

KRIBIOLISA® Anti-Pembrolizumab (KEYTRUDA™) ELISA

REF : KBI2084

Ver 1.6

RUO

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

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

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REF KBI2084

 96 tests

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Introduction:

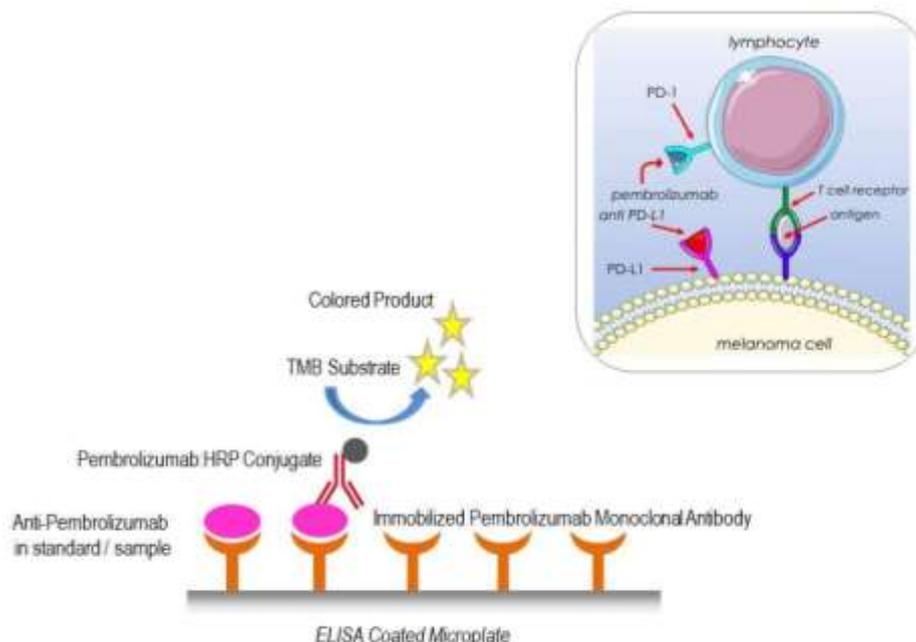
Pembrolizumab, sold under the brand name KEYTRUDA, is a humanized antibody used in cancer immunotherapy that treats melanoma, lung cancer, head and neck cancer, Hodgkin lymphoma, stomach cancer, and cervical cancer. Pembrolizumab was approved for medical use in the United States in 2014. In 2017, the 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® Anti-Pembrolizumab ELISA is used as an analytical tool for quantitative determination of Anti-Pembrolizumab in human serum and plasma.

Principle:

The method employs the sandwich enzyme immunoassay technique. Pembrolizumab is pre-coated onto microwells. Samples or standards are pipetted into microwells and antibodies to Pembrolizumab present in the standards or sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated Pembrolizumab is pipetted and incubated. After washing microwells in order to remove any nonspecific binding, the ready to use substrate 3,3',5,5' Tetra Methyl Benzidine (TMB) solution is added to microwells and color develops proportionally to the amount of Anti-Pembrolizumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



PRINCIPLE OF THE KRIBIOLISA™ ANTI-PEMBROLIZUMAB ELISA

Materials Provided:

Part	Description	Qty
Pembrolizumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Pembrolizumab.	1 x 96 wells
Anti-Pembrolizumab Standard lyophilized	Recombinant Anti-Pembrolizumab Standard in a buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane (lyophilized, concentrated, 1 ug/ml)	2 vials

Part	Description	Qty
Pembrolizumab:HRP Conjugate Concentrated	Pembrolizumab conjugated to Horseradish Peroxidase concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone 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 1:1000 dilution human serum and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	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. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and stores the Pembrolizumab:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Pembrolizumab:HRP Conjugate after running your assay.
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 (18-25°C). 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:

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

5. **Working 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 Anti-Pembrolizumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Pembrolizumab present in the sample.
3. Avoid assay of Samples containing sodium azide (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount of Anti-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. All steps must be performed at 37°C.
2. Pipette **100 ul of prepared Standards and diluted Samples** to 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. Pipette **100 ul of Working Pembrolizumab:HRP Conjugate** to all wells.
6. Cover the plate and incubate for 60 minutes at 37°C.
7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** as mentioned in Step 4.

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.

Interpretation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate 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 Anti-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 Anti-Pembrolizumab Concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a linear regression is best recommended for automated results.

Note:

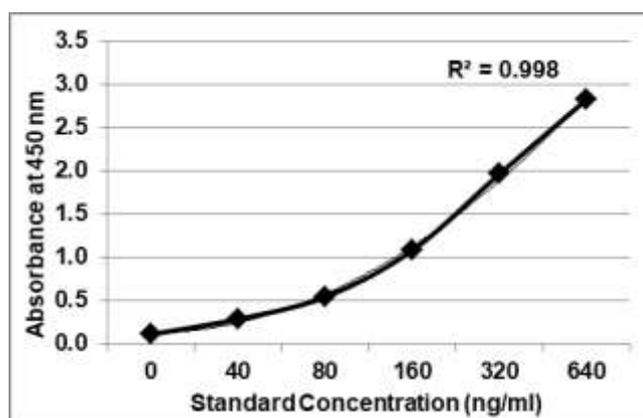
It is recommended to repeat the assay at a different dilution factor in the following cases:

- 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 (representative only)

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.112	0.104	0.108	--	--
40	0.288	0.302	0.295	42.7	106.6
80	0.544	0.538	0.541	81.4	101.8
160	1.085	1.021	1.053	156.6	97.9
320	1.968	1.981	1.974	323.2	101.0
640	2.831	2.950	2.891	638.0	99.7

Typical Graph (representative only)



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 38 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.

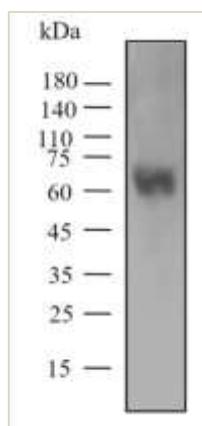
10 replicates of '0' standards were evaluated and the LOD was found to be less than 35 ng/ml.

Specificity:

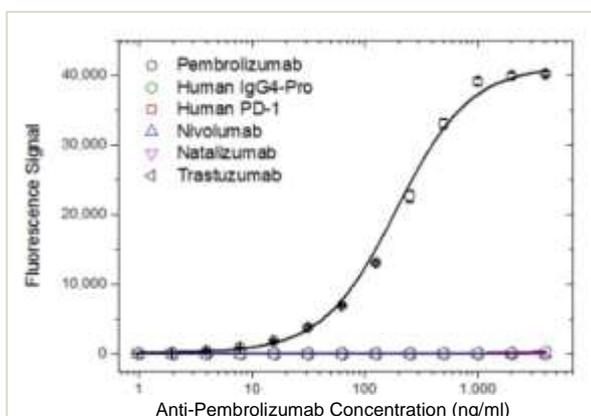
The proteins are recombinantly expressed Pembrolizumab used for capture and detection.

The calibrator / standard used in the kit is a human Anti-Pembrolizumab Antibody which is a paratope specific, inhibitory anti-idiotypic antibody that 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. These calibrators / standards used are calibrated against commercially sourced Antibody for Pembrolizumab.

Cross Reactivity:



The capture Pembrolizumab biosimilar is a humanized monoclonal antibody that blocks the interaction between PD-1 and its ligands, PD-L1 and PD-L2. It is an IgG4 kappa immunoglobulin and has a purity of >95% by SDS-PAGE and HPLC and an endotoxin limit of less than 0.75 EU/mg by LAL method.



The calibrator / standard used in the kit does not cross react to Human IgG4-Pro, Human PD-1, Nivolumab, Natalizumab or Trastuzumab.

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 (40 ng/ml), medium (160 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
Low	<12%
Medium	<10%
High	<10%

Pool	Inter Assay %CV
Low	<15%
Medium	<10%
High	<10%

Lot to Lot Variation:

Standard Concentration (ng/ml)	Lot A OD450	Lot B OD450	Lot C OD450	Mean OD450	% STD DEV	CV	% CV
0	0.058	0.085	0.126	0.09	---	---	---
40	0.331	0.24	0.268	0.28	3.8	0.17	16.6
80	0.608	0.512	0.588	0.569	4.1	0.09	8.8
160	1.112	0.967	0.913	0.997	8.4	0.1	10.3
320	1.917	2.025	1.987	1.976	4.5	0.03	2.8
640	2.81	2.768	2.757	2.778	2.3	0.01	1

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/v) 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

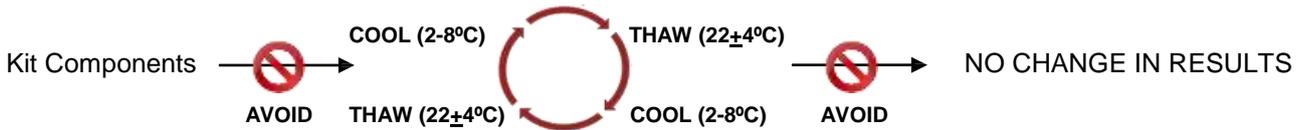


SCHEMATIC ASSAY PROCEDURE

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 prepared Standards / diluted Samples** to 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 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 450 nm with a microplate reader within of stopping reaction.

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SYMBOLS KEY

MTP	Pembrolizumab Coated Microtiter Plate (12x8 wells)
STD	Recombinant Anti-Pembrolizumab Standard, Lyophilized
HRP CONJ	Pembrolizumab:HRP Conjugate Concentrated
DETN DIL	Detection 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