ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® EVOLOCUMAB (REPATHA™) ELISA KIT (CATALOG NO. KBI1323) 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® EVOLOCUMAB (REPATHA™) ELISA (Cat No # KBI1323)**	31.01.2025

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

Introduction

This document presents a discussion of the characteristics of our **KRIBIOLISA® Evolocumab (REPATHA™) ELISA (Catalog No KBI1323)** 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 **Evolocumab.**

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

- **Assay Validation**
- **Standard Curve**
- **Pharmacokinetic Relevance**
- **Precision and Reproducibility**

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

Background

Evolocumab is a fully human monoclonal IgG2 antibody that selectively targets proprotein convertase subtilisin/kexin type 9 (PCSK9). By binding to circulating PCSK9, evolocumab prevents it from interacting with low-density lipoprotein receptors (LDLR) on hepatocytes, thereby increasing receptor recycling and enhancing clearance of LDL cholesterol from the bloodstream. Evolocumab is developed for the treatment of hypercholesterolemia, including adults with heterozygous familial hypercholesterolemia, and for patients with clinical atherosclerotic cardiovascular disease requiring additional LDL-lowering despite maximally tolerated statin therapy. The drug is administered via subcutaneous injection and was approved by the U.S. Food and Drug Administration on August 27, 2015, under the brand name Repatha, providing a potent targeted therapy for managing elevated LDL cholesterol in high-risk patients.

1. Purpose

To assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Evolocumab (REPATHA™) ELISA developed using Anti-Evolocumab as capture protein.

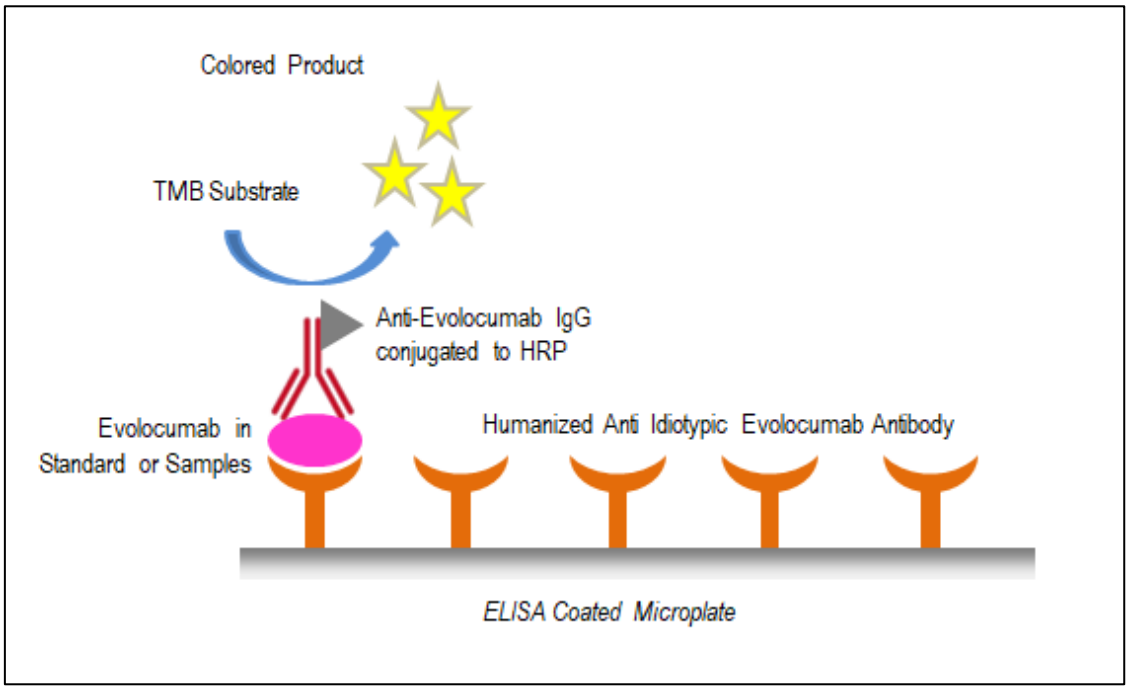
2. Experimental Design

- `A sandwich ELISA was performed using Anti-Evolocumab as capture protein.

- Standards prepared for Evolocumab.
- Assay Concentration Range: 0 - 640 ng/ml.
- Signal (% absorbance) plotted versus concentration.

The KRIBIOLISA Evolocumab ELISA employs a targeted immobilization strategy to ensure optimal presentation of recombinant human proprotein convertase subtilisin/kexin type 9 (PCSK9) on the assay plate, thereby enhancing the selective binding of evolocumab. The immobilization procedure is designed to preserve the native conformation and epitope accessibility of PCSK9, maintaining its structural integrity and functional orientation. This controlled presentation ensures that the antigen is displayed in a configuration that supports high-affinity interaction with evolocumab's LDL receptor–binding domain–specific regions.

Evolocumab's high specificity enables strong and stable antigen–antibody complex formation under plate-bound conditions. In contrast, antibodies targeting other epitopes of PCSK9 or unrelated proteins may demonstrate reduced or limited binding. This differential binding behavior reflects Evolocumab's selective recognition of PCSK9 and the preserved structural orientation of the immobilized antigen established during the coating process.



ELISA kits for Evolocumab estimation offered by KRISHGEN uses Anti Evolocumab capture proteins as above

3. Assay Validation

- IC50 Value: ~ 122 ng/ml (within 0-640 ng/mL assay range).
- LLOQ: ~ 6.77 ng/ml.

- Clinical Cmax Values*:
  - After 140 mg subcutaneous dose every 2 weeks :~15–20 µg/mL
  - After 420 mg subcutaneous dose once monthly: (≥80 kg body weight): ~60–70 µg/mL
  - At steady state (following repeated biweekly or monthly dosing): ~50–70 µg/mL

*Values are approximate and may vary depending on body weight, dosing schedule, and patient variability.

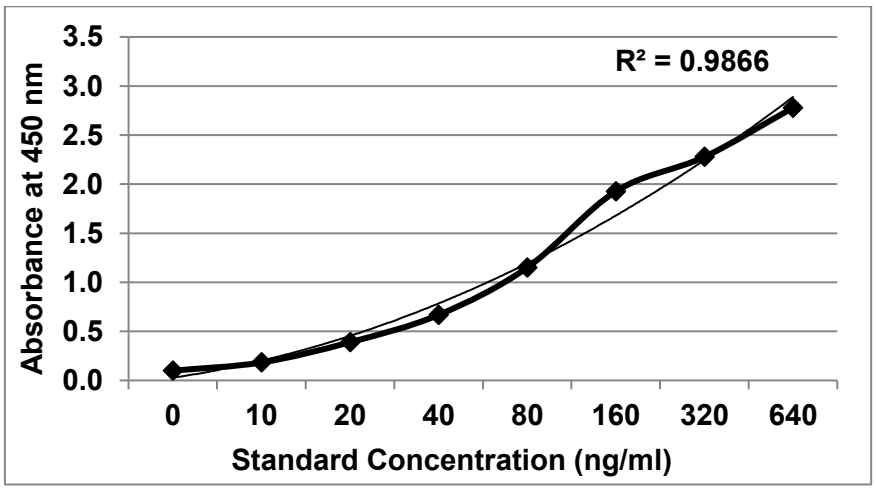
** published data*

- Precision:
  - Intra-Assay CV: <8%.
  - Inter-Assay CV: <3%.
  - Inter-Operator CV: <10%.

4. Standard Curve

Below is the standard curve for Evolocumab Sandwich ELISA assay:
Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.099	--	--
10	0.183	8.2	82.1
20	0.390	21.5	107.7
40	0.669	39.5	98.8
80	1.149	76.3	95.3
160	1.927	178.7	111.7
320	2.278	275.3	86.0
640	2.779	724.7	113.2



5. LOD and LOQ

- LOD Absorbance: (Approx ~2.23 ng/ml)
- LOQ Absorbance: (Approx ~6.77 ng/ml)

6. Pharmacokinetic Relevance

The assay is designed to cover the clinically relevant serum concentrations of Evolocumab observed following subcutaneous therapeutic dosing, making it suitable for pharmacokinetic evaluation and therapeutic monitoring.

The Evolocumab ELISA demonstrates sensitivity within the µg/mL range, which falls well within the validated assay limits, ensuring accurate quantification across clinically meaningful exposure levels. Published pharmacokinetic data for Evolocumab indicate systemic exposure consistent with therapeutic monoclonal antibodies:
Following the recommended subcutaneous dosing regimens (140 mg every 2 weeks or 420 mg once monthly), the reported initial Cmax ranges from approximately 15–20 µg/mL depending on dose and schedule.
• With repeated biweekly or monthly dosing, steady-state peak concentrations typically reach approximately 50–70 µg/mL.
• Actual exposure may vary depending on body weight, dosing frequency, and individual patient variability.
Thus:
• At clinically relevant subcutaneous doses, Evolocumab serum concentrations fall within the measurable range of the ELISA following appropriate sample dilution.
• The assay working range enables reliable differentiation across varying systemic exposure levels.
• Given the circulating concentrations associated with therapeutic dosing, routine dilution of clinical samples is recommended to ensure measurements fall within the linear dynamic range of the assay.
• The assay is therefore suitable for pharmacokinetic profiling, dose–exposure analysis, and therapeutic monitoring of Evolocumab in human serum or plasma.

7. Precision and Reproducibility

Precision was assessed by analysing 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 samples.
- Inter-assay %CV should be ≤15% for 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	1.4% to 7.8%	<3%
80	1.6 % to 4.1%	<2%
640	0.7% to 3.2%	<2%

Observations:

- Intra-assay precision was consistently less than 8% across all levels tested.
- Inter-assay precision was consistently less than 4%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:
The KRIBIOLISA® Evolocumab (REPATHA™) ELISA demonstrates excellent intra- and inter-assay precision. These results support the assay's reliability and reproducibility for routine use in pharmacokinetic and bioanalytical studies.

8. Conclusion

The KRIBIOLISA® Evolocumab (REPATHA™) ELISA is validated for sensitivity, specificity, precision, and accuracy, and is appropriate for pharmacokinetic applications in regulatory settings.

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Version 1.0, dated 31.01.2025