KRISHGEN BioSystems

KRIBIOLISA™ Relatlimab ELISA

REF : KBI1586

Ver 1.0

May also be used for estimation of Relatlimab in patient dosage of Opdualag™ (nivolumab and relatlimab-rmbw)

RUO

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

RUO	For Research Use Only	REF	Catalog Number
	Store At	LOT	Batch Code
	Manufactured By		Biologi isk
	Expiry D		Consu rating Instructions

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REF **KBI1586** Σ **96 tests**

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Introduction:

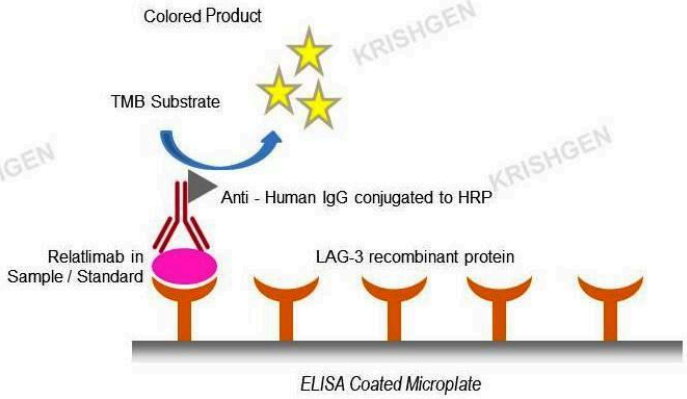
Relatlimab is a monoclonal antibody designed for the treatment of melanoma. Relatlimab is a Lymphocyte activation gene-3 (LAG-3) inhibitor. It is used in combination with nivolumab to treat melanoma. It is under development by Bristol-Myers Squibb. It is made using Chinese hamster ovary cells. The combination nivolumab/relatlimab (Opdualag) was approved for medical use in the United States in March 2022.

Intended Use:

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

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. LAG3 recombinant proteins are pre-coated onto microwells. Samples and standards are pipetted into microwells and Anti-Human LAG3 (Relatlimab) present in the sample are bound by the capture antibody. Then, an HRP (horseradish peroxidase) conjugated anti-Human IgG 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 Relatlimab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
LAG-3 recombinant protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with LAG-3 recombinant protein.	1 x 96 wells
Relatlimab Standard	Recombinant Relatlimab in a buffered protein base with preservative sodium azide – lyophilized (1 ug/ml)	2 Vials
Anti-Human IgG:HRP Conjugate	Anti-Human IgG conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative sodium azide < 0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with 1:1000 dilution human serum and preservative sodium azide < 0.01%	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	2N Sulfuric 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, Absorbent Paper, Timer
4. Wash bottle or automated microplate washer
5. Graph paper or software for data analysis

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.

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.
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 Standard Diluent to obtain a concentration of 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 640 ul of original **Standard (1 ug/ml)** with 360 ul of Standard Diluent to generate a **640 ng/ml Standard Solution**. Prepare further **Standard**s 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	640 ul Reconstituted Standard (1 ug/ml) + 360 ul Standard Diluent
320 ng/ml	Standard No.6	500 ul Standard No7 + 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.

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 Relatlimab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Relatlimab 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 Relatlimab 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 Relatlimab.
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. Pipette **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 **Anti-Human IgG: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 Semi-Log 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 relatlimab 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 Relatlimab Concentration. If samples were diluted, multiply by the appropriate dilution factor.

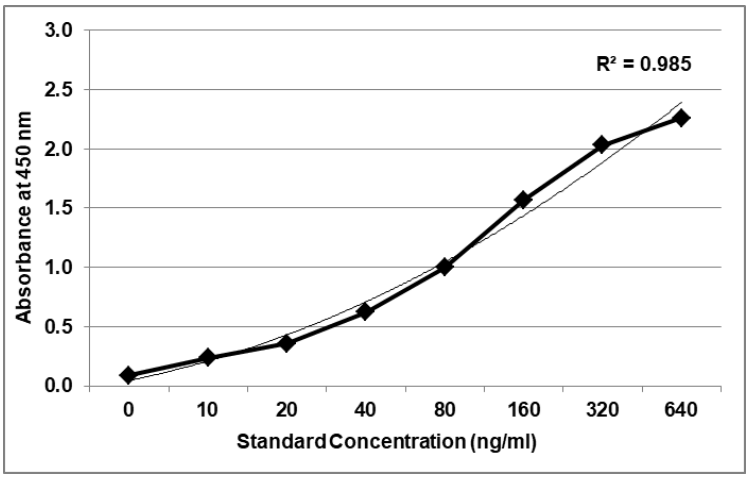
Software which is able to generate a cubic spline curve-fit or 4PL is best recommended for automated results.

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	0.087	0.088	0.088	--	--
10	0.231	0.240	0.235	11.6	115.9
20	0.360	0.354	0.357	20.8	104.0
40	0.618	0.624	0.621	41.0	102.5
80	1.002	0.998	1.000	75.8	94.7
160	1.477	1.667	1.572	162.4	101.5
320	2.033	2.027	2.030	337.2	105.4
640	2.228	2.291	2.259	599.9	93.7

Typical Graph



Abs= Absorbance @ 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 9 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 (10ng/ml), medium (160ng/ml) and high (640ng/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%	<12%
Medium	<12%	<12%
High	<10%	<10%

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.

Background:

Nivolumab / Relatlimab, sold under the brand name Opdualag, is a fixed-dose combination medication use to treat melanoma. It contains nivolumab, a programmed death receptor-1 (PD-1) blocking antibody, and relatlimab, a lymphocyte activation gene-3 (LAG-3) blocking antibody. It is given by intravenous infusion. The combination was approved for medical use in the United States in March 2022 and in the European Union in September 2022.

The KRIBIOLISA™ Relatlimab ELISA kit helps in estimating patient dosage concentrations of Relatlimab in serum and plasma.

Opdualag is the registered trade mark of Bristol Meyers Squbbs, USA.

References:

Preclinical characterization of relatlimab, a human LAG-3–blocking antibody, alone or in combination with nivolumab
K Thudium, M Selby, JA Zorn, G Rak, XT Wang… - BioRxiv, 2022 - biorxiv.org

LAG-3 blockade with relatlimab (BMS-986016) restores anti-leukemic responses in chronic lymphocytic leukemia
C Sordo-Bahamonde, S Lorenzo-Herrero… - Cancers, 2021 - mdpi.com

AK129, an anti-PD1/LAG-3 bi-specific antibody for cancer therapy
Z Huang, X Pang, T Zhong, C Jin, N Chen, D Xia… - Cancer Research, 2022 - AACR

Characterization of a novel anti-human lymphocyte activation gene 3 (LAG-3) antibody for cancer immunotherapy
X Yu, X Huang, X Chen, J Liu, C Wu, Q Pu, Y Wang… - MAbs, 2019 - Taylor & Francis

Phase IB trial of induction nivolumab or nivolumab/relatlimab prior to concurrent chemoradiation plus nivolumab or nivolumab/relatlimab in patients with …

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

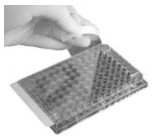
3. Pipette **100 ul** prepared **Standards** / diluted **Samples** into each well.

4. Cover plate  and incubate for 

at 37°C.

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

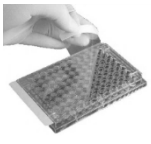
6. Pipette **100 ul Anti-Human IgG:HRP Conjugate** into each well.

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.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Relatlimab equivalent
1A 2A	zero std zero std			
1B 2B	10 ng/ml 10 ng/ml			
1C 2C	20 ng/ml 20 ng/ml			
1D 2D	40 ng/ml 40 ng/ml			
1E 2E	80 ng/ml 80 ng/ml			
1F 2F	160 ng/ml 160 ng/ml			
1G 2G	320 ng/ml 320 ng/ml			
1H 2H	640 ng/ml 640 ng/ml			
3A 4A	Sample Sample			
3B 4B	Sample Sample			

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KRISHGEN BIOSYSTEMS™, GENLISA™, DHARMAPLEX™, GENBULK™, GENLISA™, KRISHZYME™, KRISHGEN™,

	L MTP combinant protein Coated Microtiter Plate (12x8 wells)
	R STD o Standard
	HRP CONJ G:HRP Conjugate
	1X SAMP DIL luent
	1X STD DIL Diluent
	20X WASH BUF ffer
	SUB TMB te
	SOLN STOP
	Consult Instructions for Use
	C REF umber
	Expiration Date
	Storage Temperature

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SYMBOLS KEY