

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN *BioSystems*

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® ANTI-DENOSUMAB (PROLIA™ / XGEVA™) ELISA KIT (CATALOG NO. KBI2026) 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® ANTI-DENOSUMAB (PROLIA™ / XGEVA™) ELISA (Cat No # KBI2026)	31.12.2024

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



Introduction

This document presents a discussion of the characteristics of our **KRIBIOLISA® Anti-Denosumab (PROLIA™ / XGEVA™) ELISA (Catalog No KBI2026)** 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 **Anti-Denosumab**.

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

Denosumab is a fully human monoclonal IgG2 antibody that inhibits RANKL, a key regulator of osteoclast activity, thereby reducing bone resorption and increasing bone strength. It is indicated for the treatment of osteoporosis in postmenopausal women and men at high fracture risk, prevention of skeletal-related events in patients with bone metastases, and treatment of giant cell tumor of bone. Administered via subcutaneous injection, denosumab was approved by the U.S. Food and Drug Administration on June 1, 2010, under the brand name Prolia, offering a targeted therapy to prevent bone loss and related complications.

1. Purpose

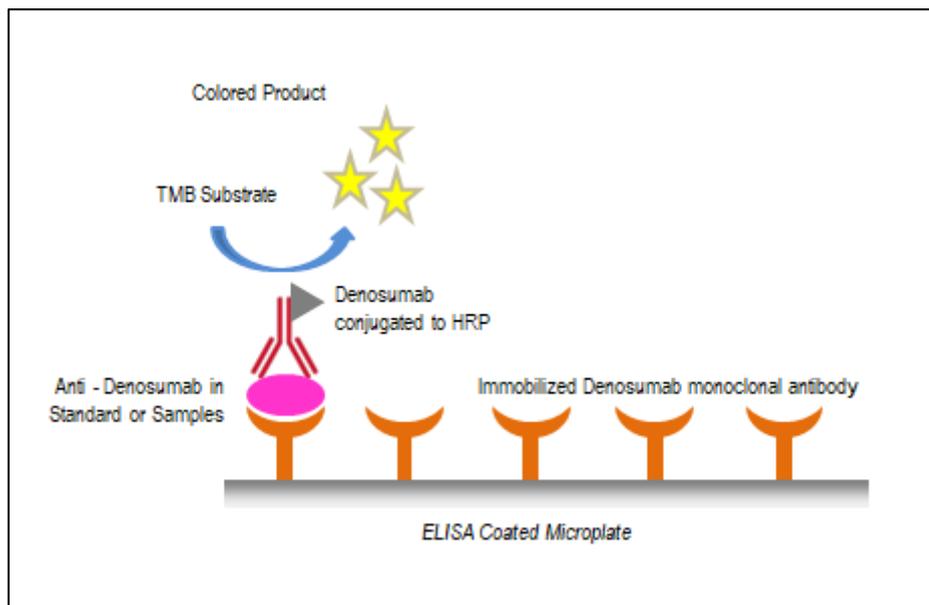
To assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Anti-Denosumab (PROLIA™ / XGEVA™) ELISA developed using Denosumab monoclonal antibody as capture protein.

2. Experimental Design

- A sandwich ELISA was performed using denosumab monoclonal antibody as the capture reagent.
- Standards were prepared using denosumab reference material.
- Assay concentration range: 0–1000 ng/mL.
- Signal (% absorbance) was plotted versus concentration.

The KRIBIOLISA Anti-Denosumab ELISA employs a targeted immobilization strategy to ensure optimal presentation of denosumab on the assay plate, thereby enhancing the selective binding of anti-denosumab antibodies. The immobilization procedure is designed to preserve the native conformation and epitope accessibility of denosumab, maintaining its structural integrity and functional orientation. This controlled presentation ensures that denosumab is displayed in a configuration that supports high-affinity interaction with anti-denosumab binding domains.

Anti-denosumab antibodies demonstrate strong and stable antigen–antibody complex formation under these plate-bound conditions. In contrast, antibodies directed against unrelated therapeutic monoclonal antibodies such as Amivantamab, or other non-specific human IgG molecules, may exhibit reduced or negligible binding. This differential binding behavior reflects the high specificity of anti-denosumab antibodies for denosumab and the preserved structural orientation of the immobilized molecule established during the coating process.



ELISA kits for Anti Denosumab estimation offered by KRISHGEN uses Denosumab monoclonal antibody capture proteins as above

3. Assay Validation

- IC50 Value: ~ 826.2 ng/ml (within 0-1000 ng/mL assay range).
- LLOQ: ~ 58.05 ng/ml.
- Clinical Cmax Values*:
 - After administration of denosumab (60 mg subcutaneous dose): anti-denosumab antibodies, when detected, are generally present at low concentrations (typically in the low ng/mL range).

- After higher-dose administration (120 mg subcutaneous dose): anti-denosumab antibody levels remain low and do not demonstrate dose-dependent increases.
- At steady state (following repeated 6-monthly or monthly dosing): no clinically meaningful accumulation of anti-denosumab antibodies has been reported.

* Values are approximate and based on published clinical immunogenicity data. The incidence of treatment-emergent anti-denosumab antibodies has been reported to be <1%, and detected antibody levels have not been associated with altered pharmacokinetics, safety, or efficacy of denosumab.

* *published data*

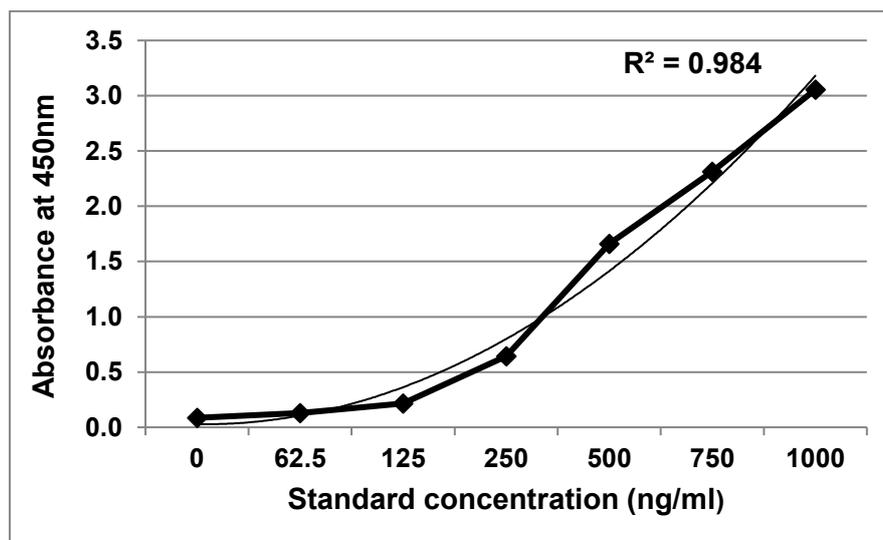
- Precision:

- Intra-Assay CV: <6.10%.
- Inter-Assay CV: <10.10%.
- Inter-Operator CV: <10%.

4. Standard Curve

Below is the standard curve for Anti –Denosumab Sandwich ELISA assay:
Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Recovery
0	0.086	--	--
62.5	0.131	66.5	106.4
125	0.215	107.0	85.6
250	0.643	247.3	98.9
500	1.659	520.8	104.2
750	2.313	719.9	96.0
1000	3.055	1017.1	101.7



5. LOD and LOQ

- LOD Absorbance: (Approx ~45.95 ng/ml)
- LOQ Absorbance: (Approx ~58.05 ng/ml)

6. Pharmacokinetic Relevance

The assay is designed to detect and quantify anti-denosumab antibodies within clinically relevant concentration ranges observed in subjects receiving denosumab therapy, making it suitable for immunogenicity assessment and therapeutic monitoring. The Anti-Denosumab ELISA demonstrates high sensitivity within the ng/mL range, which falls well within the validated assay range, ensuring accurate detection and quantification of low-titer anti-drug antibodies (ADAs).

Published clinical immunogenicity data for denosumab indicate a low incidence of treatment-emergent anti-denosumab antibodies, consistent with fully human monoclonal antibody therapeutics:

- Following therapeutic subcutaneous dosing (60 mg every 6 months or 120 mg monthly), treatment-emergent anti-denosumab antibodies have been detected in <1% of patients, typically at low titers.
- Detected antibody concentrations are generally transient and occur at low levels (commonly within the low ng/mL range).
- No meaningful increase in antibody incidence or titer has been observed with repeated dosing or at steady state.
- No neutralizing anti-denosumab antibodies with clinically significant impact on pharmacokinetics, safety, or efficacy have been consistently reported.

Thus:

- At clinically relevant dosing regimens, anti-denosumab antibody levels—when present—fall within the measurable sensitivity range of the ELISA without requiring extensive dilution.
- The assay working range enables reliable detection of low-level ADA responses and differentiation between negative and positive samples.
- Given the typically low circulating levels of anti-denosumab antibodies, minimal or no dilution of clinical samples is generally sufficient to maintain measurements within the linear dynamic range of the assay.
- The assay is therefore suitable for immunogenicity assessment, ADA monitoring, and supportive clinical evaluation of denosumab therapy in human serum or plasma.

7. Precision and Reproducibility

Precision was assessed by analysing three standard concentrations (62.5 ng/ml, 500 ng/ml, and 1000 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
62.5	4.5% to 10.1%	<10%
500	1.5% to 2.3%	<2.5%
1000	0.1% to 1.2%	<2%

Observations:

- Intra-assay precision was consistently less than 10.1% across all levels tested.
- Inter-assay precision was consistently less than 10%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:

The KRIBIOLISA® Anti - Denosumab (PROLIA™ / XGEVA™) 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® Anti- Denosumab (PROLIA™ / XGEVA™) ELISA is validated for sensitivity, specificity, precision, and accuracy, and is appropriate for pharmacokinetic applications in regulatory settings.

----- *** -----