KRIBIOLISA™ Emicizumab (HEMLIBRA™) ELISA

REF : KBI1297

Ver 2.1

RUO

Enzyme Immunoassay for the Quantitative Determination of Emicizumab 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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KBI1297 **96 tests** **REF** Σ

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Introduction:

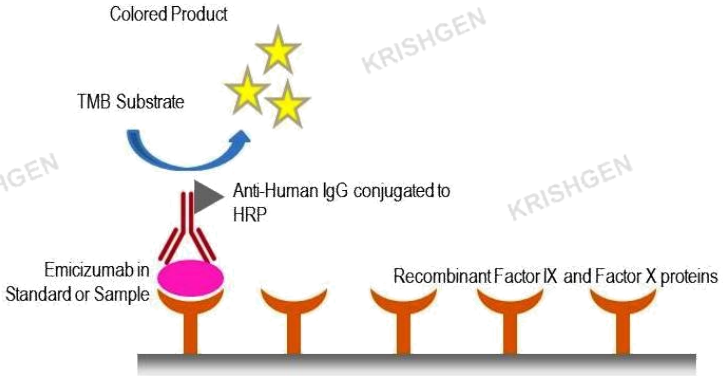
Emicizumab (trade name Hemlibra) is a humanized bispecific antibody for the treatment of haemophilia A. Emicizumab binds to both the activated coagulation factor IX and to factor X, mediating the activation of the latter. This is normally the function of coagulation factor VIII, which is missing in haemophilia A patients.

Intended Use:

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

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Factor IX and Factor X protein are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Emicizumab present in the sample are bound by the capture antibody. Then, a goat Anti-Human IgG HRP (horseradish peroxidase) 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 Emicizumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Factor IX + Factor X protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Factor IX and Factor X protein.	1 x 96 wells
Emicizumab Standard	Emicizumab Standard in a buffered protein base with preservative sodium azide– lyophilized (100ug/ml)	2 vials
Goat Anti-Human IgG:HRP Conjugate	Goat Anti-Human IgG: HRP Conjugate with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative thiomersol < 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	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.

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. 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 - 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 1000ul of Standard Diluent to obtain a concentration of 100ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Prepare **Standards** as per the below table. Use the Standard Diluent as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
100 ug/ml	Lyophilized Standard	Lyophilized Standard in the Kit + 1000 ul of Standard Diluent (1X)
50 ug/ml	Standard No.7	500ul Reconstituted Standard (100 ug/ml) + 500ul Standard Diluent (1X)
25 ug/ml	Standard No.6	500ul Standard No.7 + 500ul Standard Diluent (1X)
12.5 ug/ml	Standard No.5	500ul Standard No.6 + 500ul Standard Diluent (1X)
6.25 ug/ml	Standard No.4	500ul Standard No.5 + 500ul Standard Diluent (1X)
3.125 ug/ml	Standard No.3	500ul Standard No.4 + 500ul Standard Diluent (1X)
1.563 ug/ml	Standard No.2	500ul Standard No.3 + 500ul Standard Diluent (1X)
0.781 ug/ml	Standard No.1	500ul Standard No.2 + 500ul Standard Diluent (1X)
0 ug/ml	Standard No.0	Only Standard Diluent (1X)

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 Emicizumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Emicizumab 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 Emicizumab 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 Emicizumab.
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. 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 120 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 **Goat Anti-Human IgG:HRP Conjugate** into each well.
6. Cover the plate and incubate for 180 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 Emicizumab 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 Emicizumab Concentration.

If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic

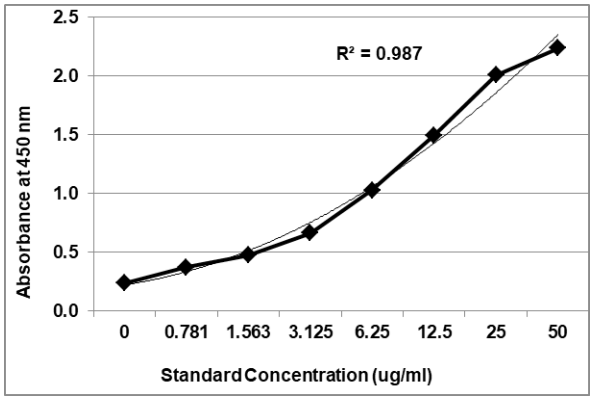
spline curve-fit or 4PL (2nd order) is best recommended for automated results.

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 50 ug/ml standard.

Typical Data

Standard Concentration (ug/ml)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.237	--	--
0.781	0.373	0.91	116.5
1.563	0.473	1.69	108.4
3.125	0.663	3.08	98.6
6.25	1.026	6.15	98.4
12.5	1.494	12.13	97.0
25	2.004	27.24	109.0
50	2.233	46.61	93.2

Typical Graph



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 and the Assay Guidance Manual.

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 0.5 ug/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 (0.781ug/ml), medium (6.25ug/ml) and high (50ug/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%	<15%
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.

References:

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T Kitazawa, K Esaki, T Tachibana, S Ishii… - Thrombosis and …, 2017 - thieme-connect.com

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N Ramamurthy, C Kucharski… - … and Practice in …, 2020 - medically.gene.com

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Laboratory monitoring in emicizumab-treated persons with hemophilia A
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Detailed analysis of anti-emicizumab antibody decreasing drug efficacy, using plasma samples from a patient with hemophilia A
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A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors
G Young, RI Liesner, T Chang, R Sidonio, J Oldenburg… - Blood, 2019 - ashpublications.org

Emicizumab prophylaxis in patients who have hemophilia A without inhibitors
J Mahlangu, J Oldenburg, I Paz-Priel… - … England Journal of …, 2018 - Mass Medical Soc

Low immunogenicity of emicizumab in persons with haemophilia A
C Schmitt, T Emrich, S Chebon, E Fernandez… - …, 2021 - Wiley Online Library

SCHEMATIC ASSAY PROCEDURE

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

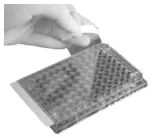
Components (2-8ºC) → 30 mins RT Before Use → 

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

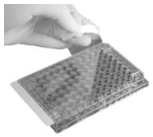
3. Pipette **100 ul** prepared **Standards** / diluted **Samples** into each well.

4. Cover plate  and incubate for 

at 37°C.

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

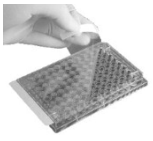
6. Pipette **100 ul Goat Anti-Human IgG:HRP Conjugate** into each well.

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	ug/ml Emicizumab equivalent
1A 2A	zero std zero std			
1B 2B	0.781 ug/ml 0.781 ug/ml			
1C 2C	1.563 ug/ml 1.563 ug/ml			
1D 2D	3.125 ug/ml 3.125 ug/ml			
1E 2E	6.25 ug/ml 6.25 ug/ml			
1F 2F	12.5 ug/ml 12.5 ug/ml			
1G 2G	25 ug/ml 25 ug/ml			
1H 2H	50 ug/ml 50 ug/ml			
3A 4A	Sample			
3B 4B	Sample			

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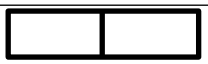
	**Factor IX + Factor X protein Coated Microtiter Plate (12 x 8 wells)**
	**Emicizumab Standard , lyophilized**
	**Goat Anti-Human IgG:HRP Conjugate**
	**(1X) Standard Diluent**
	**(1X) Sample Diluent**
	**(20X) Wash Buffer**
	**TMB Substrate**
	**Stop Solution**
	**Consult Instructions for Use**
	**Catalog Number**
	**Expiration Date**
	**Storage Temperature**

SYMBOLS KEY