KRIBIOLISA® Eculizumab (SOLIRIS™) ELISA

REF : KBI1024

Ver 4.8

RUO

Enzyme Immunoassay for the quantitative determination of Eculizumab in serum, plasma and cell culture supernatant

RUO	For Research Use	REF	Catalog Number
	Store At 	LOT	Batch Code
	Manufactured By		Biologiisk
	Expiry D		Consurating 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 KBI1024  96 tests

Krishgen Biosystems Private Limited
For US/Europe Customers: toll free +1(888)-970-0827 | tel +1(562)-568-5005
For Asia/India Customers: tel +91(22)-49198700
Email: sales1@krishgen.com | http://www.krishgen.biz / www.krishgenbio.com

Introduction:

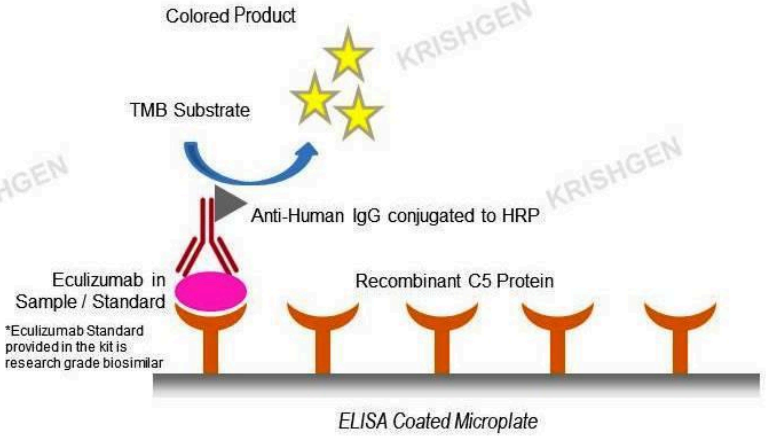
Eculizumab, sold under the trade name SOLIRIS™, is a medication used to treat paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). It is a humanized monoclonal antibody functioning as a terminal complement inhibitor. In people with PNH, it reduces both the destruction of red blood cells and need for blood transfusion, but does not appear to affect the risk of death. Eculizumab was the first drug approved for each of its uses, and its approval was granted on the basis of small clinical trials.

Intended Use:

The KRIBIOLISA® Eculizumab (SOLIRIS™) ELISA is used as an analytical tool for quantitative determination of Eculizumab in serum, plasma and cell culture supernatant.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Recombinant C5 protein is pre-coated onto microwells. Samples and standards are pipetted into microwells and human Eculizumab present in the sample are bound by the capture antibody. Then, a 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 3,3',5,5' Tetra Methyl Benzidine (TMB) solution is added to microwells and color develops proportionally to the amount of Eculizumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Recombinant C5 protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Recombinant C5 protein	1 x 96 wells
Eculizumab Standard	Recombinant Eculizumab in a buffered protein base with preservative preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane – lyophilized (1 ug/ml)	2 vials
Anti-Human IgG:HRP Conjugate concentrated	Anti-Human IgG conjugated to Horseradish Peroxidase concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane	12 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolinone and 0.02% bromonitrodioxane with 1:1000 dilution human serum	10 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 store the Anti-Human IgG: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-Human IgG: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.

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.

Test Sample Preparation: For Serum / Plasma - 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 Standard lyophilized vial with 1 ml of Standard Diluent (1X) 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 (1X) 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 (1X) 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 (1 ug/ml) + 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)

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

5. **Working Anti-Human IgG: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 Eculizumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Eculizumab 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 Eculizumab 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 Eculizumab.
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-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)** 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 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 Eculizumab 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 Eculizumab Concentration. If samples were diluted, multiply by the appropriate dilution factor.

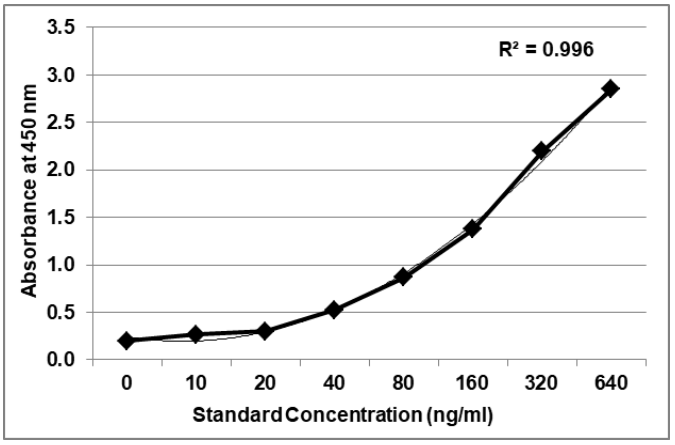
Software which is able to generate a cubic spline curve-fit or a polynomial curve (2nd order) is best recommended for automated results.

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 640 ng/ml standard.

Typical Data (representative only)

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.209	0.194	0.201	--	--
10	0.291	0.241	0.266	11.0	110.5
20	0.320	0.283	0.302	15.8	79.0
40	0.578	0.477	0.527	42.7	106.7
80	0.874	0.864	0.869	83.2	104.0
160	1.497	1.251	1.374	152.7	95.4
320	2.278	2.120	2.199	329.0	102.8
640	2.767	2.938	2.852	633.5	99.0

Typical Graph (representative only)



Abs = absorbance at 450 nm

Quality Control:

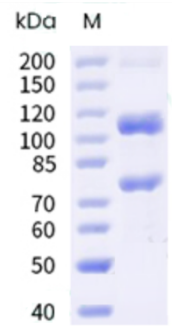
It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

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 10 ng/ml.

Specificity:
The Eculizumab capture protein used in the kit is specific for Eculizumab. The protein was expressed in HEK293 cells using a DNA sequence encoding the human C5 (NP_001726.2) (Gln19-Cys1676). Loaded Eculizumab on ProA Biosensor, can bind this capture protein with an affinity constant of 1.85 nM as determined in BLI assay (Sartorius Octet Red384).



The recombinant Eculizumab capture protein consists of 1677 amino acids and predicts a molecular mass of 188.8 kDa. The C5 single chain can be processed into the disulfide-linked heterodimer of α and β chain with predicted molecular mass of 114 KDa and 74.8 kDa.

The Standards / Calibrators used in the kit are monoclonal antibodies, anti-idiotypic and specific for Eculizumab. They are Eculizumab biosimilar producted as a recombinant humanized monoclonal IgG2/4kappa antibody in CHO cells and purified by chromatography. The calibrators / standards used are calibrated against commercially sourced (SOLIRIS™).

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 (10 ng/ml), medium (80 ng/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	<10%	<10%
Medium	<5%	<5%
High	<5%	<5%

Lot to Lot Variation:

Standard Concentration (ng/ml)	Lot A OD450	Lot B OD450	Lot C OD450	Lot D OD450	Mean OD450	% STD DEV	CV	% CV
0	0.199	0.131	0.225	0.179	0.185	---	---	---
10	0.475	0.454	0.413	0.435	0.447	1.8	0.07	7.0
20	0.702	0.654	0.746	0.799	0.701	6.2	0.07	6.6
40	0.856	0.867	0.959	0.925	0.894	4.0	0.06	6.3
80	1.383	1.399	1.525	1.499	1.436	5.8	0.05	5.4
160	1.997	1.987	1.923	2.011	1.969	3.7	0.02	2.0
320	2.489	2.339	2.587	2.515	2.472	10.4	0.05	5.1
640	2.675	2.626	2.669	2.675	2.657	2.2	0.01	1.0

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:

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Eculizumab for treating patients with paroxysmal nocturnal hemoglobinuria…Martí-Carvajal AJ, Anand V, Cardona AF, Solà I…Cochrane Database Syst Rev…2014… Cochrane Library

Variable Eculizumab Clearance Requires Pharmacodynamic Monitoring to Optimize Therapy for Thrombotic Microangiopathy after Hematopoietic Stem Cell Transplantation…Jodele S, Fukuda T, Mizuno K, Vinks AA, Laskin BL, Goebel J, Dixon BP, Chima RS, Hirsch R, Teusink A, Lazear D, Lane A, Myers KC, Dandoy CE, Davies SM... Biol Blood Marrow Transplant….2016….Elsevier

SCHEMATIC ASSAY PROCEDURE

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

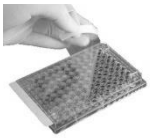
Components (2-8ºC) → 30 mins RT Before Use → 

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

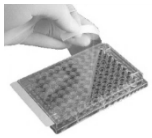
4.  Pipette **100 ul prepared Standards / diluted Samples** into the respective wells.

5. Cover plate  and incubate for 

at 37°C.

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

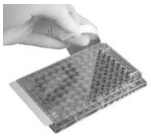
7.  Pipette **100 ul working Anti-Human IgG:HRP Conjugate** into each well.

8. Cover plate  and incubate for 

at 37°C.

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

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

11. Cover plate  and incubate for 

at 37°C.

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

13. Read absorbance at 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450 nm	Mean Absorbance	ng/ml Eculizumab 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			

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SYMBOLS **KEY**

	R MTP ant C5 protein Microtiter Plate (12x8 wells)
	E STD b Standard, Lyophilized
	HRP CONJ gG:HRP Conjugate Concentrated
	1X STD DIL Diluent
	1X SAMP DIL luent
	DETN DIL uent
	20X WASH BUF ffer
	SUB TMB te
	SOLN STOP
	Consult Instructions for Use
	C REF Number
	Expiration Date
	Storage Temperature