KRIBIOLISA™ Anti-Obinutuzumab (GAZYVA) ELISA

REF : KBI2058

Ver 1.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Antibodies to Obinutuzumab 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 KBI2058 Σ 96 tests

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Introduction:

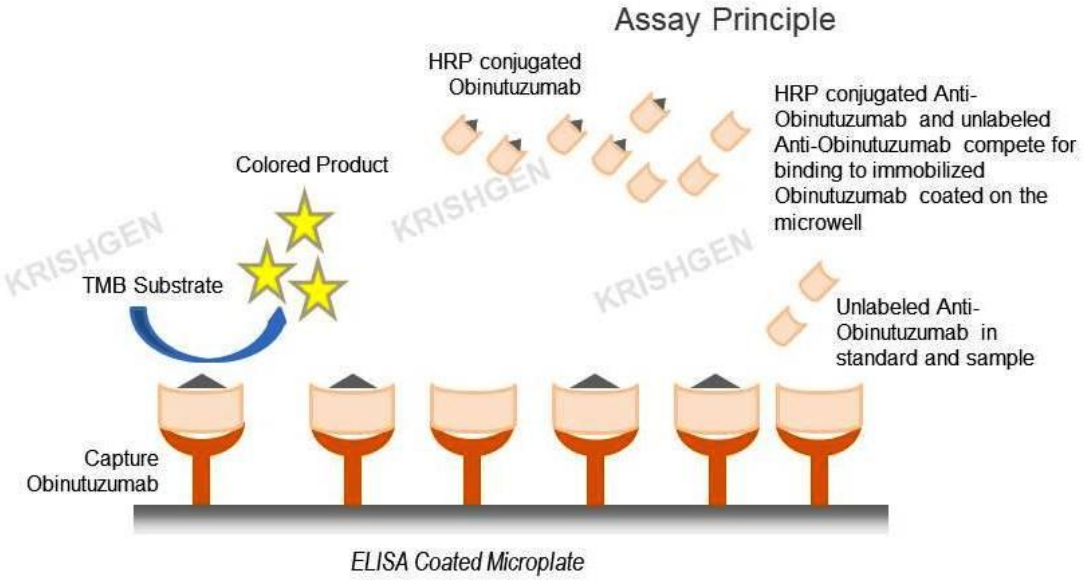
Obinutuzumab, sold under the brand name Gazyva is a humanized anti-CD20 monoclonal antibody used as a treatment for cancer. It was originated by GlycArt Biotechnology AG and developed by Roche. It is used for treatment for chronic lymphocytic leukaemia (CLL) and follicular lymphoma.
Anti-Drug Antibodies (ADA) may induce unwanted side effects in biopharmaceuticals. Hence, ADA has been subjected to increase in scrutiny by the regulatory authorities using immunogenicity safety studies. ADA has been observed in pre-clinical and clinical studies, resulting in significant changes in toxicology, pharmacokinetics and efficacy.
These effects result from the generation of drug-induced (neutralizing) autoantibodies against Obinutuzumab. This ELISA kit detects antibodies for Anti-Obinutuzumab and may be used for monitoring immunogenicity.

Intended Use:

The KRIBIOLISA™ Anti-Obinutuzumab (GAZYVA) ELISA is used as an analytical tool for quantitative determination of Anti-Obinutuzumab in human serum and plasma.

Principle:

The method employs the quantitative competitive enzyme immunoassay technique.Obinutuzumab is pre-coated onto microwells. Samples, standards and HRP conjugated Obinutuzumab antibody are pipetted into microwells and incubated. Plates are washed with wash buffer. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color the color developed is inversly proportionally to the amount of Anti-Obinutuzumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



PRINCIPLE OF THE KRIBIOLISA™ ANTI-OBINUTUZUMAB (GAZYVA) ELISA

Materials Provided:

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

Part	Description	Qty
Obinutuzumab monoclonal antibody:HRP Conjugate	Obinutuzumab monoclonal antibody 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 protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	50 ml
(1X) Standard Diluent	Buffered protein base with 1:10 dilution human serum and protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	10 ml
(20X) Wash Buffer	25 ml/vial of a 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. 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.

For Cell Culture Supernatant **-** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80ºC. Avoid repeated freeze-thaw cycles.

Preparation Before Use:

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

Serum and Plasma Test Sample preparation - Samples have to be diluted 1:10 (v/v), e.g. for 1:10 (50 ul sample + 450 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 500 ul of original **Standard (1 ug/ml)** with 500 ul of Standard Diluent (1X) to generate a **500 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 (1X)
500 ng/ml	Standard No.8	500 ul Reconstituted Standard (1 ug/ml) + 500 ul Standard Diluent (1X)
250 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
31.25 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
15.6 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
7.8 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
3.9 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)

Use the Standards as soon as possible upon reconstitution. Discard balance standard after use.

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-Obinutuzumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Obinutuzumab 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 Anti-Obinutuzumab 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 Anti-Obinutuzumab.
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. Pipette **100 ul** of **Standards** and **diluted Samples** to the respective wells.

2. Pipette **100 ul** of **Anti-Obinutuzumab:HRP Conjugate** to all wells.

3. Seal plate and incubate for 1 hour at 37ºC.

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. All the washes should be performed similarly.

5. Pipette **100 ul** of **TMB Substrate solution**.

6. Incubate in the dark for 30 minutes at Room Temperature.

7. Stop reaction by adding **100 ul** of **Stop Solution** to each well.

8. Read absorbance at 450 nm within 30 minutes of stopping reaction.

Calculation of Results:

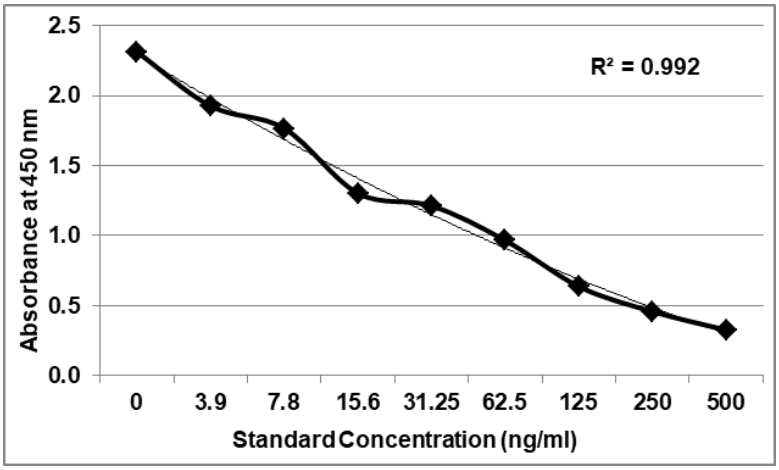
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using graph paper, plot the average value (absorbance 450 nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Anti- Obinutuzumab 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-Obinutuzumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or a polynomial curve (2^{nd} 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 500 ng/ml standard

Typical Data

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	2.313	0.0	--
3.9	1.926	3.5	89.5
7.8	1.765	6.4	81.4
15.6	1.302	22.8	146.5
31.25	1.212	28.5	91.3
62.5	0.965	52.8	84.4
125	0.636	134.0	107.2
250	0.456	260.4	104.2
500	0.325	502.3	100.5

Typical Graph



Abs = absorbance @450nm

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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 2.0 ng/ml

Specificity:
The kit uses Obinutuzumab to capture and detect the antibodies specific to Obinutuzumab.

Linearity:
Standards provided in the kit will be used for measuring the linearity range of Anti-Obinutuzumab 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 (3.9 ng/ml), medium (62.5 ng/ml) and high (500 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%	<12%
Medium	<10%	<10%
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.

SCHEMATIC ASSAY PROCEDURE

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

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

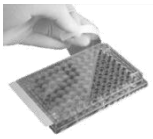
Thaw at Room Temperature (18-24ºC) → USE NOW

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 Standards / diluted Samples** into the respective wells.

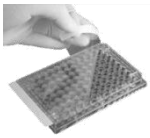
4.  Pipette **100 ul Anti-Obinutuzumab:HRP Conjugate** into each well.

5. Cover plate  and incubate for 

at 37°C.

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

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

8. Cover plate  and incubate for 

at Room Temperature.

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

10. 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 Anti-Obinutuzumab equivalent
1A 2A	zero std zero std			
1B 2B	3.9 ng/ml 3.9 ng/ml			
1C 2C	7.8 ng/ml 7.8 ng/ml			
1D 2D	15.6 ng/ml 15.6 ng/ml			
1E 2E	31.25 ng/ml 31.25 ng/ml			
1F 2F	62.5 ng/ml 62.5 ng/ml			
1G 2G	125 ng/ml 125 ng/ml			
1H 2H	250 ng/ml 250 ng/ml			
3A 4A	500 ng/ml 500 ng/ml			
3B 4B	Sample			
3C 4C	Sample			

LIMITED WARRANTY

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SYMBOLS KEY

MTP	Obinutuzumab Coated Microtiter Plate (12 x 8 wells)
STD	Anti-Obinutuzumab Standard
HRP CONJ	Conjugate Horseradish Peroxidase
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