

KRIBIOLISA® Durvalumab (IMFINZI™) ELISA

REF : KBI1034

Ver 1.0

RUO

Enzyme Immunoassay for the quantitative determination of Durvalumab (IMFINZI™) 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 KBI1034 96 tests**Krishgen Biosystems Private Limited**

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Introduction:

Durvalumab is a fully human IgG1 kappa monoclonal antibody that selectively targets and blocks programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumour-mediated immune evasion. By binding with high affinity to PD-L1 expressed on tumour and antigen-presenting cells, durvalumab prevents its interaction with PD-1 and CD80 receptors, thereby blocking inhibitory checkpoint signalling and restoring T-cell-mediated anti-tumour immune responses. This leads to reactivated cytotoxic T-cell activity and enhanced immune-mediated destruction of tumour cells. Durvalumab is approved in multiple major markets, including the United States and the European Union, for the treatment of conditions such as unresectable stage III non-small cell lung cancer, extensive-stage small cell lung cancer, locally advanced or metastatic urothelial carcinoma, and biliary tract cancer.

Intended Use:

The KRIBIOLISA® Durvalumab (IMFINZI™) ELISA is intended for use as an analytical tool for the quantitative determination of Ramucirumab in human serum and plasma.

Principle:

The method employs a sandwich enzyme-linked immunosorbent assay (ELISA) technique. Recombinant Human PD-L1 (Fc Chimera) antigen is pre-coated onto microwells. Samples or standards are added to the microwells, and Durvalumab present in the samples binds specifically to the immobilized antigen. After incubation, unbound components are removed by washing, and an HRP (horseradish peroxidase)-conjugated Anti-Durvalumab antibody is added, which binds to the captured Durvalumab, forming an antigen-antibody-antibody "sandwich" complex. Following a second washing step to remove any unbound conjugate, the ready-to-use substrate 3,3',5,5'-Tetramethylbenzidine (TMB) is added, producing a color reaction proportional to the amount of Durvalumab in the sample. The reaction is stopped with a stop solution, and absorbance is measured at 450 nm to determine the concentration of Durvalumab.

Materials Provided:

Part	Description	Qty
Recombinant Human PD-L1 (Fc Chimera) antigen Coated Microtiter Plate	96 well polystyrene microplate (12 x 8 wells) coated with Recombinant Human PD-L1 (Fc Chimera) antigen.	12 x 8 wells
Durvalumab Standard	Standard in a buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane (lyophilized, 1 ug/ml)	2 vials
Anti-Human IgG (Fc-specific): HRP Conjugate, concentrated	Anti-Human IgG (Fc-specific): conjugated to Horseradish Peroxidase, concentrated.	1 vial
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	10 ml
(1X) Sample Diluent	Buffered protein base with 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 with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	12 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 store the Durvalumab Standard concentrated at -20°C. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Anti-Human IgG (Fc-specific): 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. 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 - Samples have to be diluted 1:1000 (v/v), e.g. 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 -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 (1X) Standard Diluent to obtain a concentration of 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Prepare **Standards** as per the below table. Use the (1X) 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 (1X)
640 ng/ml	Standard No.7	640 ul Reconstituted Standard + 360 ul Standard Diluent (1X)
320 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
160 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
80 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
40 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
20 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
10 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Only Standard Diluent (1X)

5. **Working Anti-Human IgG (Fc-specific): 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 Durvalumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Durvalumab 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. Thus if the Durvalumab concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
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 Durvalumab.
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 compromise 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 off 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 Anti-Human IgG (Fc-specific): 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.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Semi-Log graph paper, plot the average value (absorbance 450 nm) 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 Ramucirumab 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 Ramucirumab Antigen 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:

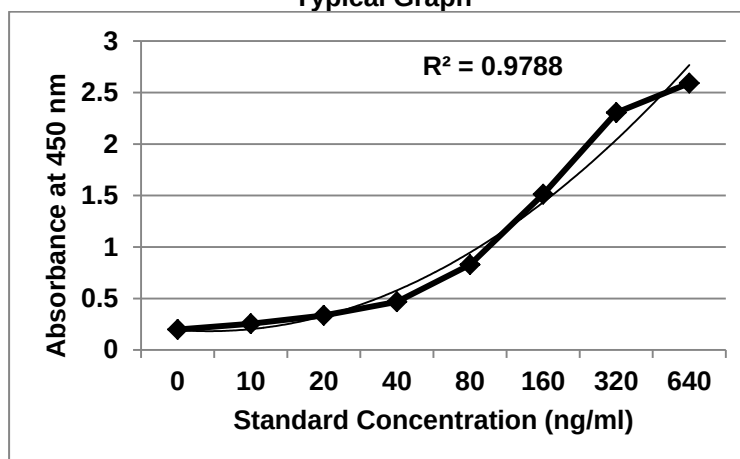
It is recommended to repeat the assay at a different dilution factor in the following cases:

- 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)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.198	-	-
10	0.255	14.0	90.0
20	0.336	40.2	128.6
40	0.468	67.0	107.2
80	0.828	124.5	99.6
160	1.512	242.1	96.8
320	2.305	536.8	107.4
640	2.591	922.3	92.2

Typical Graph



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 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 8 ng/ml.

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 (2.5 ng/ml), medium (10 ng/ml) and high (40 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	<5%	<5%
High	<5%	<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.
- Source materials maybe derived from Mouse 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.

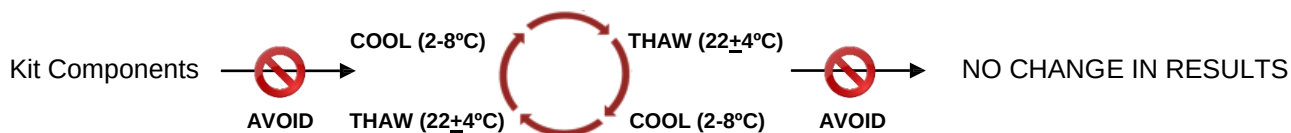


SCHEMATIC ASSAY PROCEDURE

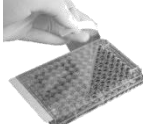
1. Remove all components, 30 minutes before adding into the assay plate.



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

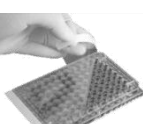


3.  Pipette **100 ul prepared Standards / diluted Samples** to the respective wells.

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

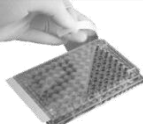
5.  Aspirate and wash wells 4 times with **Wash Buffer (1X)**.

6.  Pipette **100 ul Anti-Human IgG (Fc-specific): HRP Conjugate** into each well.

7.  Cover plate and incubate for  **60 min** 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  **30 min** at 37°C.

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

12. Read absorbance at 450 nm with a  microplate reader within  **15 min** of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance
1A	Zero Std.		
2A	Zero Std.		
1B	Standard No.1		
2B	Standard No.1		
1C	Standard No.2		
2C	Standard No.2		
1D	Standard No.3		
2D	Standard No.3		
1E	Standard No.4		
2E	Standard No.4		
1F	Standard No.5		
2F	Standard No.5		
1G	Standard No.6		
2G	Standard No.6		
1H	Standard No.7		
2H	Standard No.7		
3A	Sample		
4A	Sample		
3B	Sample		
4B	Sample		

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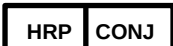
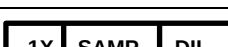
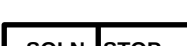
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SYMBOLS KEY

	Recombinant Human PD-L1 (Fc Chimera) antigen Coated Microtiter Plate (12 x 8 wells)
	Durvalumab Standard, Lyophilized
	Anti-Human IgG (Fc-specific): HRP Conjugate concentrated
	Detection Diluent
	(1X) Standard Diluent
	(1X) Sample Diluent
	(20X) Wash Buffer
	TMB Substrate
	Stop Solution
	Consult Instructions for Use
	Catalog Number
	Expiration Date
	Storage Temperature