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Anti-Eculizumab (SOLIRIS™) ELISA

REF : KBI2024

Ver 3.1

RUO

Enzyme Immunoassay for the Quantitative Determination of Anti-Eculizumab 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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96 tests



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Introduction:

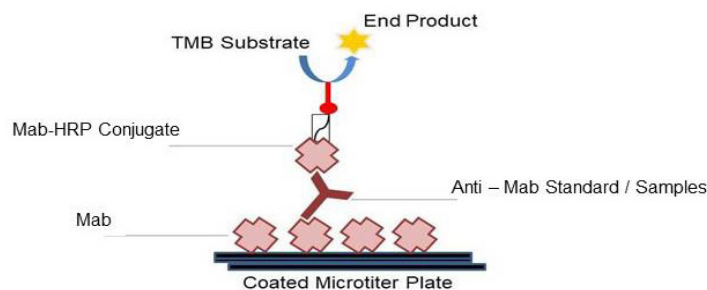
Eculizumab, sold under the trade name Soliris, is a medication used to treat paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). It is a humanized monoclonal antibody functioning as a terminal complement inhibitor. In people with PNH, it reduces both the destruction 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. 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 Eculizumab and can be responsible for allergic reaction, or even anaphylactic shock. This ELISA kit detects antibodies for Anti-Eculizumab and may be used for monitoring immunogenicity.

Intended Use:

The Anti-Eculizumab (SOLIRIS™) ELISA is used as an analytical tool for quantitative determination of Anti-Eculizumab in serum and plasma.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Eculizumab is pre-coated onto microwells. Samples and standards are pipetted into microwells and antibodies to Eculizumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated Eculizumab 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 Anti-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
Eculizumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Eculizumab monoclonal antibody.	1 x 96 wells
Anti-Eculizumab Standard	Recombinant Eculizumab in a buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane - lyophilized, concentrated (1 ug/ml)	2 vials
Eculizumab:HRP Conjugate	Eculizumab conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	2 x 50 ml
Standard Diluent	Buffered protein base with 1:100 dilution human serum and protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	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
4. Wash bottle or automated microplate washer
5. Standard (mm/mm) graph paper or software for data analysis
6. Timer
7. Absorbent Paper

Handling/Storage:

1. All reagents should be stored at 2°C to 8°C 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 (18-25°C). 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 -80°C. 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 1:1000 (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 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	Lyophilized Standard	Lyophilized Standard provided in the Kit + 1 ml of Standard Diluent
640 ng/ml	Standard No.6	320 ul Reconstituted Standard (1 ug/ml) + 180 ul Standard Diluent
320 ng/ml	Standard No.5	250 ul Standard No.6 + 250 ul Standard Diluent
160 ng/ml	Standard No.4	250 ul Standard No.5 + 250 ul Standard Diluent
80 ng/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent
40 ng/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent
20 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 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-Eculizumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Eculizumab present in the sample.
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-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. A standard curve is required for each assay. All steps must be performed at 37°C
2. Pipette **100 ul** of **Standards** or **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. Pipette **100 ul** of **Eculizumab:HRP Conjugate** into the respective wells.
6. Cover the plate and incubate for 60 minutes at 37°C.
7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** same as 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 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 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-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 Anti-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.

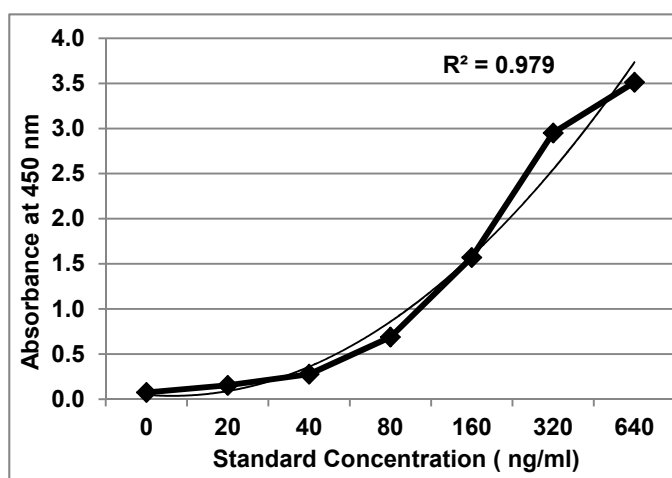
Note:

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

Standard Concentration (ng/ml)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.074	--	--
20	0.153	21.0	104.9
40	0.277	43.4	108.4
80	0.688	84.3	105.4
160	1.571	154.1	96.3
320	2.951	334.6	104.6
640	3.513	607.0	94.8

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.

Sensitivity:

Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 18 ng/ml

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 12 ng/ml

Specificity:

The proteins are recombinantly expressed Eculizumab used for capture and detection. The calibrators / standards used are calibrated against commercially sourced Antibody for Eculizumab.

Linearity:

Standards provided in the kit will be used for measuring the linearity range of Anti-Eculizumab 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 (20 ng/ml), medium (80 ng/ml) and high (640 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	<10%	<10%
Medium	<5%	<5%
High	<5%	<5%

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.

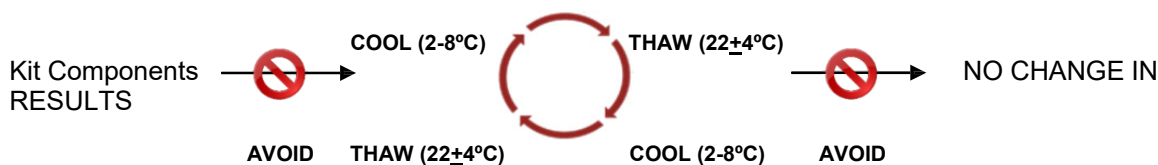


SCHEMATIC ASSAY PROCEDURE

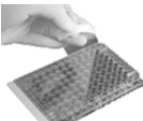

1. Remove all components, 30 minutes before adding into the assay plate.





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

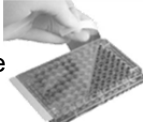




3.  Pipette **100 ul Standards / Samples** into each well.

4.  Cover plate and incubate for  at 37°C.

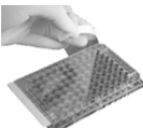

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X)**.

6.  Pipette **100 ul Eculizumab: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 Anti-Eculizumab equivalent
1A 2A	zero std zero std			
1B 2B	20 ng/ml 20 ng/ml			
1C 2C	40 ng/ml 40 ng/ml			
1D 2D	80 ng/ml 80 ng/ml			
1E 2E	160 ng/ml 160 ng/ml			
1F 2F	320 ng/ml 320 ng/ml			
1G 2G	640 ng/ml 640 ng/ml			
1H 2H	Sample Sample			
1I 2I	Sample Sample			

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SYMBOLS KEY

MTP	Eculizumab Microtiter Plate (12x8 wells)
STD	Anti-Eculizumab Standard lyophilized
HRP CONJ	Conjugate Horseradish Peroxidase
SAMP DIL	Sample Diluent
STD DIL	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

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