

# ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,  
BIOPHARMA APPLICATIONS

**KRISHGEN BioSystems**

OUR REAGENTS, YOUR RESEARCH

## Background

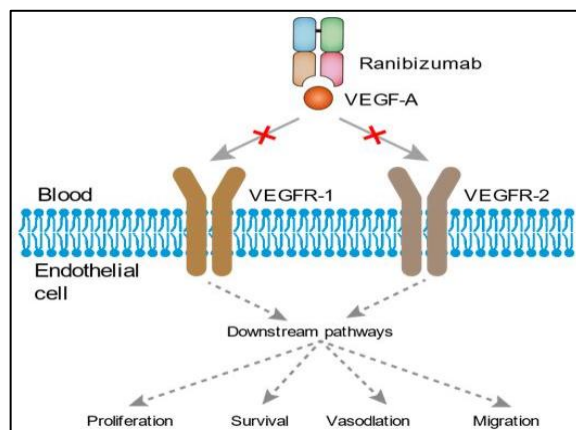
### 1. Introduction to Ranibizumab

Ranibizumab is a recombinant humanized monoclonal antibody fragment (Fab) that specifically targets vascular endothelial growth factor-A (VEGF-A), a key regulator of angiogenesis and vascular permeability. By binding to VEGF-A, Ranibizumab inhibits its interaction with VEGF receptors on vascular endothelial cells, thereby reducing abnormal blood vessel formation and vascular leakage. It plays a crucial role in the treatment of retinal disorders associated with excessive VEGF activity, including neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and macular edema following retinal vein occlusion (RVO). Due to its specific anti-VEGF activity and clinical importance, Ranibizumab is widely used in ophthalmology and has emerged as an important therapeutic agent and biomarker-related analyte in pharmacokinetic, pharmacodynamic, bioanalytical, and immunogenicity research.

### 2. Significance in Drug Discovery Research

#### 2.1 Target Identification and Validation

- Ranibizumab specifically targets vascular endothelial growth factor-A (VEGF-A), a key mediator of angiogenesis and vascular permeability involved in pathological blood vessel formation and retinal vascular leakage.
- Elevated VEGF-A activity is associated with retinal vascular disorders such as neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and macular edema following retinal vein occlusion (RVO).
- Inhibition of VEGF-A signalling is a validated therapeutic strategy for controlling abnormal angiogenesis and vascular leakage, making Ranibizumab an established anti-VEGF therapeutic agent in ophthalmic disease management and an important analyte for pharmacokinetic, pharmacodynamic, and bioanalytical studies.



#### 2.2 Assay Development

- Ranibizumab is used in the evaluation and characterization of anti-VEGF therapeutic agents through:
  - **ELISA or MSD assays** for quantitative measurement of Ranibizumab concentration in biological samples such as serum, plasma, and ocular matrices.
  - **Cell-based bioassays** to assess VEGF-A inhibition and the ability of Ranibizumab to suppress VEGF-mediated endothelial cell proliferation, migration, or vascular permeability.

- **VEGF-A/VEGFR pathway-based assays** to evaluate the inhibition of VEGF-A binding and downstream signaling, supporting potency, pharmacodynamic, and comparative bioanalytical studies.

### **2.3 Biomarker for Efficacy and Safety**

- Ranibizumab levels are correlated with therapeutic exposure and can be monitored to evaluate drug distribution, clearance, and systemic exposure following treatment.
- Serves as a pharmacokinetic and pharmacodynamic (PK/PD) analyte for assessing therapeutic response and VEGF-A pathway inhibition, particularly in ophthalmic disease studies involving neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and retinal vein occlusion (RVO).
- Monitoring Ranibizumab concentrations can support efficacy and safety assessment by evaluating systemic drug exposure and helping characterize potential accumulation, altered clearance, or unexpected exposure during repeated dosing and long-term treatment.

## **3. Relevance in Biopharmaceutical Development**

### **3.1 Monoclonal Antibodies and Biosimilars**

- Ranibizumab is a recombinant humanized monoclonal antibody Fab fragment developed to specifically bind vascular endothelial growth factor-A (VEGF-A) and inhibit VEGF-mediated angiogenesis and vascular permeability.
- Ranibizumab and its biosimilar products are evaluated for the treatment of retinal vascular disorders, including neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and retinal vein occlusion (RVO).
- Biologic development involves:
  - VEGF-A–Ranibizumab binding assays to evaluate binding affinity, specificity, and inhibition of VEGF-A interaction with its receptors.
  - Cell-based bioactivity assays to assess inhibition of VEGF-mediated endothelial cell proliferation, migration, and other biological responses.
- Comparability, potency, pharmacokinetic, and stability studies are performed to demonstrate similarity, consistency, and product quality during biosimilar development and for preclinical and regulatory submissions.

### **3.2 PK/PD Studies**

- Ranibizumab concentrations, along with pharmacodynamic markers such as free VEGF-A levels and retinal vascular outcomes, are used to evaluate drug exposure, target engagement, and therapeutic activity in clinical and bioanalytical studies.
- These studies focus on dose optimization, pharmacokinetic characterization, systemic exposure, and durability of VEGF-A inhibition during repeated Ranibizumab administration, supporting the assessment of therapeutic efficacy and safety.

### 3.3 Safety Evaluation

- **Ranibizumab inhibits VEGF-A activity, and its safety profile is primarily associated with ocular and systemic effects** related to anti-VEGF treatment, including potential increases in intraocular pressure, ocular inflammation, and rare systemic vascular events.
- **Quantification of Ranibizumab and monitoring of VEGF-related biomarkers** are important for evaluating drug exposure, pharmacodynamic effects, and potential safety concerns, particularly during repeated or long-term treatment.
- Safety profiling includes monitoring of ocular parameters, systemic adverse events, intraocular pressure, and relevant clinical biomarkers to assess tolerability and potential treatment-related toxicity.

## 4. Importance in Cell and Gene Therapy (CGT)

### 4.1 Cytokine Release Syndrome (CRS) Monitoring

- Ranibizumab is not directly involved in cytokine release syndrome (CRS) and is not typically used as a biomarker for CRS monitoring, as its primary mechanism is inhibition of VEGF-A rather than modulation of immune-cell activation.
- Monitoring Ranibizumab concentrations and VEGF-A levels can support assessment of drug exposure and target engagement, particularly in pharmacokinetic and pharmacodynamic studies of anti-VEGF therapies.
- Safety monitoring during Ranibizumab treatment focuses on ocular and systemic adverse events, including intraocular pressure, ocular inflammation, and rare systemic vascular events, rather than cytokine markers such as IL-6 or IFN- $\gamma$ .

### 4.2 Therapeutic and Pharmacodynamic Biomarker

- Quantification of Ranibizumab helps assess drug exposure, target engagement, and VEGF-A pathway inhibition in pharmacokinetic (PK) and pharmacodynamic (PD) studies.
- Ranibizumab concentration can serve as a biomarker of therapeutic exposure and treatment response in ophthalmic diseases such as neovascular age-related macular degeneration (nAMD), diabetic macular edema (DME), and retinal vein occlusion (RVO).

### 4.3 Genetic Modulation

- Ranibizumab is not directly used for genetic engineering of CAR-T or NK cells; however, VEGF-A inhibition is being investigated as a strategy to modulate the tumor microenvironment and angiogenesis in combination with advanced cancer therapies.

- Anti-VEGF strategies may be incorporated into combination approaches to reduce tumor vascularization, improve immune-cell infiltration, and potentially enhance the efficacy of cell-based cancer therapies.
- Ranibizumab and VEGF-A pathway modulation can support translational research evaluating the interaction between angiogenesis, vascular permeability, and therapeutic cell trafficking in advanced cancer and regenerative medicine platforms.

## Scope of Validation

KRIBIOLISA® Ranibizumab (LUCENTIS™) ELISA (Catalog No KBI1029) 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).
- Parallelism and Matrix Effect.
- Disease State Recovery

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](mailto:sales1@krishgen.com)

## Intended Use of the ELISA

The KRIBIOLISA® Ranibizumab (LUCENTIS™) ELISA kit is intended to measure the Ranibizumab in serum, plasma, cell culture supernatant and other biological fluids.

## 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.

## 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 IL-21 Range (ng/ml) | Recommended ELISA Range (ng/ml) |
|--------------------------------------|------------------------------|---------------------------------|
| Post-dose Systemic Exposure          | ~0.5–2 ng/ml                 | 0.125–10                        |
| Pharmacokinetic (PK) Studies         | ~0.1–2 ng/ml                 | 0.125–10                        |
| Repeated-Dose / Accumulation Studies | ~0.5–3 ng/ml                 | 0.125–10                        |
| Biosimilar Comparability Studies     | ~0.1–3 ng/ml                 | 0.125–10                        |
| Low-Level / Elimination Phase        | <0.5 ng/ml                   | 0.125–10                        |

Note: Ranibizumab is administered intravitreally and has relatively low systemic exposure compared with its local ocular exposure. Therefore, a sensitive ELISA with a lower quantification limit around 0.125 ng/ml is useful for detecting low systemic concentrations during PK, bioavailability, biosimilar, and drug-clearance studies. The 10 ng/ml upper limit provides adequate coverage for diluted samples and samples with higher measured concentrations.

The KRIBIOLISA® Ranibizumab (Lucentis™) ELISA kit is developed using an assay range of 0.125–10 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 1600 fold dilution and the values are within the acceptable range.

## 2. Specificity and Selectivity

### 2.1 Specificity

The capture antibody and detection antibody are both specific to Ranibizumab and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant Ranibizumab.

### 2.2 Selectivity

The ELISA is designed to specifically detect Ranibizumab and is expected to show no or low cross-reactivity with structurally unrelated therapeutic monoclonal antibodies and antibody-based biologics commonly present in biological samples.

### 2.3 LOD, LOQ and IC<sub>50</sub>

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

(3 $\sigma$  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

(10 $\sigma$  criterion is most common).

LOQ for PrecisionBind Human IL-21 ELISA – 0.13 ng/ml

IC<sub>50</sub> in ELISA (Half Maximal Inhibitory Concentration)

IC<sub>50</sub> = 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<sub>50</sub> for KRIBIOLISA® Ranibizumab ELISA = ~ 3.4 ng/ml

Summary:

| Parameter        | Value (ng/ml) |
|------------------|---------------|
| LOD              | 0.11 ng/ml    |
| LOQ              | 0.13 ng/ml    |
| IC <sub>50</sub> | ~3.4 ng/ml    |

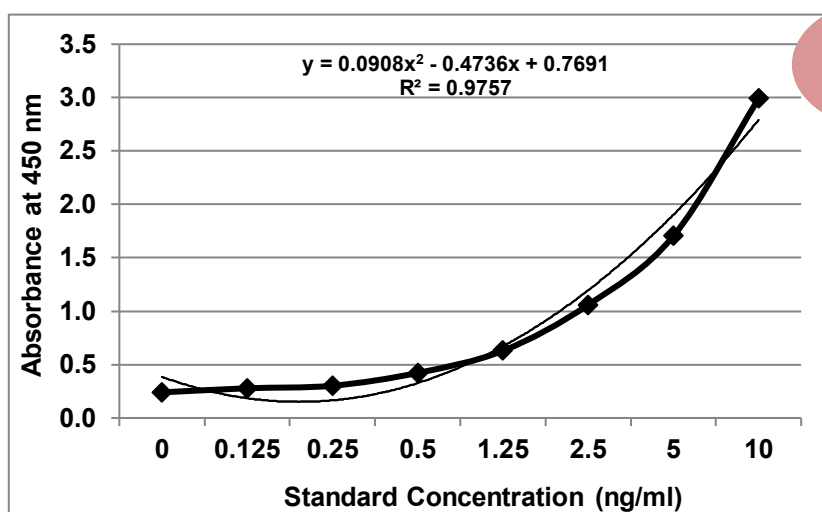


Regulatory Note:

LOD S/N  $\geq$  3:1, LOQ  $\geq$  10:1, %CV  $\leq$  20%      \*S/N = Signal / Noise Ratio

### 3. Linearity and Range

| Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration | % Recovery |
|--------------------------------|-----------------|----------------------------|------------|
| 0                              | 0.240           | -                          | -          |
| 0.125                          | 0.278           | 0.1                        | 99.0       |
| 0.25                           | 0.302           | 0.2                        | 75.6       |
| 0.5                            | 0.422           | 0.5                        | 107.3      |
| 1.25                           | 0.629           | 1.2                        | 94.4       |
| 2.5                            | 1.059           | 2.6                        | 104.7      |
| 5                              | 1.703           | 4.9                        | 98.7       |
| 10                             | 2.990           | 10.0                       | 100.1      |
| Low QC control (0.125 ng/ml)   | 0.298           | 0.24                       | 96         |
| Mid Control (1.25 ng/ml)       | 0.628           | 1.2                        | 94.2       |
| High QC control (10 ng/ml)     | 2.905           | 9.7                        | 96.6       |



Polynomial 2<sup>nd</sup> Order

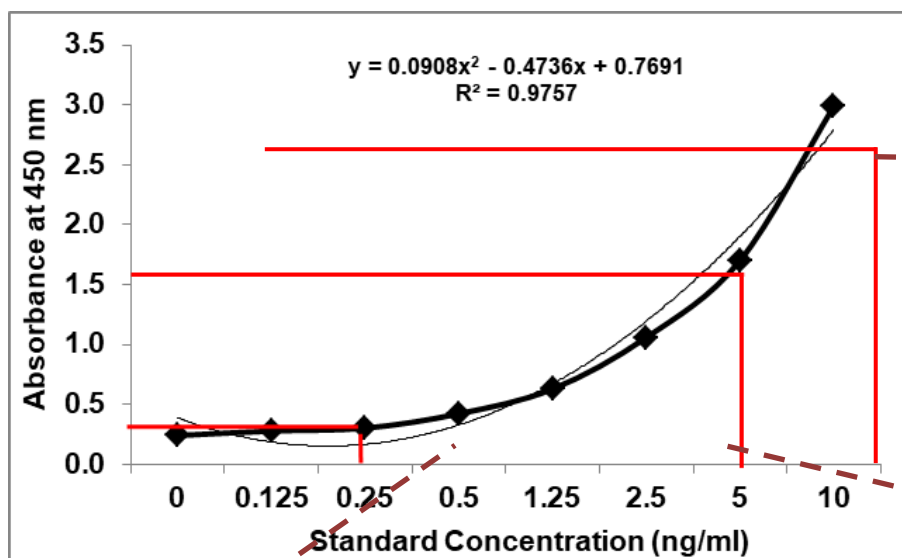
R<sup>2</sup> = 97.5%

Accuracy %

Intra CV%

Inter CV%

Figure 1. Standard Curve Accuracy — %



QC High

99.6%

1.2%

0.42%

QC Mid

99.6%

1.2%

0.42%

QC Low

103.2%

3.4%

2.31%

Recovery Across the Assay Range

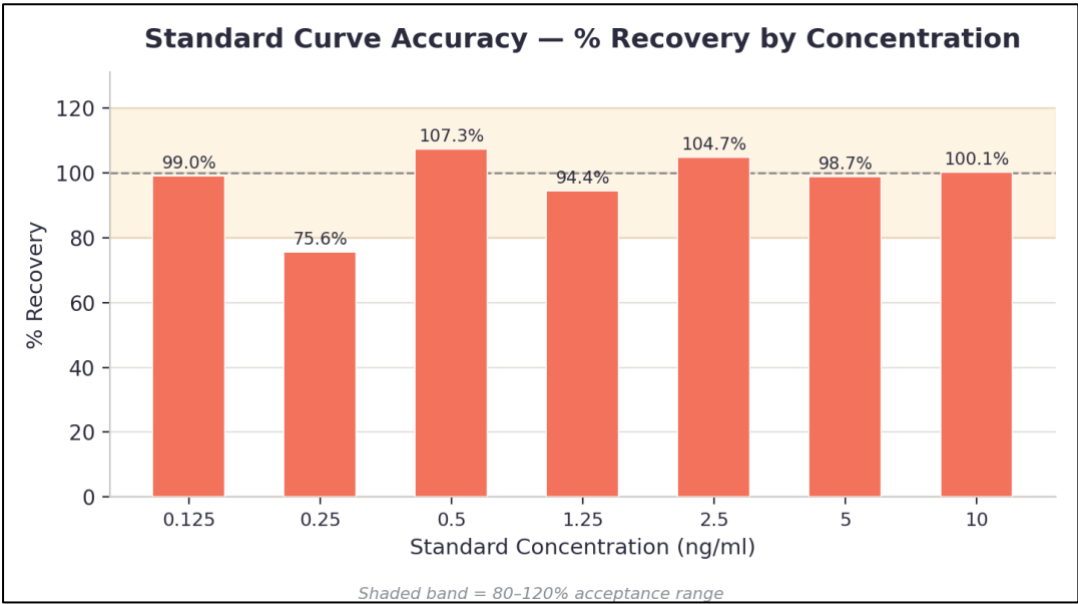
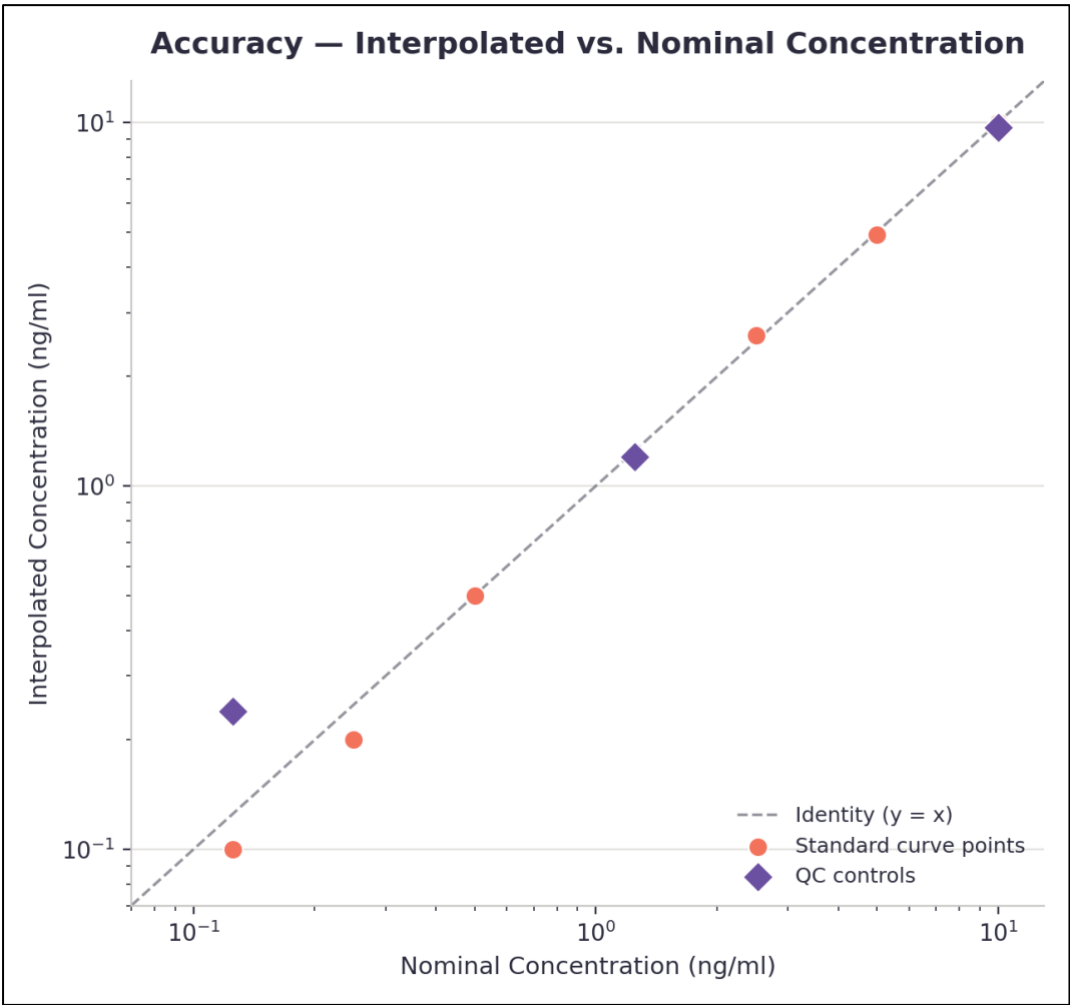


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

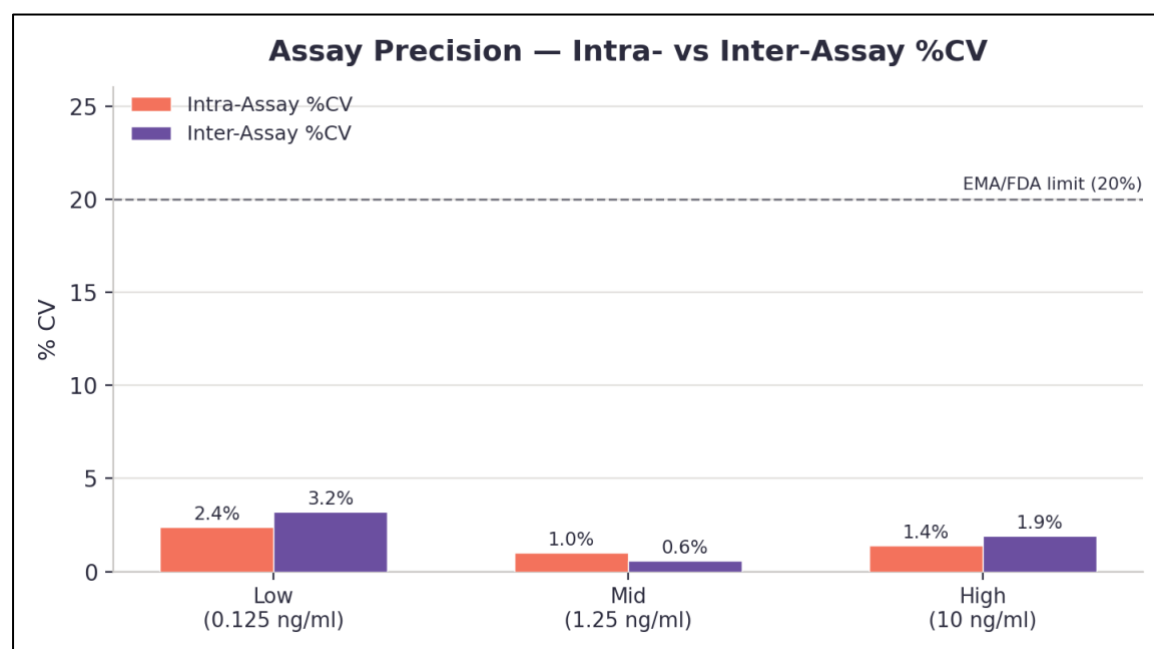
##### A) Intra-Assay:

| Standard (ng/ml) | Mean OD450 | SD   | %CV |
|------------------|------------|------|-----|
| 0.125            | 0.299      | 0.59 | 2.4 |
| 1.25             | 0.624      | 0.51 | 1.0 |
| 10               | 2.865      | 3.36 | 1.4 |

##### B) Inter assay:

| Standard (ng/ml) | Mean OD450 | SD   | %CV |
|------------------|------------|------|-----|
| 0.125            | 0.294      | 0.78 | 3.2 |
| 1.25             | 0.622      | 0.33 | 0.6 |
| 10               | 2.904      | 4.52 | 1.9 |

Figure 3. Intra- vs Inter-Assay Precision (%CV)



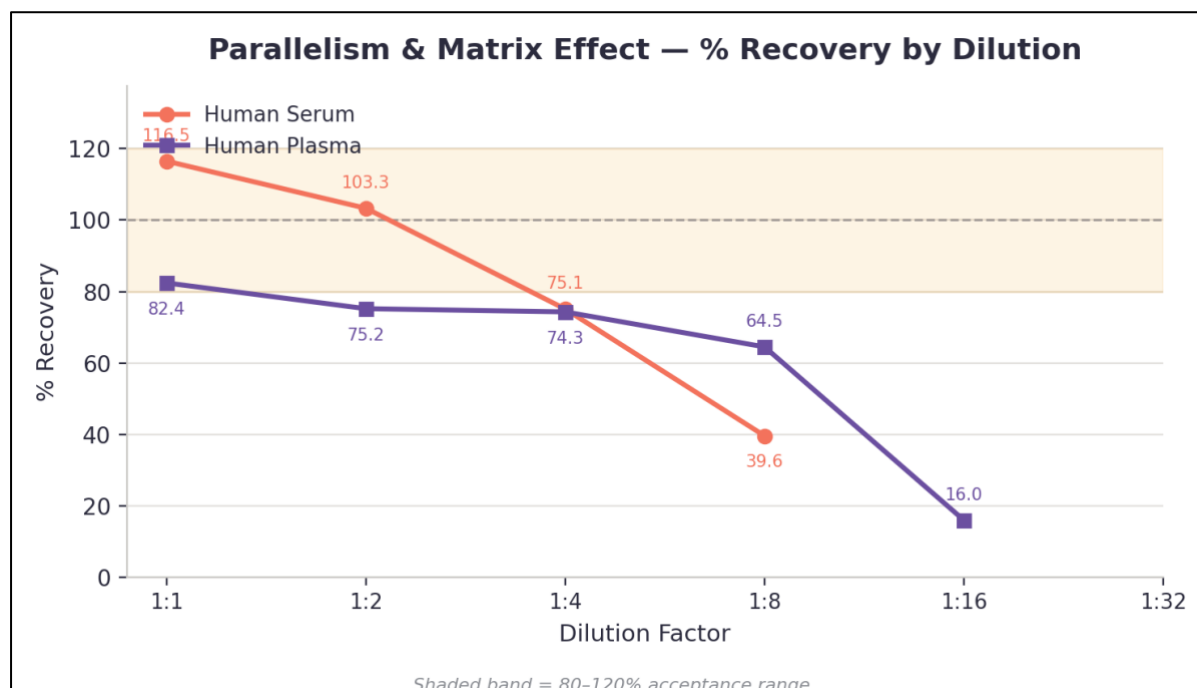
## 5. Parallelism and Matrix Effect

Sample Dilution factor – Human Serum and Human Plasma samples have been tested.

| Serum Dilution | Expected Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery |
|----------------|-----------------------------------------|-----------------|------------------------------------|------------|
| 1:1            | 5                                       | 1.939           | 5.8                                | 116.5      |
| 1:2            | 2.5                                     | 1.049           | 2.6                                | 103.3      |
| 1:4            | 1.25                                    | 0.553           | 0.9                                | 75.1       |
| 1:8            | 0.625                                   | 0.323           | 0.2                                | 39.6       |
| 1:16           | 0.313                                   | 0.195           | -                                  | -          |
| 1:32           | 0.156                                   | 0.156           | -                                  | -          |

| Plasma Dilution | Expected Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration (ng/ml) | % Recovery |
|-----------------|-----------------------------------------|-----------------|------------------------------------|------------|
| 1:1             | 5                                       | 1.890           | 4.1                                | 82.4       |
| 1:2             | 2.5                                     | 1.021           | 1.9                                | 75.2       |
| 1:4             | 1.25                                    | 0.554           | 0.9                                | 74.3       |
| 1:8             | 0.625                                   | 0.315           | 0.4                                | 64.5       |
| 1:16            | 0.313                                   | 0.203           | 0.1                                | 16.0       |
| 1:32            | 0.156                                   | 0.178           | -                                  | -          |

Figure 4. Parallelism & Matrix Effect — % Recovery by Dilution (Serum vs. Plasma)



## Results:

Parallelism was demonstrated between the diluted serum and plasma samples and the standard curve, with recovery values remaining within the acceptable range across the lower to intermediate dilution levels.

- i. % Recovery were generally consistent for serum and plasma samples up to the 1:4 dilution, indicating minimal matrix interference and reliable analyte detection within the recommended working range. Reduced recovery was observed at higher dilution factors, particularly beyond the 1:8 dilution, which may be attributed to decreased analyte concentration approaching the lower limit of quantification.
- ii. The KRIBIOLISA® Ranibizumab (LUCENTIS™) ELISA kit was tested for matrix effect on human serum and human plasma samples

## 6. Disease State Sample Recovery

Recovery was assessed by analysing diseased plasma (n=3) and diseased serum samples (n=1) across multiple dilution factors (1:2 to 1:32) against calibration standards. This study was conducted to evaluate potential matrix effects and the ability of the assay to accurately recover Ranibizumab in clinically relevant diseased plasma and diseased serum matrices.

### Sample Details:

Diseased-state sample validation was performed using four clinically relevant human biological matrix samples (recovered plasma and off-the-clot serum) from patients with Type 2 diabetes. The samples included both male and female subjects, with ages ranging from 49 to 54 years, and were of African American and Hispanic demographics. All samples were reported as diabetic and pathogen-negative. These samples were evaluated to assess the assay performance and suitability in a clinically relevant diseased-state matrix.

| Sample ID     | Disease Type                         | Patient details<br>Ethnicity<br>Age/Sex | Diabetic /Smoker | Pathogen | Medi-<br>cation | Blood Type/BMI | Anticoa-<br>gulant |
|---------------|--------------------------------------|-----------------------------------------|------------------|----------|-----------------|----------------|--------------------|
| PL1206<br>22C | Human Plasma<br>(Type 2<br>Diabetic) | African<br>American<br>52/<br>Female    | Yes/No           | Negative | Nil             | A+/25.1        | K2<br>EDTA         |

| Sample ID        | Disease Type                      | Patient details<br>Ethnicity<br>Age/Sex | Diabetic /Smoker | Pathogen | Medication | Blood Type/<br>BMI | Anticoagulant |
|------------------|-----------------------------------|-----------------------------------------|------------------|----------|------------|--------------------|---------------|
| PL1206<br>22D    | Human Plasma<br>(Type 2 Diabetic) | Hispanic<br>49/<br>Male                 | Yes/No           | Negative | Nil        | O-/28.3            | K2<br>EDTA    |
| PL1206<br>22F    | Human Plasma<br>(Type 2 Diabetic) | Hispanic<br>54/<br>Female               | Yes/Yes          | Negative | Nil        | O+/35.2            | K2<br>EDTA    |
| 803569<br>167388 | Human Serum,<br>Type 2 Diabetic)  | African American<br>50/Male             | Yes/No           | Negative | Nil        | Nil                | Nil           |

#### Acceptance Criteria:

- Recovery within 70–130% is generally considered acceptable for ligand-binding assays.
- Consistency across dilutions indicates minimal matrix interference.

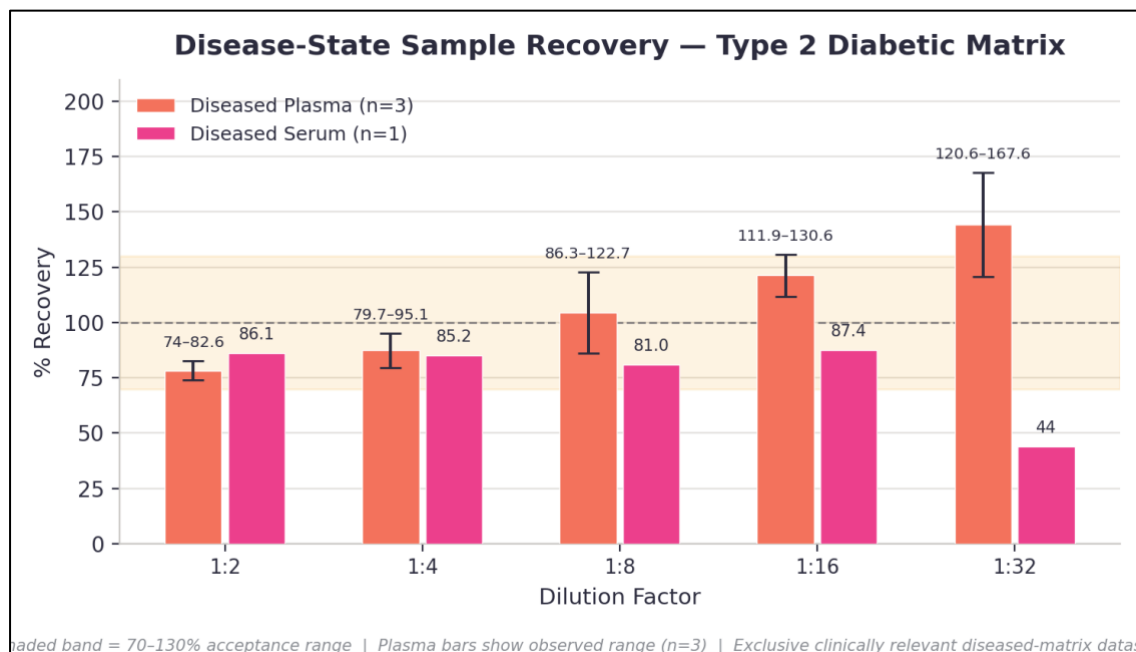
#### *Diseased State Sample Impact on Ranibizumab Recovery*

| Sample Matrix                | 1:2 Dilution (%) | 1:4 Dilution (%) | 1:8 Dilution (%) | 1:16 Dilution (%) | 1:32 Dilution (%) |
|------------------------------|------------------|------------------|------------------|-------------------|-------------------|
| <b>Diseased Plasma (n=3)</b> | 74 – 82.6        | 79.7 – 95.1      | 86.3 – 122.7     | 111.9 – 130.6     | 120.6 – 167.6     |
| <b>Diseased Serum (n=1)</b>  | 86.1             | 85.2             | 81.0             | 87.4              | 44                |

#### Observations:

- Recovery of Ranibizumab in diseased plasma and diseased serum samples was predominantly within the acceptance range (70–130%), demonstrating good assay accuracy and minimal matrix interference across the evaluated dilution levels.
- Consistent recovery was observed across the 1:2 to 1:16 dilution range in both matrices, indicating robust assay performance and reliable analyte detection under diseased sample conditions.
- The recovery profile demonstrates that the assay effectively quantifies Ranibizumab in complex biological matrices, with acceptable performance maintained across a broad range of sample dilutions.
- Minor deviations observed at the highest dilution level are likely attributable to increased dilution-related variability and do not impact the overall suitability of the assay within its recommended working range.
- The results support the suitability of the assay for accurate, precise, and reproducible quantification of Ranibizumab in diseased plasma and diseased serum samples for bioanalytical and pharmacokinetic applications.

**Figure 5. Disease-State Sample Recovery — Diseased Plasma vs. Diseased Serum**



## 7. Sample Handling and Storage Conditions

### A) Sample collection and handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

**Cell Culture Supernatant:** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at temperature  $<-20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

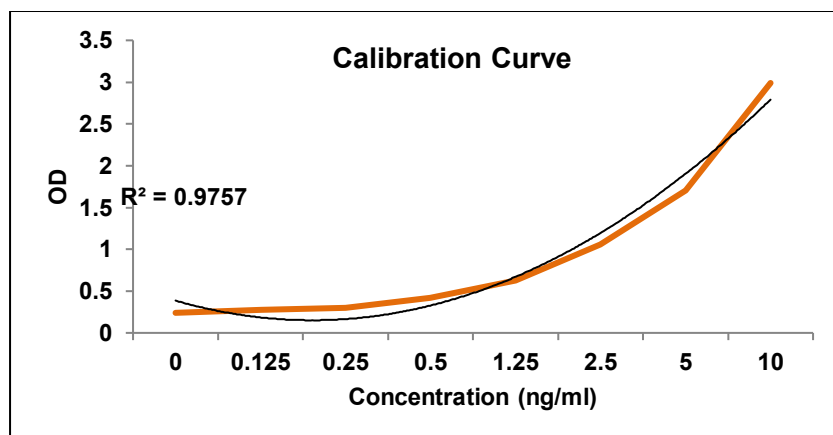
**Serum:** Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at  $1000 \times g$ . Remove serum layer and assay immediately or store serum samples at temperature  $<-20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

**Plasma:** Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at  $1000 \times g$  within 30 minutes of collection. Assay immediately or store plasma samples at temperature  $<-20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles.

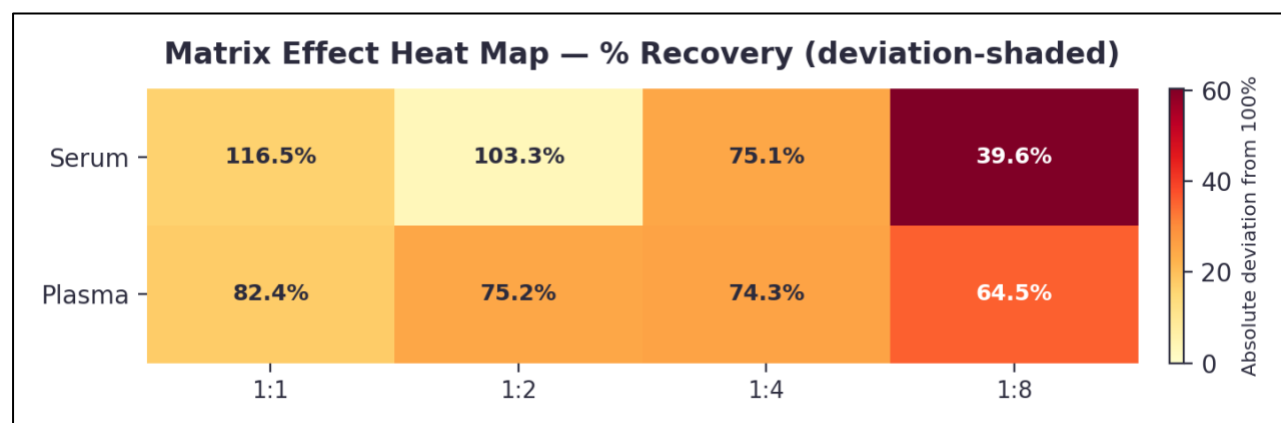
### B) Storage conditions:

- Store main kit components at  $2-8^{\circ}\text{C}$ .
- Store recombinant lyophilized standard at  $2-8^{\circ}\text{C}$ . Upon reconstitution aliquot standards into polypropylene vials and store at  $-20^{\circ}\text{C}$  as per assay requirements. Do not freeze thaw for more than two times.
- Before using, bring all components to room temperature ( $18-25^{\circ}\text{C}$ ). Upon assay completion return all components to appropriate storage conditions.

## Graphs, Maps and Appendices:



## Matrix Effect Heat Map



|        | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
|--------|-----|-----|-----|-----|------|------|
| 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%              | -                                       |
| Total Error (TE)         | TE < 30% (40% at LLOQ and ULOQ) | -                                       |
| Specificity/Interference | Recovery 100 ± 25% <sup>2</sup> | H (null hypothesis) = 100 ± 25 %        |
| Parallelism/Linearity    | CV < 30% <sup>2</sup>           | Deviation from linearity < 20%          |
| LLOQ / LoQ               | Recovery 100 ± 25%              | TE % < 32.9%                            |

## References

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