ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA

APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® ALEMTUZUMAB ELISA KIT (CATALOG NO. KBI1012) 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® ALEMTUZUMAB ELISA KIT (CATALOG NO. KBI1012)**	30.11.2025

Approved Quality Control	Approved Product Development	Approved Operations Head
Prairna B	Atul G	K Jain

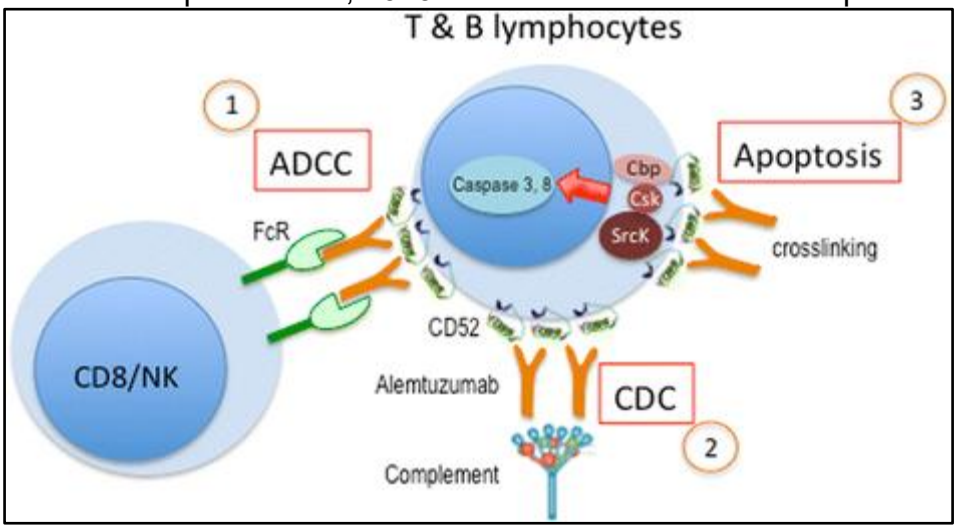
Background

1. Introduction to Alemtuzumab (LEMTRADA)

Alemtuzumab falls under the category of monoclonal antibody which targets the CD52 antigen that is present on mature lymphocytes surface. Its primary use is for treating relapsing conditions of multiple sclerosis (MS) and certain specific types of leukaemia. The primary working mechanism of Alemtuzumab is by reducing the circulating B and T lymphocytes that helps in modulation of the immune system and depleting disease activities.

Initially, Alemtuzumab was approved by the FDA on May 7, 2001 under the brand name CAMPATH for the treatment of B-cell chronic lymphocytic leukaemia (B-CLL) in patients who have been previously treated with alkylating agents and have suffered failed fludarabine therapy.

In November 14, 2014, Alemtuzumab was approved under a different brand name of LEMTRADA for treating the relapsing condition of multiple sclerosis (MS) in adults. Due to its risk of causing autoimmune diseases, infusion reactions and malignancies its applications are reserved only for patients who have shad inadequate responses for two or more MS therapies. The European Medicines Agency (EMA) approved Alemtuzumab under the brand name LEMTRADA on September 17, 2013 for its similar actions in multiple sclerosis.



2. Clinical Relevance of Alemtuzumab Monitoring

TDM or Therapeutic drug monitoring of Alemtuzumab is significant for optimization of dosage regiments, assessment of pharmacokinetics (PK) and reducing the risk of cytokine release syndrome (CRS). The aim is to measure the drug levels that benefits in correlating exposure of therapeutic response with immune reconstitution dynamics.

Scope of Validation

This document presents a discussion of the characteristics of our KRIBIOLISA™ ALEMTUZUMAB (LEMTRADA™) ELISA (Catalog No KBI1012) 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 Alemtuzumab.

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).
- Sample Handling and Storage Conditions.
- References (Alemtuzumab C_{max} 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 a preview of the assay and the characteristics of the kit and is generic in nature. We 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 assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA Alemtuzumab Sandwich ELISA developed using CD52 capture protein, which mimics the role of target epitope bound by Alemtuzumab.

Principle of the Assay

This ELISA is a sandwich immunoassay. Antibodies are coated on 96 well plates. The antigen protein present in sample and standard respectively bind to the coated wells. The wells are washed and an antibody:HRP Conjugate is added which binds to the bound complex in the well.

Washing is performed to remove any unbound material. TMB substrate is added and the enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is directly proportional to the amount of antigen protein present in the standard or samples.

Experimental Design

- A Sandwich ELISA was performed using recombinant human CD52 protein as the capture antigen.
- Standards prepared for Alemtuzumab.

- Assay Concentration Range: 0 - 3000 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The specific CD52 immobilization strategy used in the KRIBIOLISA Alemtuzumab ELISA enhances the binding efficiency of Alemtuzumab while reducing at most cross-reactivity with other monoclonal antibodies, supporting the assay's high specificity and suitability for clinical and bio analytical applications.

The KRIBIOLISA Alemtuzumab ELISA employs a targeted immobilization protocol for optimization of CD52 antigen presentation on the assay plate, preserving and restoring its native orientation and epitope accessibility. This specialized and strategic configuration influences high-affinity binding of Alemtuzumab, whose variable region is designed in a way so that it can recognize and bind a specific extracellular epitope of CD52.

On the other hand, monoclonal antibodies such as Rituximab and Ofatumumab do not target CD52 and thus show minimal to very poor or no binding under these assay conditions. This distinct binding profile of Alemtuzumab develops from the epitope-targeting precision and the controlled orientation of immobilization of CD52, validating high assay specificity and robustness

Validation Parameters and Acceptance Criteria

1. Alemtuzumab C_{max} Values and Recommended ELISA Range

This table summarizes Alemtuzumab C_{max} levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected Alemtuzumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Post low-dose (e.g., 3–5 mg) infusion	300-500	0–1000
Standard induction dose (12–15 mg)	1000-1500	0–2000
Multiple-dose regimens (e.g., CLL therapy)	3000-5000 (can peak <7000)	0-10,000

Application	Expected Alemtuzumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Cytokine release monitoring in gene therapy trials	500–5000	0–10,000

Note: Assay sensitivity <100 ng/ml recommended for baseline detection; upper limit ≥10,000 ng/ml advised for CRS monitoring.

The KRIBIOLISA Alemtuzumab ELISA kit is developed using an assay range of 0 - 3000 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 6400 fold dilution and the values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity

The detection and capture antibodies that are used in the Alemtuzumab ELISA are a highly specific monoclonal anti-idiotype antibody that typically recognizes the unique variable regions of Alemtuzumab. They showcase a high affinity for both the intact therapeutic monoclonal antibody (Alemtuzumab) and its humanized recombinant form, depicting precise detection without interference from the endogenous IgG or other therapeutic antibodies.

2.2 Selectivity

The ELISA exhibits minimal to no cross-reactivity with endogenous human IgG, anti-CD52 natural antibodies, or other monoclonal antibodies (e.g., Rituximab, Trastuzumab, or Bevacizumab). It also shows no binding to soluble CD52 antigen or unrelated Fc fragments, confirming high assay selectivity.

2.4 Clinical C_{max} Value*:

3014 ng/ml

2.5 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 Alemtuzumab ELISA = 14.6 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).

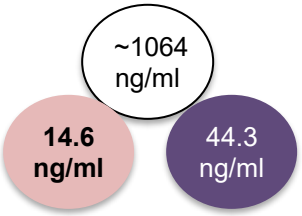
LOQ for KRIBIOLISA Alemtuzumab ELISA – 44.3 ng/ml

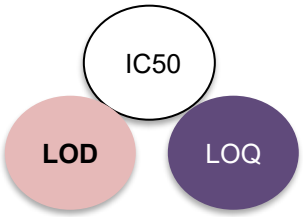
IC_{50} in ELISA (Half Maximal Inhibitory Concentration)
IC_{50} = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

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 Alemtuzumab ELISA = ~1064 ng/ml
Summary:

Parameter	Value (ng/ml)
LOD	14.6 ng/ml
LOQ	44.3 ng/ml
IC_{50}	1064 ng/ml

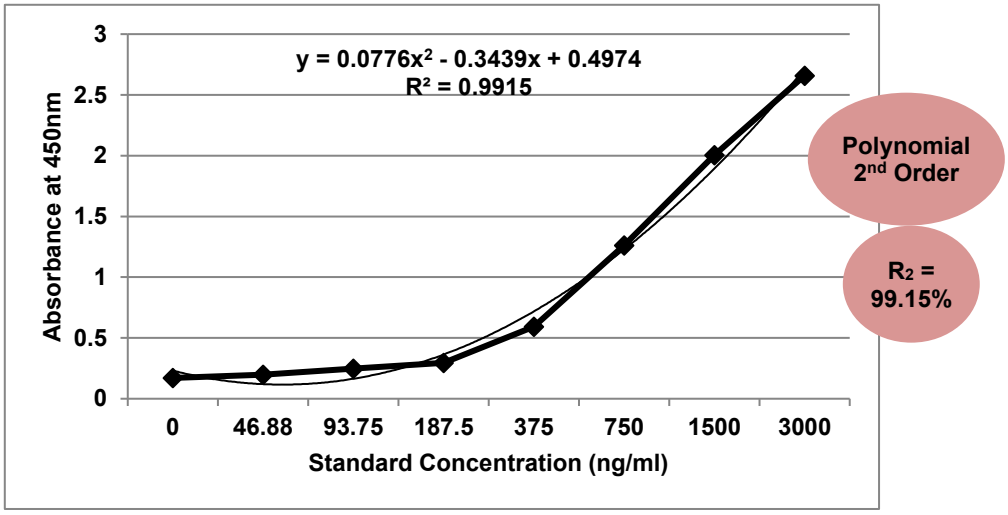


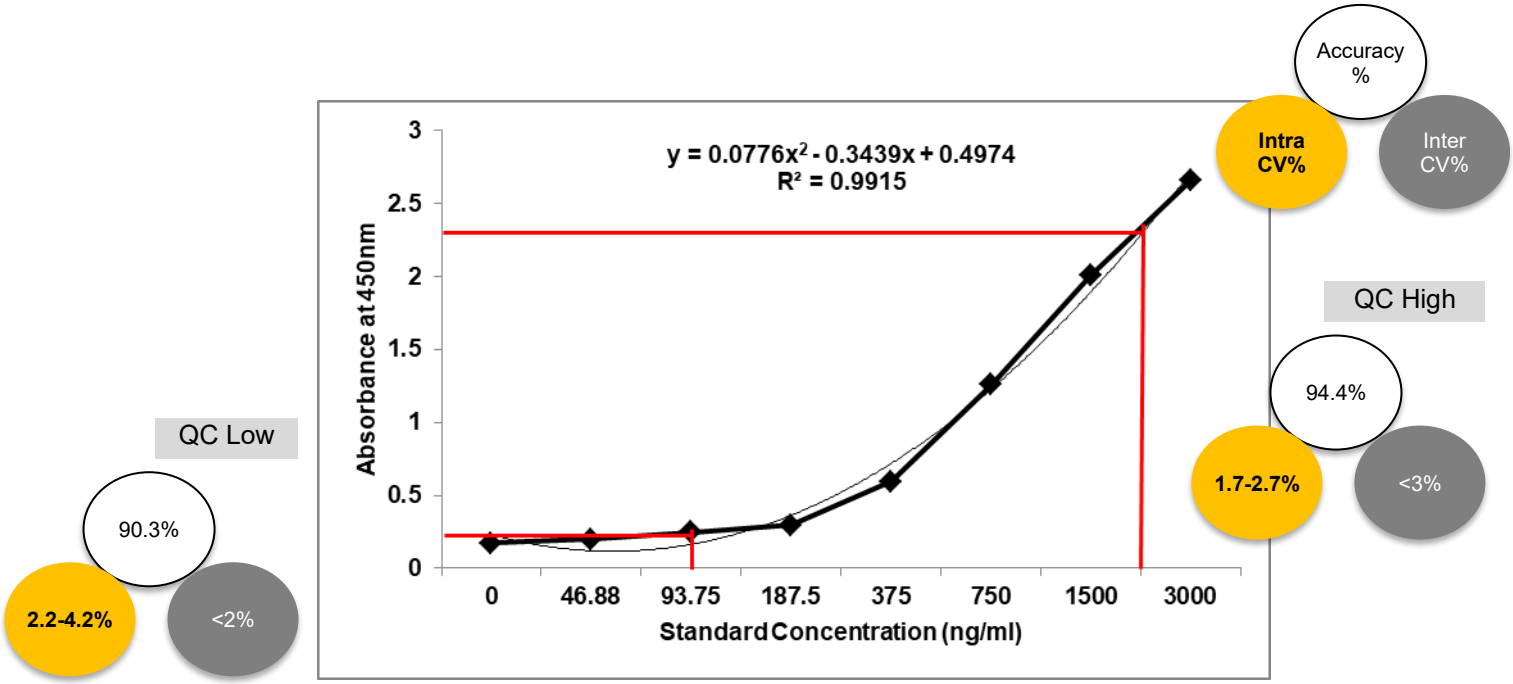


Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% **S/N = Signal / Noise Ratio*

3. Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
0	0.171	--	--
46.88	0.198	60.5	129.0
93.75	0.249	111.6	119.0
187.5	0.295	168.4	89.8
375	0.594	354.5	94.5
750	1.261	781.1	104.1
1500	2.006	1459.4	97.3
3000	2.659	3053.3	101.8
Positive Control (2500 ng/ml)	2.369	2102.0	84.1
Low QC Control (93.75 ng/ml)	0.212	84.7	90.3
High QC Control (2000 ng/ml)	2.273	1888.2	94.4





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

Precision was assessed by analyzing three standard concentrations (46.875 ng/ml, 375 ng/ml, and 3000 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
46.875	2.2% to 4.2%	<2%
375	1.3% to 2.1%	<4%
3000	1.7% to 2.7%	<3%

Conclusion:

The KRIBIOLISA Alemtuzumab 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. Pharmacokinetic Relevance

The assay covers the clinical C_{max} concentrations of Alemtuzumab following therapeutic dosing. Thus, it is suitable for pharmacokinetic evaluation and therapeutic monitoring. The Alemtuzumab ELISA demonstrated an IC_{50} value of approximately 1064 ng/ml. This IC_{50} falls well within the validated assay range of 0 to 3000 ng/ml, ensuring that the assay is suitably sensitive for detection and quantification across clinically relevant concentrations.

7. Parallelism

Serial dilutions of a high-concentration sample were prepared at dilutions of 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400 for both human serum and human plasma. Each dilution was assayed using the KRIBIOLISA Alemtuzumab 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)	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:200	1500	1361.1	90.7	110.2
1:400	750	644.8	86.0	116.3
1:800	375	344.3	91.8	108.9
1:1600	188	165.2	88.1	113.5
1:3200	93.75	116.0	123.8	80.8
1:6400	46.88	58.9	125.6	79.6

B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:200	1500	1409.5	94.0	106.4
1:400	750	671.1	89.5	111.7
1:800	375	471.4	125.7	79.6
1:1600	188	224.3	119.6	83.6
1:3200	93.75	88.6	94.5	105.8
1:6400	46.88	48.9	104.3	95.9

Results:

i. Parallelism is generally maintained across the 1:200 to 1:6400 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 Alemtuzumab ELISA kit was tested for matrix effect on human serum, plasma and physiological buffer 7.4 to mimic tear fluid samples.

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 KRIBIOLISA Alemtuzumab ELISA without significant loss of accuracy.

6. 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 + 1:100 human serum)
- Assay buffer spiked with human serum (buffer + 1:100 human plasma)

Samples were tested across the standard curve range (0–3000 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	0.157	0.168	0.8	4.8
46.88	0.170	0.199	2.1	11.2
93.75	0.186	0.254	4.8	21.9
187.5	0.203	0.300	6.9	27.3
375	0.278	0.640	25.6	55.7
750	0.600	1.289	48.8	51.6
1500	1.398	2.012	43.4	25.5
3000	2.295	2.640	24.4	9.9

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:100 Human Plasma)	% Standard Deviation	% CV
0	0.157	0.141	1.1	7.4
46.88	0.170	0.154	1.1	6.9
93.75	0.186	0.173	0.9	5.0
187.5	0.203	0.211	0.6	2.9
375	0.278	0.325	3.3	10.9
750	0.600	0.616	1.2	1.9
1500	1.398	1.375	1.6	1.2
3000	2.295	2.323	1.9	0.8

Results:

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

Conclusion:

The KRIBIOLISA Alemtuzumab ELISA demonstrates excellent performance in the presence of human serum and plasma.

7. Sample Handling and Storage Conditions

A.) 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.

For Serum - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 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.

For Plasma - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 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.

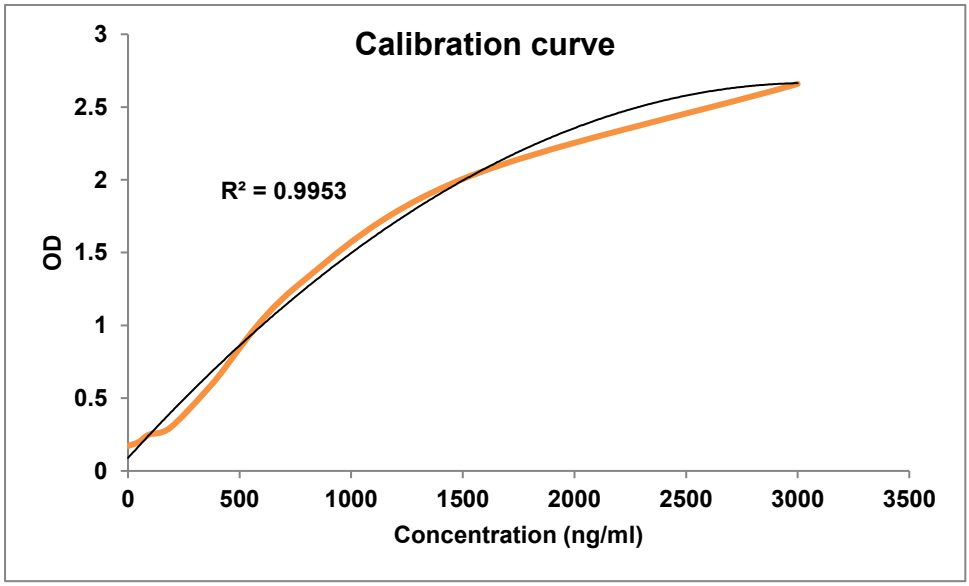
B.) Handling/Storage:

1. All reagents should be stored at 2 - 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.

C). Health Hazard Warnings:

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

3. **Graphs, Maps and Appendices:**



Matrix Effect Heat Map

	1:200	1:400	1:800	1:1600	1:3200	1:6400
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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