# ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA

APPLICATIONS

KRISHGEN BioSystems

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**VALIDATION OF KRIBIOLISA® ECULIZUMAB ELISA KIT (CATALOG NO. KBI1024) AS PER FDA/ICH GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION**

*This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline recommended by FDA/ICH.*

**Document History**

| First Codification | History | Date |
|---|---|---|
| **Version#1** | **VALIDATION DATA OF KRIBIOLISA® ECULIZUMAB ELISA KIT (CATALOG NO. KBI1024)** | 31.12.2025 |
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| Approved Quality Control | Approved Product Development | Approved Operations Head |
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| Prairna B | Atul G | K Jain |

## Background

### 1. Introduction to Eculizumab (SOLIRIS™)

Eculizumab is a humanized monoclonal IgG2/4κ antibody that specifically targets complement component C5, a central effector molecule in the terminal complement cascade. By binding with high affinity to C5, Eculizumab prevents its enzymatic cleavage into C5a and C5b, thereby blocking the formation of C5b-9 (the membrane attack complex, MAC). This inhibition protects host cells from complement-mediated lysis and uncontrolled inflammatory activation. Clinically, Eculizumab is used as a life-saving therapy in complement-driven disorders such as paroxysmal nocturnal hemoglobinuria (PNH), atypical haemolytic uremic syndrome (aHUS), generalized myasthenia gravis (gMG), and neuromyelitis optica spectrum disorder (NMOSD).

The antibody was strategically engineered to neutralize terminal complement activation while preserving upstream complement functions essential for immune surveillance. This selective blockade offers sustained inhibition of complement-mediated cytotoxicity with a well-established safety and efficacy profile. Because its mechanism targets a common final pathway rather than disease-specific antigens, Eculizumab is broadly applicable across multiple complement-Dysregulated diseases and is increasingly explored for additional indications involving uncontrolled complement activation.

Eculizumab plays a pivotal role in immunology, haematology, and neurology research, aiding studies on complement biology, inflammatory signalling, and complement-associated tissue injury. Its clinical monitoring utilizes assays such as CH50 functional complement tests, ELISA, flow cytometry, and biomarkers that reflect haemolysis or complement activation. In research settings, it serves as a tool for investigating complement-mediated pathology, therapeutic complement blockade, and mechanisms of cell lysis prevention.

Commercially available worldwide, Eculizumab remains one of the most impactful complement-inhibiting biologics. It continues to be evaluated in next-generation trials, combination therapies, and expanded clinical applications aimed at improving outcomes in rare, severe immune-mediated disorders.

Eculizumab received its first regulatory approval from the U.S. Food and Drug Administration (FDA) in March 2007 under the brand name SOLIRIS, for the treatment of paroxysmal nocturnal hemoglobinuria (PNH)—a rare, life-threatening hematologic disorder characterized by complement-mediated haemolysis. Its indications later expanded based on strong clinical evidence demonstrating effective terminal complement (C5) inhibition and meaningful improvement in patient outcomes. Over the years, eculizumab was approved for atypical haemolytic uremic syndrome (aHUS), generalized myasthenia gravis (gMG) in anti-AChR–positive patients, and neuromyelitis optica spectrum disorder (NMOSD) in AQP4-IgG–positive patients, establishing it as a cornerstone therapy across multiple severe complement-driven diseases.

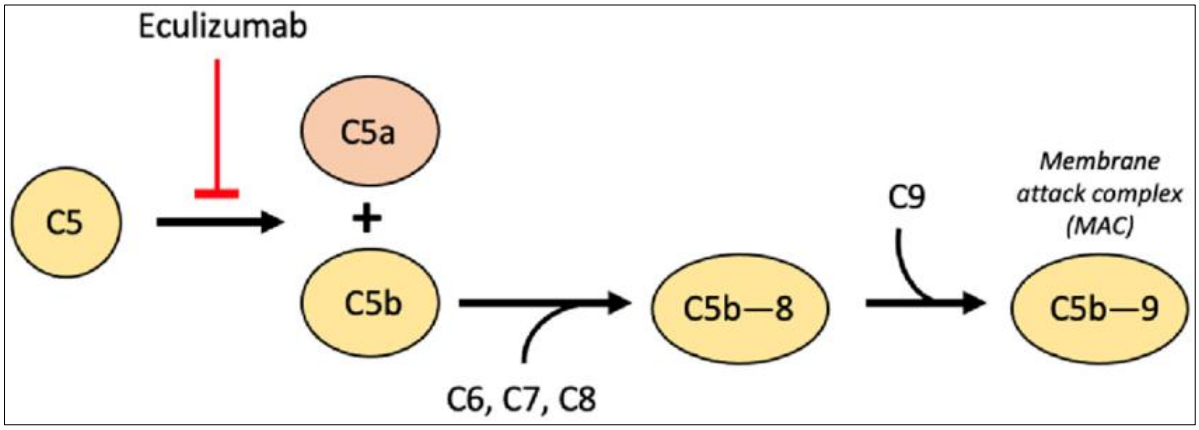



**Figure 1: Immune response to Eculizumab.**

### 2. Clinical Relevance of Eculizumab-Based Monitoring

Therapeutic drug monitoring (TDM) of Eculizumab is critical for ensuring effective complement inhibition, especially in patients showing variable disease activity, altered complement turnover, genetic polymorphisms in complement components, or physiological conditions that modify antibody clearance. Regular quantification of circulating Eculizumab levels enables precise assessment of its pharmacokinetic (PK) behavior, degree of C5 blockade, and the sustained suppression of terminal complement activation—key determinants of therapeutic efficacy and prevention of breakthrough hemolysis or inflammatory relapse.

Evaluating drug exposure through TDM also helps correlate Eculizumab concentrations with clinically meaningful outcomes such as stabilization of hemolysis in PNH, normalization of platelet and renal parameters in aHUS, reduction of neuromuscular symptoms in gMG, and prevention of relapses in NMOSD. This information guides optimized dosage intervals, identifies patients requiring intensified dosing due to high complement turnover, and supports decisions regarding switching to long-acting complement inhibitors.

Ultimately, monitoring Eculizumab levels enables a personalized therapeutic approach by aligning complement inhibition with patient-specific disease biology, complement activation profiles, and treatment responsiveness. This ensures maximal suppression of pathogenic complement activity while minimizing risks such as breakthrough hemolysis, relapse episodes, or unnecessary drug exposure—thereby improving overall clinical outcomes and long-term disease control.

## Scope of Validation

This document presents a discussion of the characteristics of our KRIBIOLISA® Eculizumab ELISA KIT (CATALOG NO. KBI1024) kit considered by us during the validation of this kit in accordance with ICH Q2 (R1) guidelines. The document is prepared based on tests run in our laboratory and does not necessarily seek to cover the testing that may be required at user's end for registration in, or regulatory submissions. The objective of this validation is to demonstrate that it is suitable for its intended purpose - detection of Eculizumab.

Validation characteristics considered by us in accordance with the guidelines are listed below:

- Specificity and Selectivity.
- Sensitivity (LOD & LOQ).
- Linearity and Range.
- Accuracy and Precision (Intra/Inter-Assay).
- Matrix Effect (serum, plasma).
- Accelerated stability study.
- Sample Handling and Storage Conditions.
- References Eculizumab Values and Recommended ELISA Range).

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get recommend that the user performs at the minimum; the spike and recovery assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

For any queries or support on the data and its performance, please contact us at sales1@krishgen.com.

## Intended Use of the ELISA

To evaluate the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Eculizumab ELISA KIT, designed specifically to quantify free, active Eculizumab molecules with high sensitivity and specificity, enabling accurate monitoring of terminal complement inhibition status. This assay supports clinical research, therapeutic drug monitoring (TDM), dose individualization, and assessment of treatment efficacy in complement-mediated disorders. It facilitates precise evaluation of pharmacokinetics, detection of Subtherapeutic exposure, and monitoring of breakthrough complement activation, thereby strengthening clinical decision-making in conditions such as PNH, aHUS, gMG, and NMOSD.

## Principle of the Assay

This ELISA is based on a sandwich immunoassay format. C5 Protein Human, Recombinant (His & FLAG Tag) capture antibodies are immobilized onto 96-well microplate wells. Eculizumab present in the standards and test samples specifically binds to the coated antibodies during incubation. After a wash step to remove unbound substances, an Anti-Human IgG:HRP-conjugated detection antibody is introduced, which binds to the captured Eculizumab, forming a stable antibody–antigen–antibody complex. Following additional washing to eliminate excess conjugate, TMB substrate is added, allowing HRP to catalyze a colorimetric reaction. The reaction is terminated by adding stop solution, producing a

yellow color. The resulting optical density (OD), measured at 450 nm, is directly proportional to the concentration of Eculizumab present in the samples or standards.

## Experimental Design

- A Sandwich ELISA was performed using C5 Protein Human, Recombinant (His & FLAG Tag) capture antibodies as the capture antibody.
- Standards were prepared using purified Eculizumab reference material.
- Assay Concentration Range: 0 - 640 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The optimized antibody-coating and detection strategy employed in the KRIBIOLISA® Eculizumab ELISA ensures efficient capture of free, pharmacologically active drug molecules while minimizing background signals from endogenous IgG, complement proteins, or other serum components. This highly specific design delivers excellent assay sensitivity and selectivity, making it ideal for research use as well as clinical monitoring of complement inhibition status in patients receiving Eculizumab therapy.

The KRIBIOLISA® Eculizumab ELISA utilizes a quantitative sandwich immunoassay format based on the selective interaction between drug-specific monoclonal antibodies that recognize Eculizumab with high affinity. C5 Protein Human, Recombinant (His & FLAG Tag) capture antibodies are pre-coated onto microwells to act as capture molecules. Patient samples and Eculizumab standards are added, permitting the drug to bind to the immobilized capture antibodies. An Anti-Human IgG:HRP-conjugated detection antibody is then applied to form a stable antibody–antigen–antibody complex. After washing to remove unbound material, TMB substrate is added, generating a colorimetric signal proportional to the amount of Eculizumab present in the sample. The reaction is stopped using stop solution, and absorbance is measured at 450 nm, providing a reliable quantitative determination of circulating Eculizumab levels.

### Validation Parameters and Acceptance Criteria

#### 1. Eculizumab Values and Recommended ELISA Range

This table summarizes Eculizumab levels across different therapies and suggested corresponding ELISA working ranges.

| Application | Expected Eculizumab Range (ug/ml) | Recommended ELISA Range (ug/ml) |
|---|---|---|
| Post low-dose / induction phase (initial complement blockade) | 20–60 | 0–100 |
| Standard therapeutic maintenance dosing (routine clinical treatment cycles) | 50–150 | 0–200 |

| Application | Expected Eculizumab Range (ug/ml) | Recommended ELISA Range (ug/ml) |
|---|---|---|
| High complement turnover states or intensified dosing regimens (PNH, aHUS, NMOSD exacerbations) | 120–300 | 0–300 |
| Pharmacokinetic monitoring / complement blockade-response evaluation | 150–400 | 0–500 |

Note: Assay sensitivity <2 ng/mL is recommended for baseline or trough-level detection of eculizumab, while an upper quantification limit ≥200–300 ng/mL is advised for monitoring therapeutic exposure, complement blockade status, and potential breakthrough hemolysis in high-dose or intensified regimens.

The KRIBIOLISA® Eculizumab ELISA kit is developed using an assay range of 0 - 640 ng/ml with the dilutional linearity accuracy to measure responses as per the application table above on patient $C_{max}$ values. The kit has also been validated upto 64000 fold dilution and the values are within the acceptable range.

## 2. Specificity and Selectivity

### 2.1 Specificity

The capture and detection antibodies used in the Eculizumab ELISA are monoclonal antibodies that specifically recognize the intact humanized IgG2/4κ anti-C5 therapeutic antibody without cross-reacting with endogenous human immunoglobulins or complement proteins. These assay antibodies are engineered to target unique idiotype or Fab-region epitopes of Eculizumab, ensuring high-affinity binding to the drug molecule in its native, functional conformation.

The optimized specificity profile enables selective detection of Eculizumab in complex biological matrices—including serum, plasma, or cell-culture supernatants—while demonstrating minimal interference from structurally related complement-targeting therapeutics, complement component C5, C5a fragments, or unrelated immunoglobulins. This degree of molecular discrimination ensures accurate quantification of circulating Eculizumab even in patients with elevated complement turnover or inflammatory background, provided the drug's idiotype integrity is maintained.

### 2.2 Selectivity

The ELISA demonstrates minimal to no cross-reactivity with endogenous human IgG subclasses, recombinant antibodies, or unrelated therapeutic monoclonal antibodies. It effectively excludes molecules that do not share the unique idiotype or antigenic determinants of Eculizumab, including native complement component C5, its activation fragments (C5a, C5b), or other complement pathway proteins circulating in patient samples.

The assay maintains high selectivity in complex biological matrices such as serum, plasma, or cell-culture supernatants, showing negligible interference from complement factors, inflammatory mediators, cytokines, heterophilic antibodies, or IgG-binding serum proteins. This stringent selectivity ensures reliable and accurate quantification of Eculizumab without false-positive signals arising from structurally similar complement-targeting agents or immunologically active matrix components.

### 2.3 LOD, LOQ and $IC_{50}$

LOD (Limit of Detection)
The lowest analyte concentration that can be reliably distinguished from blank/background noise but not necessarily quantified precisely.

Statistically:
LOD = Mean of Blank + 3X SD of Blank
(3σ criterion is most common).
LOD for KRIBIOLISA® Eculizumab ELISA = 2.60 ng/ml

LOQ (Limit of Quantitation)
The lowest analyte concentration that can be quantified with acceptable accuracy and precision.

Statistically:
LOQ = Mean of Blank + 10X SD of Blank
(10σ criterion is most common).

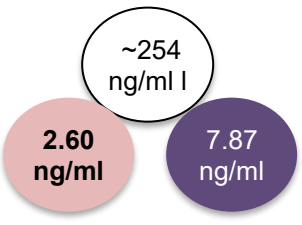
LOQ for KRIBIOLISA® Eculizumab ELISA – 7.87 ng/ml

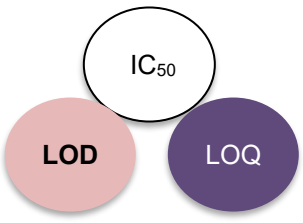
$IC_{50}$ in ELISA (Half Maximal Inhibitory Concentration)
$IC_{50}$ = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

In ELISA, this is commonly used for:
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

$IC_{50}$ for KRIBIOLISA® Eculizumab ELISA = ~254 ng/ml
Summary:

| Parameter | Value (ng/ml) |
|---|---|
| LOD | 2.60 ng/ml |
| LOQ | 7.87 ng/ml |
| $IC_{50}$ | 254 ng/ml |

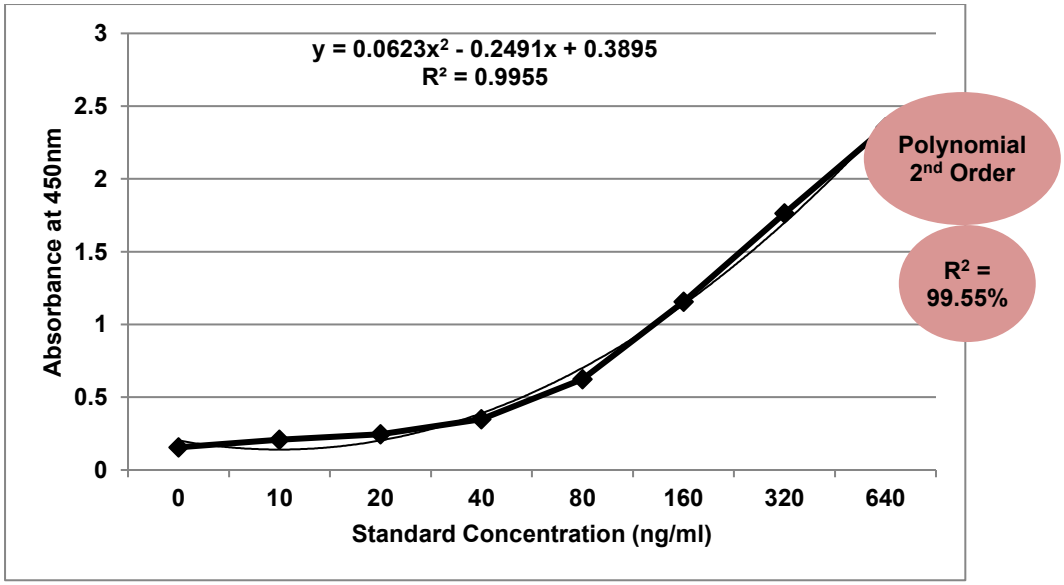


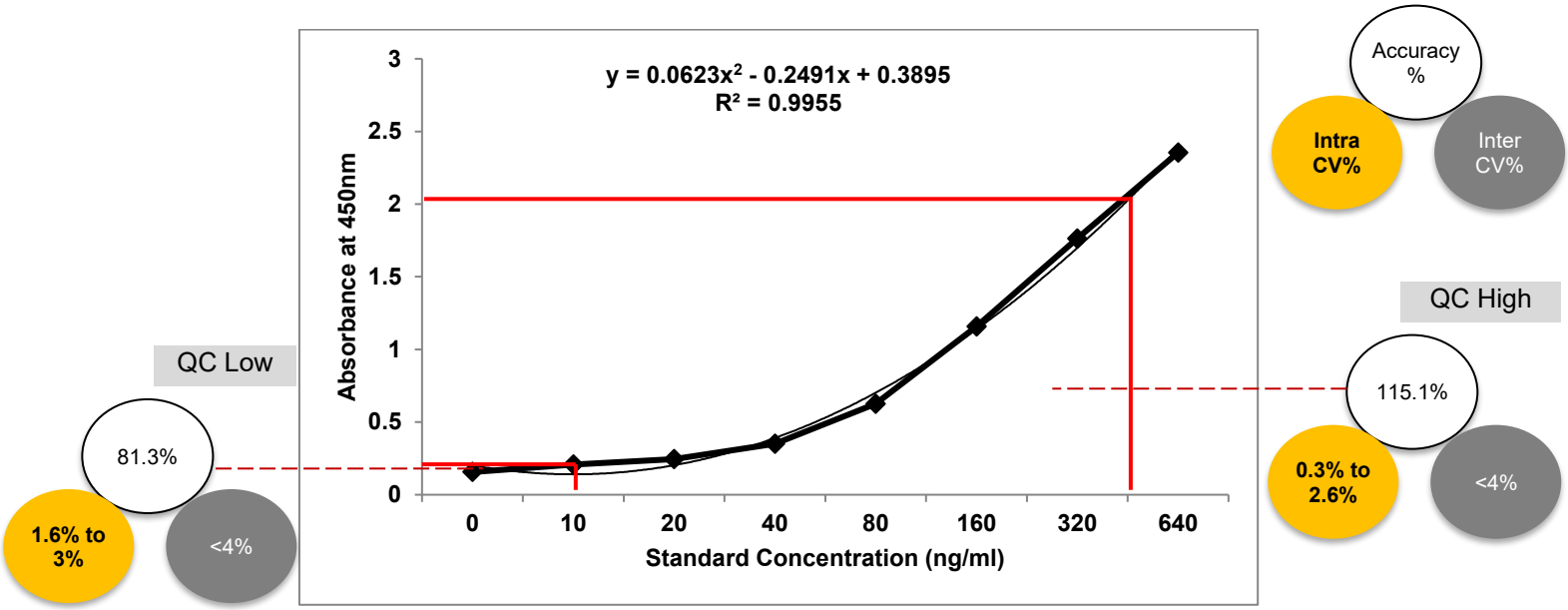



Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20%  **S/N = Signal / Noise Ratio*

### 3. Linearity and Range

| Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery |
|---|---|---|---|
| 0 | 0.156 | -- | -- |
| 10 | 0.208 | 13.3 | 132.5 |
| 20 | 0.245 | 20.4 | 101.8 |
| 40 | 0.35 | 37.7 | 94.3 |
| 80 | 0.625 | 78.4 | 98.1 |
| 160 | 1.157 | 164.9 | 103 |
| 320 | 1.763 | 313.5 | 98 |
| 640 | 2.355 | 646 | 100.9 |
| Positive Control (600 ng/ml) | 2.258 | 552.5 | 92.1 |
| Low QC Control (10 ng/ml) | 0.223 | 16.3 | 81.3 |
| High QC Control (500 ng/ml) | 1.912 | 368.2 | 115.1 |






## 4. Precision and Reproducibility (Intra/Inter-Assay)

Precision was assessed by analyzing three standard concentrations (10 ng/ml, 80 ng/ml, and 640 ng/ml). Each concentration was tested in triplicate across three independent assay runs. %CV (Coefficient of Variation) was calculated within runs (intra-assay precision) and across runs (inter-assay precision).

Acceptance Criteria:

- Intra-assay %CV should be ≤15% for QC samples.
- Inter-assay %CV should be ≤15% for QC samples.
- %CV at LLOQ (Lower Limit of Quantitation) allowed up to 20%.

Precision Results Summary:

| Standard (ng/ml) | Intra-Assay %CV (Range) | Inter-Assay %CV |
|---|---|---|
| 10 | 1.6% to 3% | <3% |
| 80 | 1.6% to 2.6% | <3% |
| 640 | 0.3% to 2.6% | <2% |

Observations:

- Intra-assay precision was consistently less than 7% across all levels tested.
- Inter-assay precision was consistently less than 7%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:

The KRIBIOLISA® Eculizumab ELISA demonstrates excellent intra- and inter-assay precision. These results support the assay's reliability and reproducibility for routine use in pharmacokinetic and bio analytical studies.

## 5. Diluents Effect Study

Evaluation of PBS-based buffer vs Proprietary buffer revealed slight recovery differences. PBS (pH 7.4) diluent offered consistent and reliable performance across tested concentrations.

## 6. Parallelism

Serial dilutions of a high-concentration sample were prepared at dilutions of 1:2000, 1:4000, 1:8000, 1:16000, 1:32000 and 1:64000 for both human serum and human plasma. Each dilution was assayed using the KRIBIOLISA Eculizumab ELISA and compared to the standard curve.

Acceptance Criteria:

- The back-calculated concentration (interpolated) should fall within ±20% of the expected concentration across the tested range.
- % Recovery should be between 80% and 120% for most samples.

### A) Human Serum:

| Dilution | Expected Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery | % Deviation |
|---|---|---|---|---|---|
| 1:2000 | 320 | 1.768 | 315.1 | 98.5 | 98.5 |
| 1:4000 | 160 | 1.164 | 166.2 | 103.9 | 103.9 |
| 1:8000 | 80 | 0.699 | 89.4 | 111.8 | 111.8 |
| 1:16000 | 40 | 0.355 | 38.5 | 96.2 | 96.2 |
| 1:32000 | 20 | 0.256 | 22.3 | 111.6 | 111.6 |
| 1:64000 | 10 | 0.212 | 11.9 | 119 | 119 |

### B) Human Plasma:

| Dilution | Expected Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery | % Deviation |
|---|---|---|---|---|---|
| 1:2000 | 320 | 1.852 | 343.7 | 107.4 | 107.4 |
| 1:4000 | 160 | 1.208 | 155.8 | 97.3 | 97.3 |
| 1:8000 | 80 | 0.658 | 79.2 | 99.1 | 99.1 |

| Dilution | Expected Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery | % Deviation |
|---|---|---|---|---|---|
| 1:16000 | 40 | 0.34 | 38.1 | 95.3 | 95.3 |
| 1:32000 | 20 | 0.267 | 23.9 | 119.5 | 119.5 |
| 1:64000 | 10 | 0.205 | 11.9 | 119 | 119 |

Results:

i. Parallelism is generally maintained across the 1:2000 to 1:64000 dilutions.
ii. % Recovery for most dilutions falls within the acceptable range of 80–120%.
iii. No significant matrix effect observed at higher dilutions.
iv. The KRIBIOLISA® Eculizumab ELISA kit was tested for matrix effect on human serum and plasma.

Conclusion:

Parallelism was demonstrated between the diluted samples and the standard curve. This supports the validity of using sample dilutions within the working range of the Eculizumab ELISA without significant loss of accuracy.

## 7. Matrix Effect Study

Matrix effect was evaluated by comparing the assay performance of standards prepared in:

- Assay buffer (only buffer)
- Assay buffer spiked with human serum (buffer + 1:1000 human serum)
- Assay buffer spiked with human serum (buffer + 1:1000 human plasma)

Samples were tested across the standard curve range (0–640 ng/ml). Mean absorbance, % Standard Deviation, and % Coefficient of Variation (%CV) were calculated to assess the impact of the serum matrix.

### Matrix Effect Study Results

| Standard (ng/ml) | Mean Absorbance (Buffer) | Mean Absorbance (Buffer + 1:100 Human Serum) | % Standard Deviation | % CV |
|---|---|---|---|---|
| 0 | 0.121 | 0.156 | 2.54 | 18.3 |
| 10 | 0.197 | 0.208 | 0.75 | 3.7 |

| Standard (ng/ml) | Mean Absorbance (Buffer) | Mean Absorbance (Buffer + 1:100 Human Serum) | % Standard Deviation | % CV |
|---|---|---|---|---|
| 20 | 0.274 | 0.245 | 2.09 | 8 |
| 40 | 0.39 | 0.35 | 2.86 | 7.7 |
| 80 | 0.661 | 0.625 | 2.52 | 3.9 |
| 160 | 1.259 | 1.157 | 7.21 | 6 |
| 320 | 1.835 | 1.763 | 5.09 | 2.8 |
| 640 | 2.046 | 2.355 | 21.81 | 9.9 |

| Standard (ng/ml) | Mean Absorbance (Buffer) | Mean Absorbance (Buffer + 1:100 Human Plasma) | % Standard Deviation | % CV |
|---|---|---|---|---|
| 0 | 0.121 | 0.147 | 0.68 | 4.5 |
| 10 | 0.197 | 0.207 | 0.04 | 0.2 |
| 20 | 0.274 | 0.276 | 2.23 | 8.6 |
| 40 | 0.39 | 0.328 | 1.53 | 4.5 |
| 80 | 0.661 | 0.677 | 3.66 | 5.6 |
| 160 | 1.259 | 1.216 | 4.16 | 3.5 |
| 320 | 1.835 | 1.825 | 4.4 | 2.5 |
| 640 | 2.046 | 2.116 | 16.89 | 7.6 |

Results:

i. Very low %CV across all concentrations.
ii. Minimal shift in absorbance values between buffer-only and buffer + serum and buffer + plasma conditions.
iii. No significant matrix effect observed.

Conclusion:
The KRIBIOLISA® Eculizumab ELISA demonstrates excellent performance in the presence of human serum and plasma. The assay results confirm the absence of significant matrix interference, supporting its reliability for analyzing biological samples.

**8. Accelerated Stability Study:**

Accelerated stability studies in ELISA are performed to predict the shelf life and long-term stability of an ELISA kit or its individual components by exposing them to elevated stress conditions (typically higher temperatures) for a defined period.

The following table demonstrates the relation of temperature with time point and number of days:

| Accelerated Study Day (37 degrees) | Real-Time Equivalent Age (2-8 degree) | Interpretation |
|---|---|---|
| Day 0 | Present day (0 months) | Initial / release testing |
| Day 1 | 26 days (Approx. 1 month) | Early stability checkpoint |
| Day 4 | 104 days (Approx. 3.5 months | Short-term stability trend |
| Day 7 | 182 days (Approx. 6 months) | Mid-term shelf-life prediction |
| Day 14 | 364 days ( Approx. 1 year) | One-year shelf-life equivalence |

Accelerated Stability Study data:

| Standard Concentration (ng/ml) | Absorbance (Day 0) | Absorbance (Day 1) | Absorbance (Day 4) | Absorbance (Day 7) | Absorbance (Day 14) | %CV |
|---|---|---|---|---|---|---|
| 0 | 0.168 | 0.166 | 0.189 | 0.199 | 0.185 | 7.8 |
| 10 | 0.250 | 0.241 | 0.269 | 0.266 | 0.250 | 4.6 |
| 20 | 0.395 | 0.345 | 0.319 | 0.327 | 0.334 | 8.8 |
| 40 | 0.502 | 0.484 | 0.482 | 0.448 | 0.477 | 4.1 |
| 80 | 0.801 | 0.792 | 0.761 | 0.699 | 0.751 | 5.3 |
| 160 | 1.405 | 1.305 | 1.272 | 1.190 | 1.287 | 6.0 |
| 320 | 1.945 | 1.951 | 1.829 | 1.764 | 1.913 | 4.3 |
| 640 | 2.689 | 2.481 | 2.546 | 2.441 | 2.443 | 4.1 |

Results:

I. %CV is less than 15% across all days.
II. Based on the accelerated stability study results, the Eculizumab ELISA kit demonstrates satisfactory stability and robustness, supporting its viability with an extended shelf life and an assigned expiry of 1 year under recommended storage conditions

## 9. Sample Handling and Storage Conditions

### A) Specimen Collection and Handling:

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.

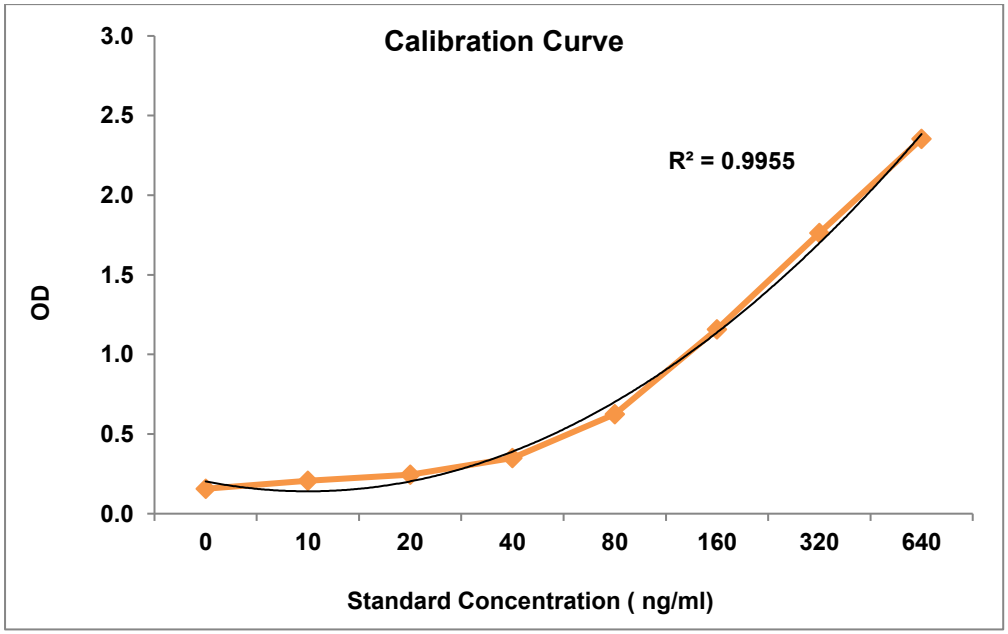
**B) Handling / Storage**:

- 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.
- All the reagents and wash solutions should be used within 12 months from manufacturing date.
- 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.
- The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

**C) Health Hazard Warnings:**

- Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
- For Research Use Only

**Graphs, Maps and Appendices:**




**Matrix Effect Heat Map**

| | 1:2000 | 1:4000 | 1:8000 | 1:16000 | 1:32000 | 1:64000 |
|---|---|---|---|---|---|---|
| Serum | | | | | | |
| Plasma | | | | | | |

**Determined Limits for Acceptance according to EMA/FDA and CLSI regulations**

| | **Limits for Acceptance (EMA/FDA)** | **Determined Limits for Acceptance (CLSI)** |
|---|---|---|
| Intra Precision | CV < 20% (25% at LLOQ) | - |
| Inter Precision | CV < 20 % (25% at LLOQ) | - |
| Accuracy at LLOQ | Recovery 100 ± 20% (100 ± 25% | - |
| Total Error (TE) | TE < 30% (40% at LLOQ and ULOQ) | - |
| Specificity/Interference | Recovery 100 ± 25% | H (null hypothesis) = 100 ± 25 % |
| Parallelism/Linearity | CV < 30% | Deviation from linearity < 20% |
| LLOQ / LOQ | Recovery 100 ± 25% | TE % < 32.9% |

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