ELISA VALIDATION GUIDE

RANIBIZUMAB ELISA FOR USE IN

DRUG DISCOVERY RESEARCH AND BIOPHARMA

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

Background

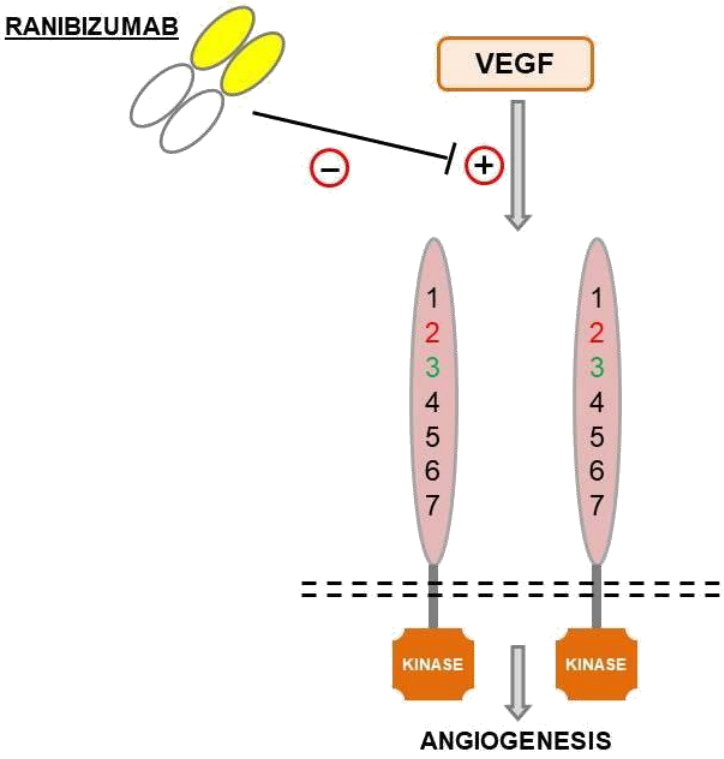
1. Introduction to Ranibizumab

Ranibizumab is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment directed against human vascular endothelial growth factor A (VEGF-A), which is a glycoprotein implicated in the pathophysiology of age-related macular degeneration.2,7 Ranibizumab is used to treat various ocular disorders with abnormal growth of blood vessels, such as neovascular (wet) age-related macular degeneration.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- Ranibizumab (anti-VEGF-A monoclonal antibody fragment) was developed to inhibit Vascular Endothelial Growth Factor-A (VEGF-A), a key molecule involved in angiogenesis and vascular permeability.
- The success of Ranibizumab validates VEGF-A as a critical therapeutic target in diseases characterized by pathological neovascularization such as age-related macular degeneration (AMD), diabetic macular edema (DME), and retinal vein occlusion (RVO).
- It provides a proof-of-concept for anti-angiogenic therapy, strengthening VEGF pathway targeting strategies for both ocular and oncologic drug discovery programs.



2.2 Assay Development

- Ranibizumab serves as a model for screening anti-inflammatory agents via:
  - ELISA (quantification of Ranibizumab)
  - Cell-based neutralization assays to assess affinity and neutralization potency against VEGF-A isoforms.
  - Surface Plasmon Resonance (SPR)

2.3 Biomarker for Efficacy and Safety

- VEGF-A levels in ocular fluids and plasma serve as pharmacodynamic biomarkers for efficacy assessment in anti-angiogenic therapy.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Biosimilar development requires:
    - Ranibizumab binding assays (affinity comparison)
    - Neutralizing potency assays (cell-based)
    - Comparability studies for regulatory submissions

3.2 PK/PD Studies

- Ranibizumab levels are monitored as a pharmacodynamic marker in clinical trials of Anatomical and functional endpoints, such as central retinal thickness (via OCT) and visual acuity (VA) improvements.

4. Importance in Eye Disorders

4.1. Targeted Therapy Against Pathological Angiogenesis

- Ranibizumab is a **humanized monoclonal antibody fragment (Fab)** that specifically binds to **Vascular Endothelial Growth Factor-A (VEGF-A)**, a key mediator of abnormal blood vessel growth and leakage in the eye.
- By neutralizing VEGF-A, it **prevents neovascularization and vascular permeability**, which are central to many retinal and choroidal diseases.

4.2. Transformative Role in Age-Related Macular Degeneration (AMD)

- Before Ranibizumab, **neovascular (wet) AMD** led to irreversible central vision loss.
- Ranibizumab therapy revolutionized management by **stabilizing and improving visual acuity** in a significant proportion of patients.
- It remains the **gold-standard treatment** for wet AMD and has set the benchmark for anti-VEGF therapies.

Scope of Validation

The KRIBIOLISA Ranibizumab (Lucentis™) ELISA (Catalog No KBI1029-3) kit is 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 Ranibizumab.

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 and CSF).
- Sample Handling and Storage Conditions.
- References (Ranibizumab Cmax 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

The KRIBIOLISA Ranibizumab (Lucentis™) ELISA kit is intended to measure the Ranibizumab in serum and plasma.

Principle of the Assay

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Ranibizumab are pre-coated onto microwells. HRP Conjugate, Samples / Standards are pipetted into microwells and human Ranibizumab present in the sample are bound by the capture antibody. 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 Ranibizumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Validation Parameters and Acceptance Criteria

1. Ranibizumab Cmax Values and Recommended ELISA Range

This table summarizes Ranibizumab Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected Ranibizumab (ng/mL)	Recommended ELISA Range (ng/ml)
Post intravitreal injection (initial systemic exposure)	1-10	0–50
Steady-state systemic levels (monthly dosing)	10-30	0–100
Treat-and-extend regimen (reduced frequency dosing)	1-20	0–100
High-dose or repeated injection monitoring (research or extended therapy)	20-50	0–200
Pharmacokinetic/therapeutic monitoring in ophthalmology clinical studies	1-50	0-200

Note: Assay sensitivity <1 ng/mL is recommended for detecting low systemic exposure levels, while an upper quantification limit of ≥200 ng/mL is advised to support extended pharmacokinetic profiling and high-concentration research applications.

The KRIBIOLISA® Ranibizumab based ELISA kit is developed using an assay range of 0 - 10 ng/ml with the dilutional linearity accuracy to measure responses as per the application table above on patient Cmax values. The kit has also been validated upto 4000 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 KRIBIOLISA® Ranibizumab ELISA are monoclonal antibodies engineered to selectively recognize the structural and functional domains unique to Ranibizumab. These antibodies exhibit high affinity for the Fab fragment configuration and VEGF-A–binding epitope region present in Ranibizumab, while demonstrating minimal cross-reactivity with endogenous human immunoglobulins, full-length monoclonal antibodies, anti-VEGF biosimilars, or related ophthalmic biologics such as Bevacizumab or Aflibercept.

This high degree of epitope-level discrimination ensures reliable detection of Ranibizumab independent of VEGF-binding status or formulation source, supporting precise quantification in both research and therapeutic drug monitoring applications.

2.2 Selectivity

The ELISA demonstrates low to negligible cross-reactivity with endogenous human antibodies, serum proteins, or non-Ranibizumab anti-VEGF therapeutics, including Bevacizumab or Aflibercept. The assay also excludes structurally unrelated monoclonal antibodies and immunoglobulin fragments, ensuring discrimination even in biologically complex samples. In addition, the method maintains high analytical selectivity in clinical matrices such as serum, plasma, and ophthalmic fluids, with minimal interference from complement proteins, binding proteins, or circulating VEGF-A. This ensures accurate and reliable quantification of Ranibizumab without non-specific signal contribution or false detection.

2.3 LOD, LOQ and IC50

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 Ranibizumab ELISA = 0.11 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).

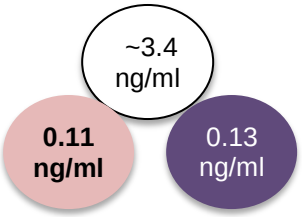
LOQ for KRIBIOLISA Ranibizumab ELISA – 0.13 ng/ml

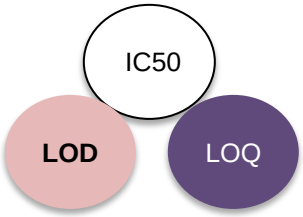
IC50 in ELISA (Half Maximal Inhibitory Concentration)
IC50 = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

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.

IC50 for KRIBIOLISA Ranibizumab ELISA = ~3.4 ng/ml
Summary:

Parameter	Value (ng/mL)
LOD	0.11 ng/ml
LOQ	0.13 ng/ml
IC50	3.4 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 (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery
0	0.267		
0.125	0.36	0.2	176.4
0.25	0.387	0.3	134.2
0.5	0.412	0.5	90.6
1.25	0.546	1.2	93.9
2.5	0.802	2.8	110.1
5	1.083	4.7	94.1
10	1.774	10.1	101
Positive Control (5 ng/ml)	1.294	5.98	119.6
Low QC control (0.5 ng/ml)	0.414	0.49	97.4
High QC control (7.5 ng/ml)	1.633	8.95	119.3

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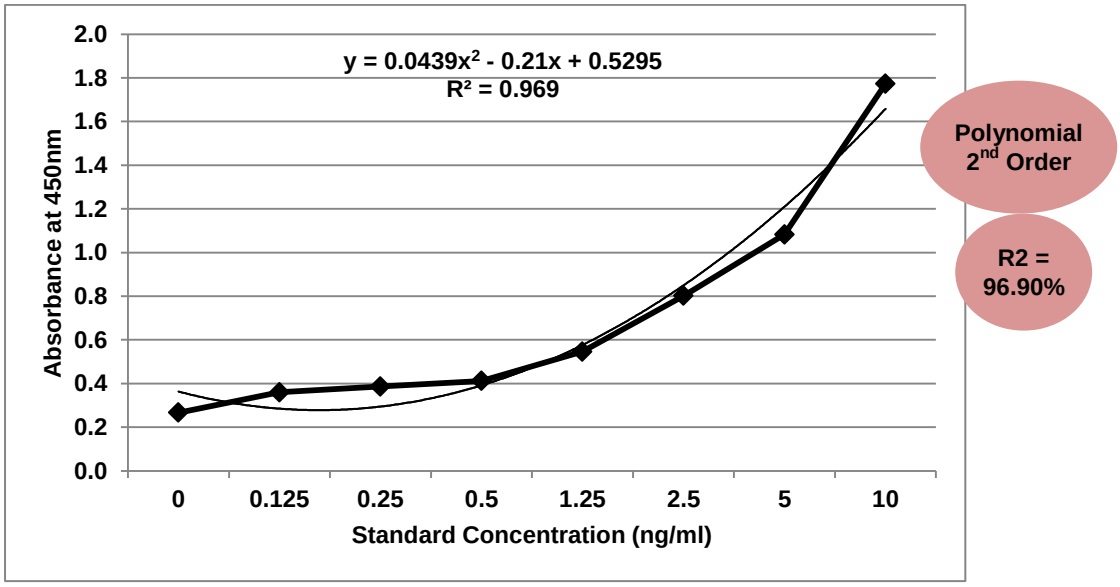

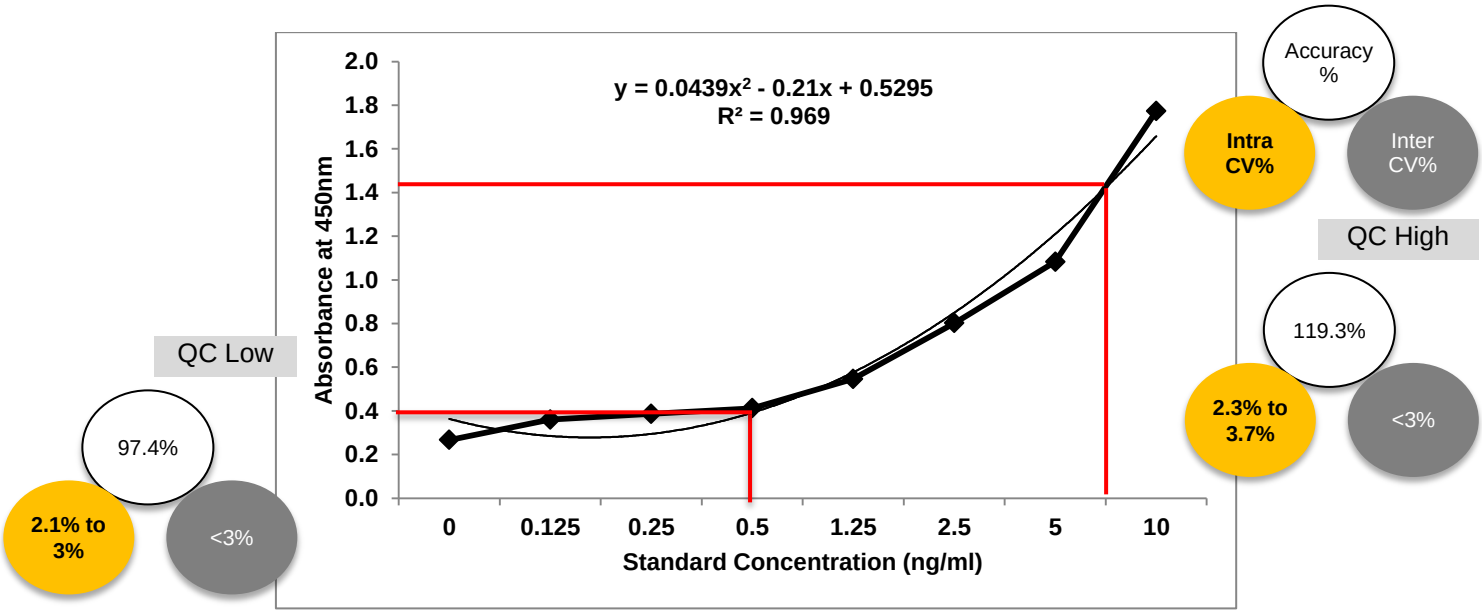

4. Accuracy and Precision (Intra / Inter-Assay)

Precision was assessed by analyzing three standard concentrations (0.125 ng/ml, 2.5 ng/ml, and 10 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%.

Results Summary:

Standard (ng/ml)	Intra-Assay %CV (Range)	Inter-Assay %CV
0.125	0.9% to 2.1%	<3%
2.5	2.1% to 2.3%	<3%
10	2.3% to 2.5%	<3%

Conclusion:

The KRIBIOLISA Ranibizumab 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 Ranibizumab 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 dilution	5	5.000	5.3	106.1	94.2
1:4000 dilution	2.5	2.500	2.2	88.0	113.6
1:8000 dilution	1.25	1.250	0.4	30.2	331.5
1:16000 dilution	0.5	0.500	--	--	--
1:32000 dilution	0.25	0.250	--	--	--
1:64000 dilution	0.125	0.125	--	--	--

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B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:2000 dilution	5	5.000	4.5	90.9	110.0
1:4000 dilution	2.5	2.500	3.1	124.8	80.1
1:8000 dilution	1.25	1.250	1.3	102.2	97.9
1:16000 dilution	0.5	0.500	0.1	23.9	523.7
1:32000 dilution	0.25	0.250	--	0.0	--
1:64000 dilution	0.125	0.125	--	0.0	--

Results:

i. Parallelism is generally maintained across the 1:2000 to 1: 4000 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 Ranibizumab 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 Ranibizumab 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 + 1:1000 human serum)
- Assay buffer spiked with human serum (buffer + 1:1000 human plasma)

Samples were tested across the standard curve range (0–10 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 + 1:1000 Human Serum)	Average	Standard Deviation	% CV
0	0.208	0.267	0.238	0.04	17.6
0.125	0.298	0.360	0.329	0.04	13.3
0.25	0.358	0.387	0.372	0.02	5.4
0.5	0.425	0.412	0.418	0.01	2.3
1.25	0.602	0.546	0.574	0.04	6.9
2.5	0.852	0.802	0.827	0.04	4.3
5	1.005	1.083	1.044	0.06	5.3
10	1.857	1.774	1.815	0.06	3.3

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:1000 Human Plasma)	Average	Standard Deviation	% CV
0	0.208	0.200	0.204	0.01	2.8
0.125	0.298	0.302	0.300	0.00	0.9
0.25	0.358	0.347	0.353	0.01	2.2
0.5	0.425	0.439	0.432	0.01	2.3
1.25	0.602	0.623	0.613	0.01	2.4
2.5	0.852	0.914	0.883	0.04	5.0
5	1.005	1.118	1.062	0.08	7.5
10	1.857	1.815	1.836	0.03	1.6

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.

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Conclusion:

The KRIBIOLISA Ranibizumab 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.

Tear Study Validation Results

Standard Concentration (ng/ml)	Absorbance	Interpolated Concentration	% Recovery	Standard Diluents
0.25	0.378	0.30	**119.7**	**Physiological Buffer**
5	1.108	4.89	**97.8**	
0.25	0.367	0.25	**100.3**	**Tear Sample**
5	1.125	5.01	**100.3**	

7. 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:

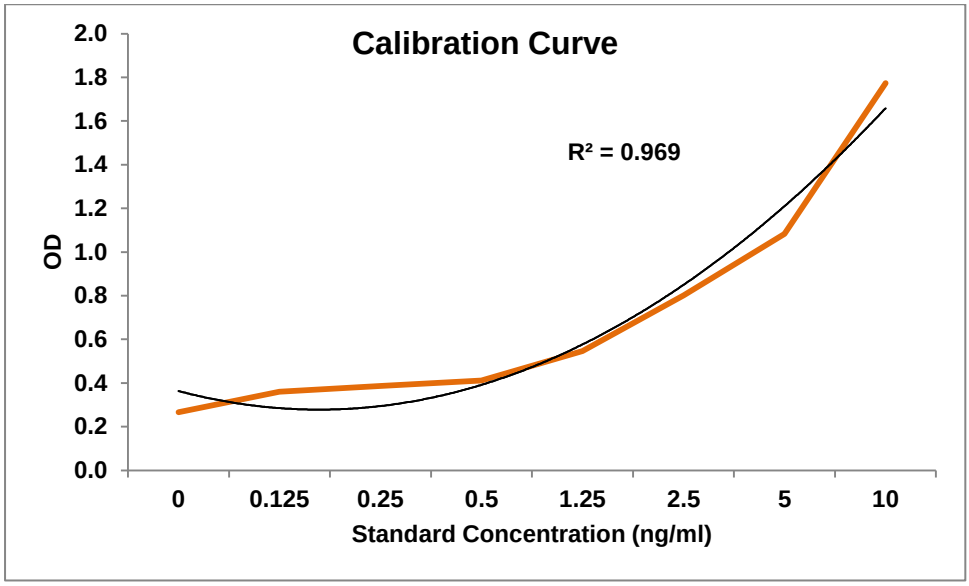
- It is advisable to aliquot and stores the Anti-Human Kappa:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Anti-Human Kappa:HRP Conjugate after running your assay.
- All the reagents and wash solutions should 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%2	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30%2	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%