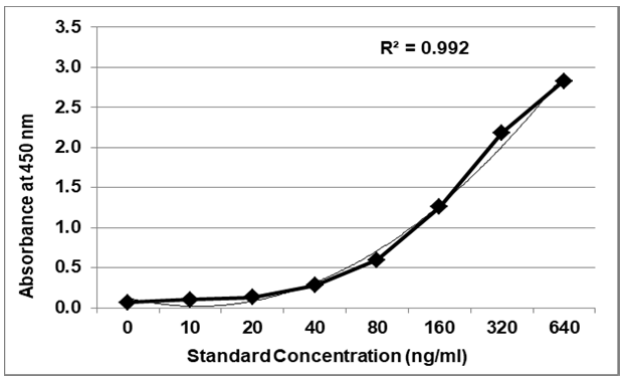
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Pembrolizumab 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 Pembrolizumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit 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 640 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.073	0.065	0.069	---	---
10	0.099	0.109	0.104	11.9	118.8
20	0.146	0.111	0.128	17.7	88.4
40	0.298	0.261	0.279	42.3	105.8
80	0.666	0.523	0.595	80.4	100.5
160	1.397	1.125	1.261	158.0	98.7
320	2.365	2.009	2.187	323.6	101.1
640	2.818	2.844	2.831	635.3	99.3

Typical Graph



Abs = absorbance at 450nm

Quality Control:

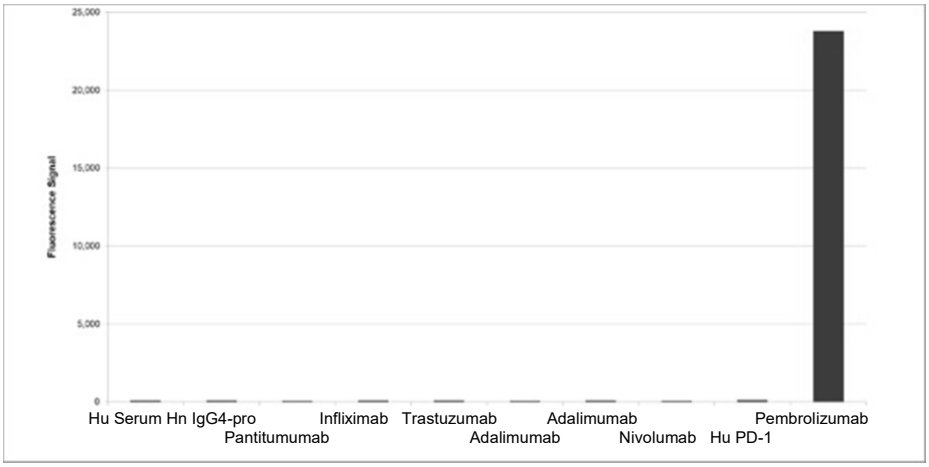
It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

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 Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 8.25 ng/ml.
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 7.75 ng/ml.

Specificity:
The capture antibody used in the kit is a paratope specific, inhibitory anti-idiotypic antibody. It specifically recognizes the free humanized monoclonal antibody pembrolizumab. The antibody does not recognize recombinant human programmed cell death 1 (PD-1) or pembrolizumab in complex with recombinant human PD-1 and is specific to measure free pembrolizumab levels in serum from patients.



The Capture antibody shows low reactivity to Panitumumab, Infliximab, Trastuzumab, Adalimumab, Nivolumab and Human PD-1. The intrinsic affinity of the monovalent form of the antibody is K_D = 0.6 nM as measured by real time, label free molecular interaction analysis on immobilized Pembrolizumab

The detection antibody used is a HRP conjugated anti-idiotypic antibody that specifically recognizes the free humanized monoclonal antibody pembrolizumab. The standards used in the kit is a monoclonal antibody specific to PD-1 / Pembrolizumab.

Note: The Standards / Calibrators have not yet been referenced and calibrated against the reference therapeutic drug. Incase of any technical difficutly when using the drug as standard, please connect with our technical team at email: sales1@krishgen.com for further optimization.

Linearity:
Standards provided in the kit will be used for measuring the linearity range of Pembrolizumab present in matrix.

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 (10 ng/ml), medium (80 ng/ml) and high (640 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	<12%	<10%
Medium	<10%	<8%
High	<10%	<8%

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.

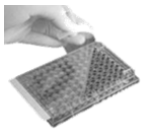
Components (2-8ºC) → 30 mins RT Before Use → 

Thaw at Room Temperature (18-24ºC) → USE NOW

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

Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

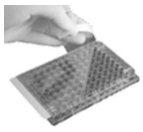
3.  Pipette **100 ul prepared Standards / diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

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

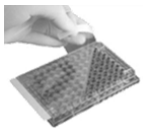
6.  Pipette **100 ul working Anti-Pembrolizumab:HRP** into each well.

7. Cover plate  and incubate for 

at 37°C

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

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

10. Cover plate  and incubate for 

at 37°C.

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

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

of stopping reaction.

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 Products; 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 Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.

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THANK YOU FOR USING KRISHGEN PRODUCT!

KRISHGEN BIOSYSTEMS | OUR REAGENTS | YOUR RESEARCH |

SYMBOLS KEY

MTP	Anti-Pembrolizumab Coated Microtiter Plate (12x8 wells)
STD	Pembrolizumab Standard
HRP | CONJ	Conjugate Horseradish Peroxidase, concentrated
1X | SAMP | DIL	(1X) Sample Diluent
1X | STD | DIL	(1X) Standard Diluent
DETN | DIL	Detection 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