KRISHGEN BioSystems
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KRIBIOLISA® Rituximab (RITUXAN™) ELISA

REF : KBI1010

Ver 4.5

RUO

This Kit has been Calibrated against an International Standard from the National Institute of Biologicals and Control (NIBSC), Potters Bar, Hertfordshire EN6 3QG, UK.

Enzyme Immunoassay for the Quantitative Determination of Rituximab 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**

For Research Use Only. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Krishgen Biosystems Private Limited is strictly prohibited.

REF KBI1010 Σ 96 tests

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Introduction:

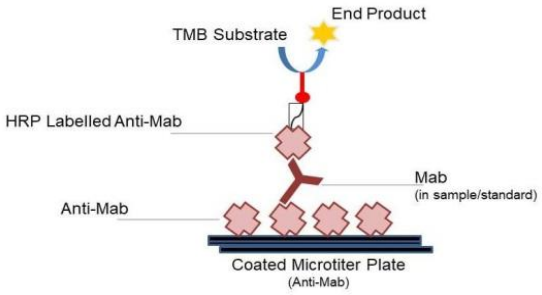
Rituximab is a monoclonal antibody which has high specificity for CD20 and it is used to treat certain autoimmune diseases and types of cancer. It is also used for non-Hodgkin's lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis, and granulomatosis with polyangiitis, idiopathic thrombocytopenic purpura, pemphigus vulgaris, myasthenia gravis and Epstein - Barr virus-positive mucocutaneous ulcers. It is given by slow injection into a vein.

Intended Use:

The KRIBIOLISA® Rituximab (RITUXAN™) ELISA is used as an analytical tool for quantitative determination of Rituximab in human serum and plasma.

Principle:

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



Materials Provided:

Part	Description	Qty
Anti-Rituximab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Rituximab monoclonal antibody	1 x 96 wells
Rituximab Standard, (lyophilized)	Recombinant Rituximab in a buffered protein base and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane – (lyophilized, concentrated 1 ug/ml)	2 vials
Anti-Rituximab:HRP Conjugate concentrated	Anti-Rituximab conjugated to Horseradish Peroxidase concentrated (0.5 mg/ml)	2 vials
Standard Diluent	Buffered protein base with preservative 0.02% methylisothiazolone and 0.02% bromonitrodioxane and 1:1000 dilution human serum	10 ml
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	12 ml
Sample Diluent	Buffered protein base with preservative 0.02% methylisothiazolone and 0.02% bromonitrodioxane	2 x 50 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal < 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 Anti-Rituximab: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 Anti-Rituximab: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 (18-25ºC). 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.

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 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 320 ul of original **Standard (1 ug/ml)** with 180 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 + 1ml of Standard Diluent
640 ng/ml	Standard No.7	320 ul Reconstituted Standard (1 ug/ml) + 180 ul Standard Diluent
320 ng/ml	Standard No.6	250 ul Standard No.7 + 250 ul Standard Diluent
160 ng/ml	Standard No.5	250 ul Standard No.6 + 250 ul Standard Diluent
80 ng/ml	Standard No.4	250 ul Standard No.5 + 250 ul Standard Diluent
40 ng/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent
20 ng/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent
10 ng/ml	Standard No.1	250 ul Standard No.2 + 250 ul Standard Diluent
0 ng/ml	Standard No.0	Only Standard Diluent

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

5. **Working Anti-Rituximab: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 Rituximab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Rituximab 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 Rituximab 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 Rituximab.
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-Rituximab: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)** 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 within 10-15 minutes after addition of Stop solution.

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.

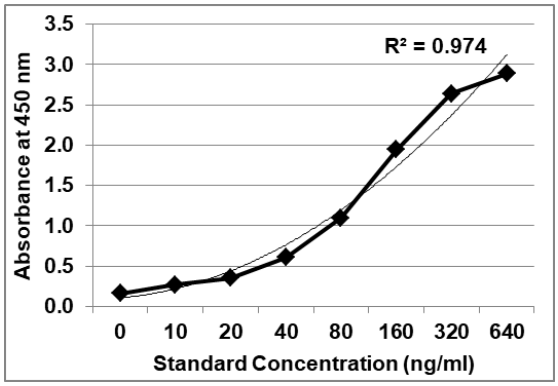
To determine the unknown Rituximab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Rituximab Concentration. If samples were diluted, multiply by the appropriate 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 (representative only)

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.163	--	--
10	0.269	6.8	68.0
20	0.349	19.6	97.9
40	0.604	42.7	106.7
80	1.096	78.6	98.3
160	1.945	158.9	99.3
320	2.636	333.7	104.3
640	2.885	599.5	93.7

Typical Graph (representative only)



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:

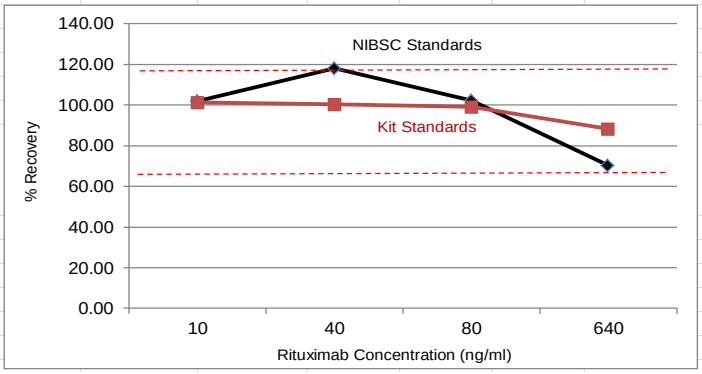
This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

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 9.4 ng/ml

Specificity:
The capture antibody is a monovalent human recombinant Fab (kappa light chain) expressed in E. coli with Rituximab as the immunogen. The intrinsic affinity of the capture antibody is K_D=0.13 nM as measured by real time, label-free molecular interaction analysis on immobilized rituximab.

The standard used in the kit is a recombinant monoclonal antibody validated against commercially available Rituximab and also NIBSC/WHO standard.

Calibration:
This Kit has been calibrated against an International Standard from the National Institute of Biologicals and Control (NIBSC), Potters Bar, Hertfordshire EN6 3QG, UK. 100 ug/1ml of supplied standard equals 1,000 IU of Rituximab. Please note that the calibration is lot specific. The Standards provided in the kit are also calibrated against commercially sourced Rituxan™ and alternate biosimilar recombinant Rituximab injection.



NIBSC Standard (ng/ml)	NIBSC Calibrator % Recovery	Kit Standard % Recovery
10	101.72	101.12
40	117.9	100.24
80	102.1	99.12
640	70.3	88.23

Linearity:
Standards provided in the kit present a linearity range of Rituximab with a regression coefficient of more than >0.9 when measured using a polynomial regression to the 2nd order.

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 (80ng/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
Low	<10%
Medium	<10%
High	<10%

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration	% STD Deviation	CV	%CV
0	0.074	0.083	0.078		--	0.6	0.08	8.0
10	0.253	0.248	0.251	16.2	161.6	0.4	0.02	1.6
20	0.331	0.349	0.340	29.0	144.9	1.3	0.04	3.7
40	0.479	0.445	0.462	44.8	112.1	2.4	0.05	5.2
80	0.730	0.710	0.720	76.9	96.1	1.4	0.02	2.0
160	1.285	1.237	1.261	149.6	93.5	3.4	0.03	2.7
320	2.194	2.185	2.190	341.9	106.9	0.6	0.00	0.3
640	2.742	2.857	2.799	620.3	96.9	8.1	0.03	2.9

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

Standard Concentration (ng/ml)	OD450 Day 1 Operator A	OD450 Day 2 Operator B	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration	% STD Deviation	CV	%CV
0	0.115	0.138	0.127	--	--	1.7	0.1	13.1
10	0.174	0.169	0.171	10.8	108.4	0.4	0.0	2.1
20	0.295	0.269	0.282	19.2	96.2	1.8	0.1	6.5
40	0.539	0.521	0.530	39.0	97.6	1.3	0.0	2.5
80	0.943	0.987	0.965	81.2	101.6	3.1	0.0	3.2
160	1.486	1.540	1.513	161.6	101.0	3.8	0.0	2.5
320	2.036	2.060	2.048	313.2	97.9	1.7	0.0	0.8
640	2.499	2.501	2.500	649.0	101.4	0.2	0.0	0.1

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.

References:

Mechanism of action of rituximab..Thomas Cerny; Bettina Borisch; Martino Introna; Peter Johnson; Andrea L Rose….Anti-Cancer Drugs. 2002…. - Wolters Kluvers

The therapeutic use of rituximab in non-Hodgkin's lymphoma….Marcus R1, Hagenbeek A..Eur J Haematol Suppl. 2007…. -Wiley

Clinical and immunological outcomes of high- and low-dose rituximab treatments in patients with pemphigus: a randomized, comparative, observer-blinded study...Kanwar AJ1, Vinay K, Sawatkar GU, Dogra S, Minz RW, Shear NH, Koga H, Ishii N, Hashimoto T. Br J Dermatol. 2014… - Wiley

Population pharmacokinetics of rituximab with or without plasmapheresis in kidney patients with antibody-mediated disease…Puisset F1, White-Koning M, Kamar N, Huart A, Haberer F, Blasco H, Le Guellec C, Lafont T, Grand A, Rostaing L, Chatelut E, Pourrat J. Br J Clin Pharmacol…. 2013… Wiley

Validation of an ELISA for the determination of rituximab pharmacokinetics in clinical trials subjects….Hampson G1, Ward TH, Cummings J, Bayne M, Tutt AL, Cragg MS, Dive C, Illidge TM…Immunol Methods. 2010… Elsevier

Rituximab in clinical practice: dosage, drug adherence, Ig levels, infections, and drug antibodies.Einarsson JT1,2, Evert M3, Geborek P3, Saxne T3, Lundgren M4, Kapetanovic MC3… Clin Rheumatol. 2017 Epub 2017 Oct…Springer

SCHEMATIC ASSAY PROCEDURE

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

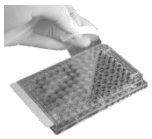
Components (2-8ºC) → 30 mins RT Before Use → 

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

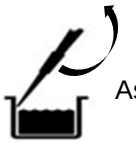
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

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

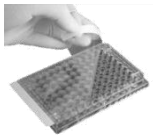
3.  Pipette **100 ul Standards** / **diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

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

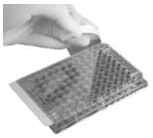
6.  Pipette **100 ul Working Anti Rituximab:HRP Conjugate** into each well.

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 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Rituximab 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			
3B 4B	Sample			

LIMITED WARRANTY

Krishgen Biosystems Private Limited 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 Private Limited, or against damages resulting from such non-Krishgen Biosystems Private Limited made products or components. Krishgen Biosystems Private Limited 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 Private Limited.

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This Limited Warranty states the entire obligation of Krishgen Biosystems Private Limited with respect to the Products. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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THANK YOU FOR USING KRISHGEN PRODUCT!

KRISHGEN BIOSYSTEMS PRIVATE LIMITED | OUR REAGENTS | YOUR RESEARCH |
RITUXAN ™ is the registered trademark of Biogen.

SYMBOLS KEY

MTP	**Anti-Rituximab Coated Microtiter Plate (12X8 wells)**
STD	**Rituximab Standard, Lyophilized**
HRP | CONJ	**Anti-Rituximab:HRP Conjugate concentrated**
DETN | DIL	**Detection Diluent**
STD | DIL	**Standard Diluent**
SAMP | DIL	**Sample 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**