

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN BioSystems
OUR REAGENTS, YOUR RESEARCH

Background

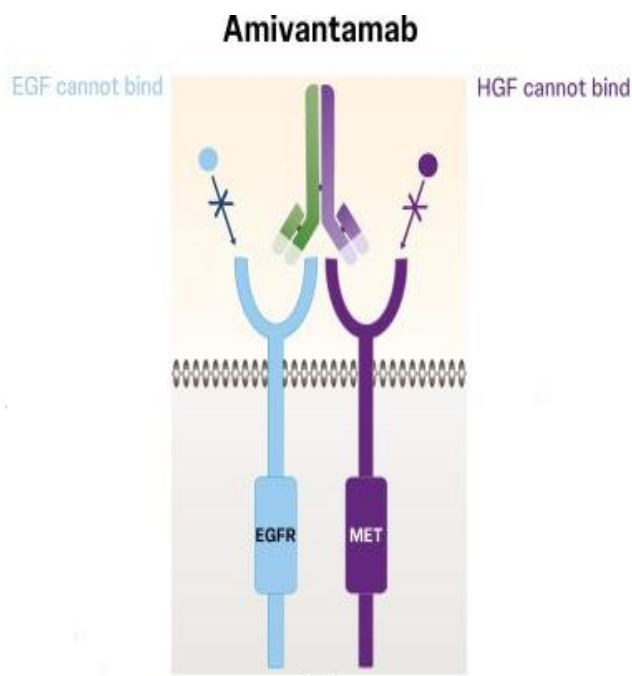
1. Introduction to Amivantamab

Amivantamab is a fully human bispecific monoclonal antibody that targets the epidermal growth factor receptor (EGFR) and mesenchymal-epithelial transition factor (MET). By simultaneously binding to these two receptors, Amivantamab can inhibit tumor growth and survival pathways associated with EGFR and MET signalling. It is particularly relevant in the treatment of certain cancers, including non-small cell lung cancer (NSCLC) with specific EGFR mutations. Consequently, Amivantamab has emerged as an important therapeutic agent and target of interest in modern oncology drug discovery and development programs.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- Amivantamab targets two important receptor pathways involved in tumor growth and progression: the epidermal growth factor receptor (EGFR) and mesenchymal-epithelial transition factor (MET).
- Dysregulation of EGFR and MET signalling is associated with the development and progression of various cancers, particularly certain types of non-small cell lung cancer (NSCLC).
- Simultaneous targeting of EGFR and MET represents an important therapeutic strategy for overcoming tumor growth and resistance mechanisms associated with these signalling pathways.



2.2 Assay Development

- Amivantamab can be evaluated during drug discovery and development using various analytical and functional assays, including:
 - ELISA for the quantification of Amivantamab or the assessment of relevant biomarkers.
 - Cell-based assays for evaluating the biological activity and inhibitory effects of Amivantamab on EGFR- and MET-mediated signalling.
 - Binding assays for assessing the interaction of Amivantamab with its target receptors.
 - Pharmacokinetic and immunogenicity assays for monitoring drug concentration and anti-drug antibody responses.

2.3 Biomarker for Efficacy and Safety

- Amivantamab-related biomarkers may be used to evaluate target engagement, pharmacodynamic activity, and therapeutic response.
- Monitoring EGFR and MET pathway-related biomarkers can support the assessment of treatment efficacy and the identification of potential resistance mechanisms.
- Pharmacokinetic, immunogenicity, and safety assessments are important components of the preclinical and clinical development of Amivantamab.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilar Development

- Amivantamab is a bispecific monoclonal antibody that simultaneously targets the epidermal growth factor receptor (EGFR) and mesenchymal-epithelial transition factor (MET).
- The development and characterization of therapeutic monoclonal antibodies and potential biosimilar products require comprehensive analytical and functional evaluation, including:
 - Binding assays to assess affinity and interaction with EGFR and MET.
 - Cell-based potency assays to evaluate the biological activity and functional effects of the antibody.
 - Physicochemical and biological comparability studies to demonstrate consistency between different product batches or candidate products.
 - Pharmacokinetic and immunogenicity assessments as part of product development and regulatory evaluation.

3.2 PK/PD Studies

- Amivantamab concentrations may be monitored during preclinical and clinical studies to characterize its pharmacokinetic profile.
- Pharmacodynamic assessments may include the evaluation of target engagement and changes in biomarkers associated with EGFR and MET signalling.
- PK/PD studies can help establish the relationship between drug exposure, biological activity, therapeutic response, and safety.

3.3 Safety Evaluation

- The safety of Amivantamab is evaluated throughout preclinical and clinical development using appropriate safety and tolerability assessments.
- Monitoring of treatment-related adverse effects, immunogenicity, and other clinically relevant safety parameters is important during therapeutic development.
- Appropriate analytical methods for the quantification of Amivantamab and the assessment of related biomarkers can support pharmacokinetic, pharmacodynamic, and safety evaluations.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Therapeutic Development and Safety Monitoring

- Amivantamab is not a primary cytokine mediator of cytokine release syndrome (CRS). However, appropriate safety monitoring is important when evaluating its use alone or in combination with other therapeutic approaches.
- The assessment of treatment-related immune responses, adverse events, and other safety parameters is important during preclinical and clinical development.

4.2 Target Engagement and Biomarker Evaluation

- Amivantamab-related studies may be relevant for evaluating EGFR and MET expression and signalling in cellular models used for therapeutic research.
- The assessment of EGFR and MET pathway-related biomarkers can support the evaluation of target engagement, biological activity, and therapeutic response.
- Quantification of Amivantamab may also support pharmacokinetic and pharmacodynamic studies during therapeutic development.

4.3 Genetic and Cellular Engineering Research

- Genetic and cellular engineering approaches may be used to develop experimental models with altered EGFR or MET expression to study the mechanism of action and therapeutic activity of Amivantamab.
- Such models can support the investigation of target dependency, treatment response, and potential resistance mechanisms associated with EGFR and MET signalling.
- These studies may contribute to the development of personalized therapeutic strategies and the identification of patient populations that may benefit from EGFR- and MET-targeted therapies.

Scope of Validation

The KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA Kit (Catalog No. KBI1130) has been considered by us during the validation of this assay in accordance with applicable FDA/ICH guidelines for bioanalytical method validation. This document is prepared based on tests performed in our laboratory and does not necessarily seek to cover all testing that may be required at the user's end for registration or regulatory submissions. The objective of this validation is to demonstrate that the assay is suitable for its intended purpose—the detection and quantification of Amivantamab in appropriate biological samples.

The validation characteristics considered by us are listed below:

- Assay Performance and Specificity.
- Standard Curve and Assay Range.
- Sensitivity, including LOD and LLOQ.
- Precision and Reproducibility.

- Pharmacokinetic Relevance.
- Disease-State Sample Recovery.
- Sample Handling and Storage Conditions.

The degree of revalidation required depends on the nature and extent of any changes made to the assay, reagents, procedure, or intended use. Certain changes may require additional validation.

Please note that this validation is performed in our laboratory and may not necessarily be duplicated in the user's laboratory. The data has been generated to provide the user with an overview of the assay performance characteristics and is generic in nature. We recommend that users perform, at a minimum, appropriate spike and recovery studies to confirm assay performance in their specific sample matrix and laboratory conditions. For more comprehensive validation, users may perform additional studies as appropriate to establish quality control parameters for their intended application.

For any queries or support regarding the data and assay performance, please contact us at sales1@krishgen.com.

Intended Use of the ELISA

The KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA Kit is intended for the quantitative measurement of Amivantamab in serum, plasma, and other suitable biological samples for research and bioanalytical applications.

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. Amivantamab Pharmacokinetic Relevance and Recommended ELISA Range

The following section summarizes the pharmacokinetic relevance of Amivantamab and the recommended working range of the KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA Kit.

Application	Expected Amivantamab Concentration	Recommended ELISA Working Range
Bioanalytical and Pharmacokinetic Studies	Concentrations may vary depending on dose, body weight, dosing schedule, and individual patient variability	0–500 ng/ml
Initial Therapeutic Exposure	Approximately 80–180 µg/ml following recommended intravenous dosing	Appropriate sample dilution recommended before analysis
Steady-State Exposure	Approximately 250–320 µg/ml following repeated dosing	Appropriate sample dilution recommended before analysis
Therapeutic Monitoring and Exposure Assessment	Variable depending on clinical dosing conditions	Samples should be diluted to fall within the validated assay range

Note: The KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA Kit has an assay concentration range of 0–500 ng/ml. Due to the relatively high circulating concentrations of Amivantamab following therapeutic administration, clinical serum or plasma samples may require appropriate dilution before analysis to ensure that the measured concentration falls within the linear dynamic range of the assay.

The assay demonstrates measurable sensitivity within the working range of approximately 3.9–500 ng/ml, with an approximate LLOQ of 3.07 ng/ml. The assay is therefore suitable for pharmacokinetic profiling, dose–exposure analysis, and bioanalytical studies following appropriate sample dilution.

2. Specificity and Selectivity

2.1 Specificity

The KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA employs a targeted ligand-binding approach using **Human EGFR as the capture protein** to facilitate the selective detection of Amivantamab.

Amivantamab is a bispecific monoclonal antibody that targets the epidermal growth factor receptor (EGFR) and mesenchymal–epithelial transition factor (MET). The assay is designed to support selective interaction between Amivantamab and the immobilized target protein.

2.2 Selectivity

The ELISA shows no or low cross-reactivity with other EGFR/MET-targeted therapeutic antibodies, such as cetuximab and panitumumab.

2.3 LOD, LOQ and IC₅₀

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 σ criterion is most common).

The approximate LOD for the KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA is 1.01 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 σ criterion is most common).

The approximate LOQ for the KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA is 3.07 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.

For the KRIBIOLISA® Amivantamab (RYBREVANT™) ELISA validation, the observed IC₅₀ value is 99.2 ng/ml

Summary:

Parameter	Value (ng/ml)
LOD	~1.01 ng/ml
LOQ	~3.07 ng/ml
IC ₅₀	~99.2 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 (ng/ml)	% Recovery
0	0.154	---	---
3.9	0.287	4.6	116.9
7.8	0.376	8.5	109.5
15.6	0.556	17.2	110.0
31.25	0.780	29.6	94.9
62.5	1.206	61.2	97.9
125	1.682	121.6	97.2
250	2.225	279.8	111.9

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
500	2.447	458.6	91.7
Low QC (7.8 ng/ml)	0.379	8.7	111.2
Mid QC (125 ng/ml)	1.652	120.4	96.3
High QC (500 ng/ml)	2.483	512.8	102.6

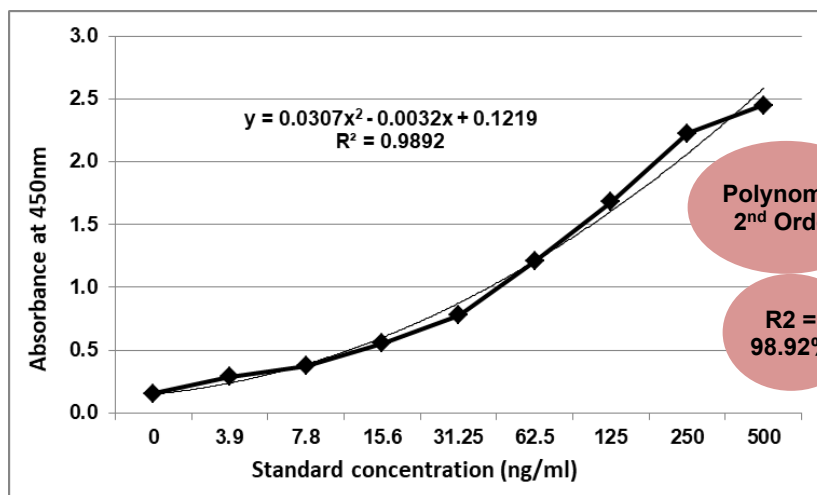
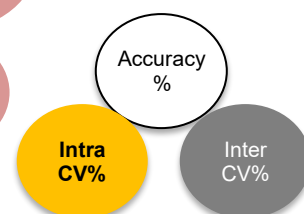
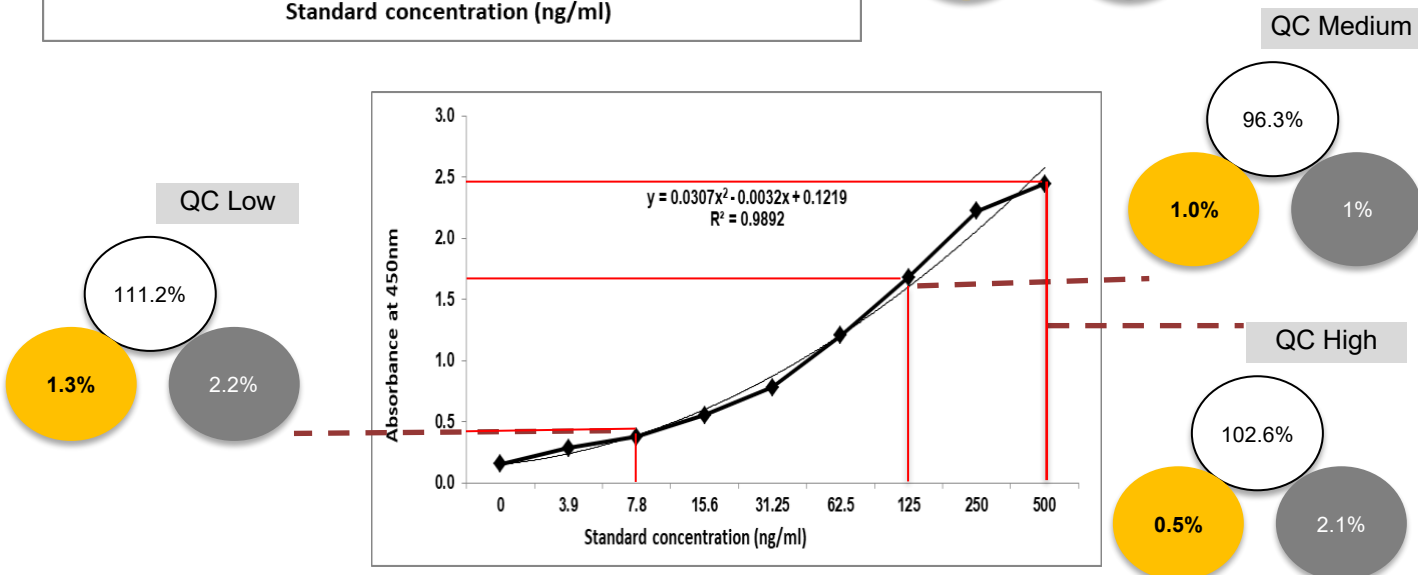


Figure 1. Standard Curve Accuracy — % Recovery Across the Assay Range

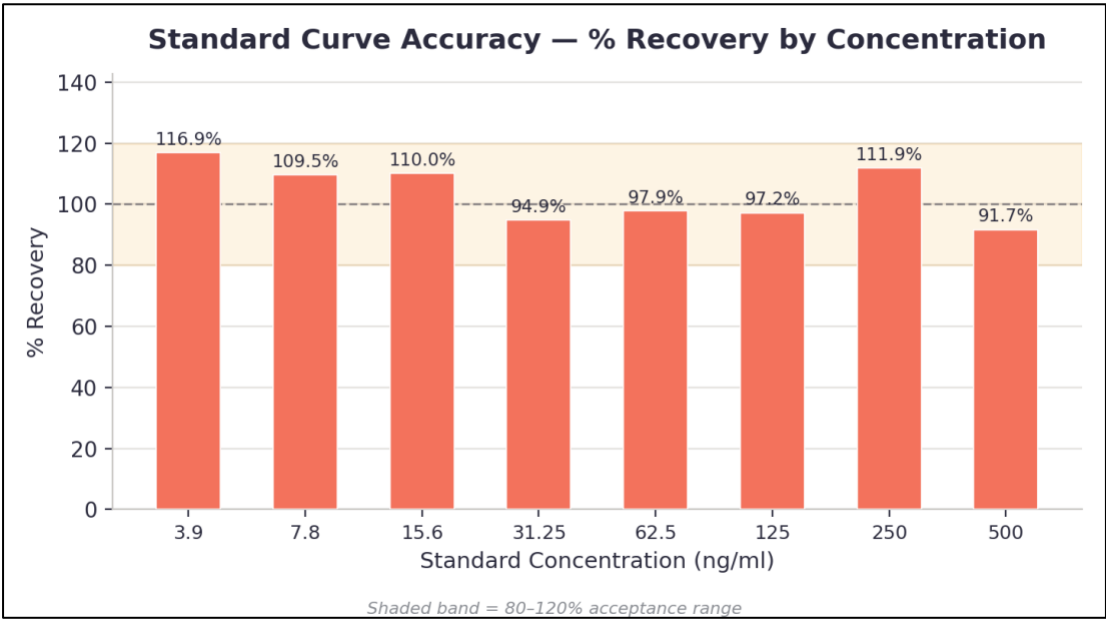
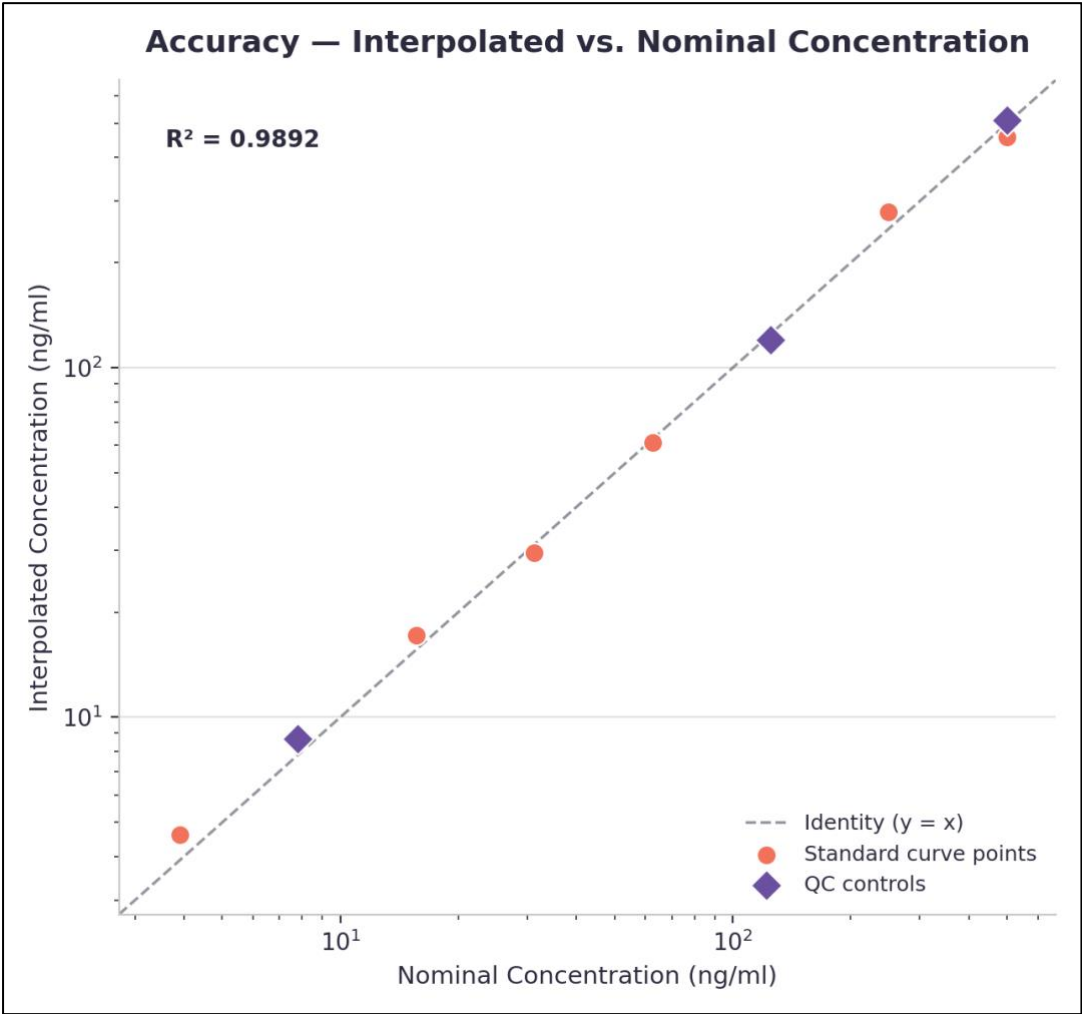


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

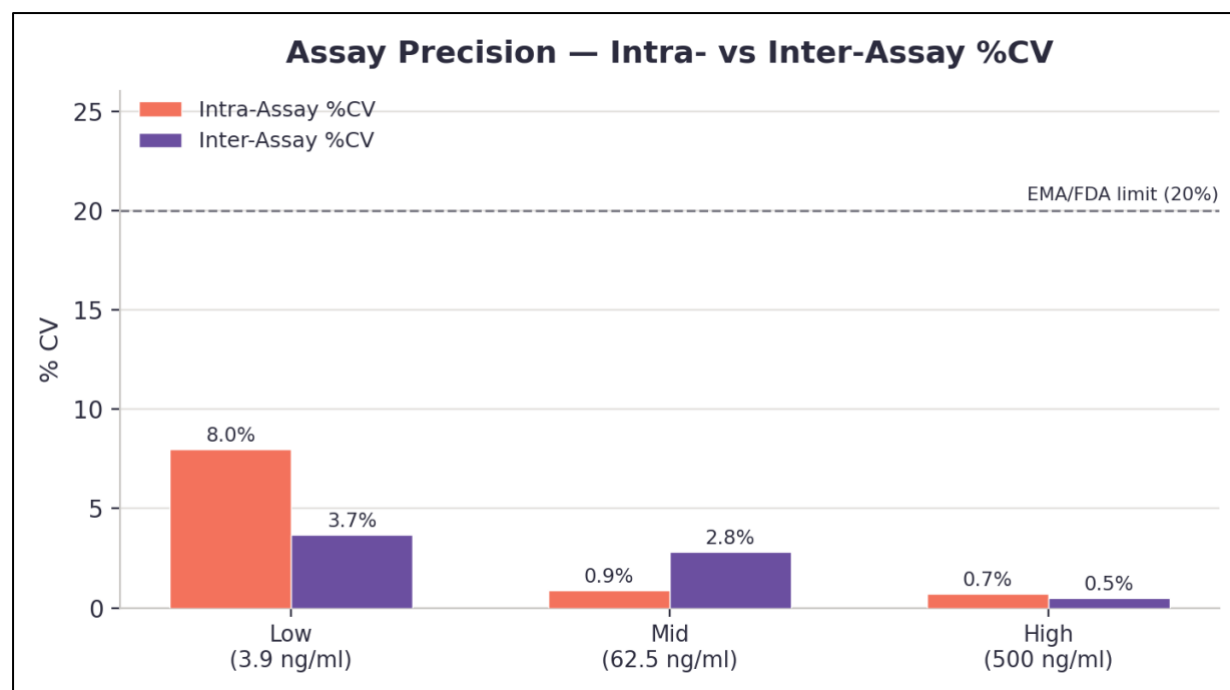
A) Intra-Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
3.9	0.263	0.02	8.0
62.5	1.123	0.01	0.9
500	2.431	0.02	0.7

B) Inter Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
3.9	0.274	0.01	3.7
62.5	1.157	0.03	2.8
500	2.439	0.01	0.5

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



4. Parallelism and Matrix Effect

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

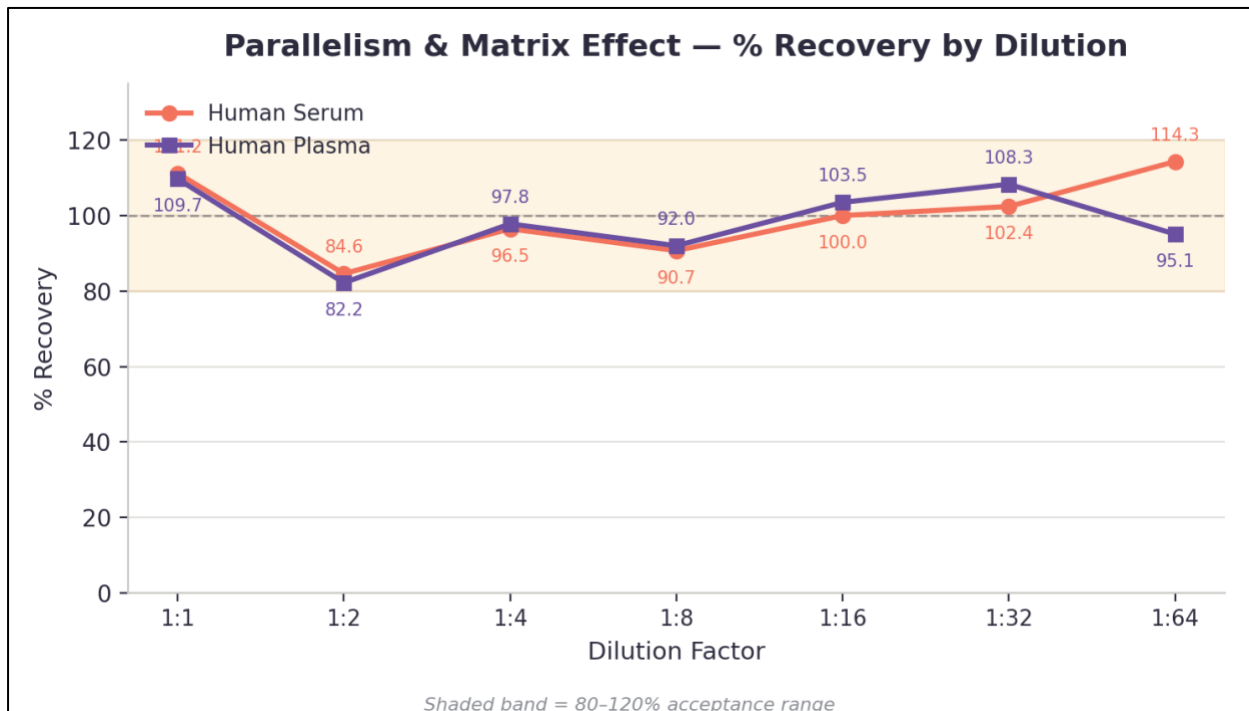
A) Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	250	2.215	277.9	111.2	101.8
1:2	125	1.589	105.8	84.6	92.6
1:4	62.5	1.195	60.3	96.5	93.0
1:8	31.25	0.758	28.3	90.7	97.1
1:16	15.6	0.524	15.6	100.0	97.8
1:32	7.8	0.364	8.0	102.4	93.4
1:64	3.9	0.285	4.5	114.3	111.0

B) Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	250	2.208	274.4	109.7	95.5
1:2	125	1.568	102.7	82.2	92.4
1:4	62.5	1.204	61.1	97.8	99.7
1:8	31.25	0.765	28.8	92.0	102.9
1:16	15.6	0.535	16.1	103.5	101.5
1:32	7.8	0.374	8.4	108.3	105.3
1:64	3.9	0.268	3.7	95.1	99.2

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



Results:

- i) Parallelism is maintained across the 1:1 to 1:64 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® Amivantamab (RYBREVANT™) ELISA Kit was tested for matrix effect on human serum and plasma samples.

5. Disease State Sample Recovery.

Sample Details:

Diseased-state sample validation was performed using four clinically relevant human plasma samples from patients with Non-Small Cell Lung Cancer (NSCLC). The samples included both male and female subjects, with ages ranging from 56 to 68 years, and all samples were of Hispanic demographic. All samples were reported as non-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 Ethnicity Age/Sex	Diabetic/ Smoker	Pathogen	Medication	Blood Type/ BMI	Anticoagulant
PL1022 19N	Lung Cancer Human Plasma (NSCLC)	Hispanic 56/Male	No/No	Negative	Nil	Nil	K2 EDTA
PL1022 19P	Lung Cancer Human Plasma (NSCLC)	Hispanic 68/Female	No/No	Negative	Nil	Nil	K2 EDTA
PL1022 19Q	Lung Cancer Human Plasma (NSCLC)	Hispanic 57/ Female	No/No	Negative	Nil	Nil	K2 EDTA
PL1022 19R	Lung Cancer Human Plasma (NSCLC)	Hispanic 63/ Male	No/No	Negative	Nil	Nil	K2 EDTA

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 Amivantamab Recovery

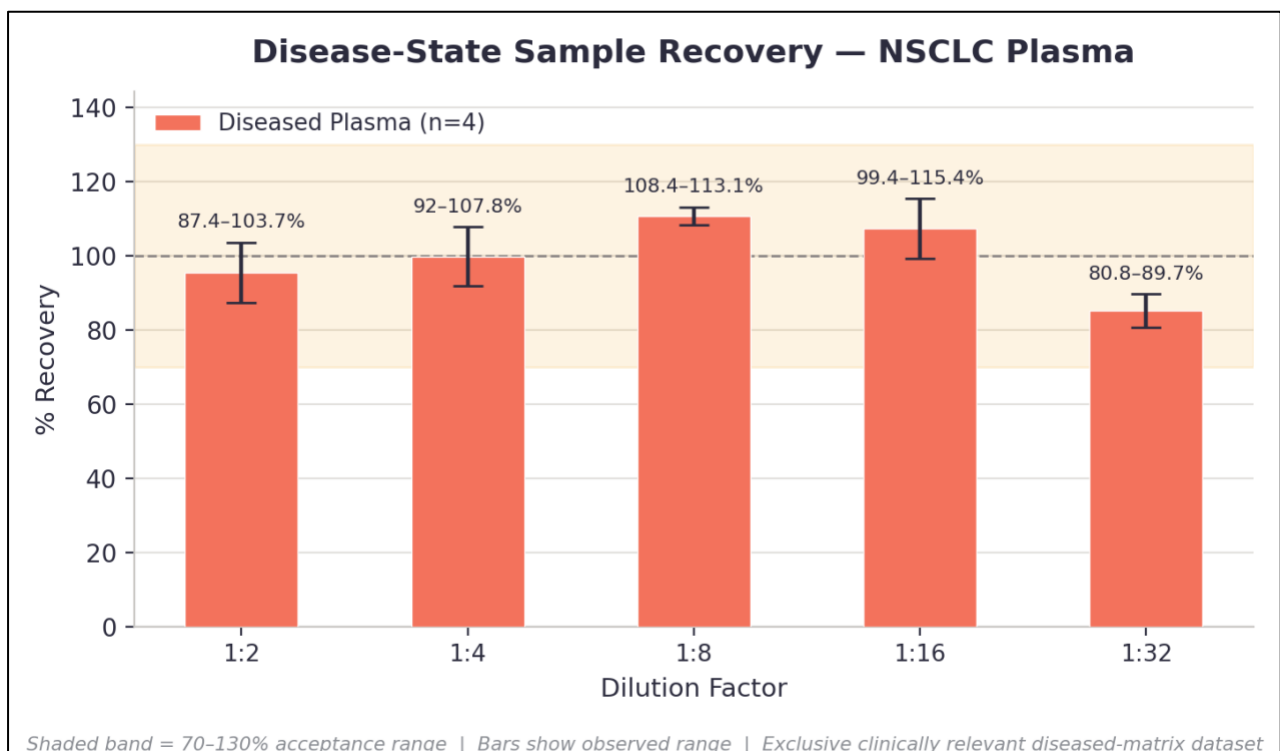
Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Plasma (n=4)	87.4 - 103.7	92 - 107.8	108.4 - 113.1	99.4 - 115.4	80.8 - 89.7

Observations:

- Recovery across all dilution levels falls within the acceptable range (70–130%), indicating minimal matrix interference in diseased plasma samples.
- Consistent recovery is observed from 1:2 to 1:16 dilutions, demonstrating robustness of the assay across a broad dilution range.
- Slight reduction in recovery at 1:32 dilution is noted; however, values remain within acceptable limits.
- The data indicate reliable and reproducible analyte recovery in diseased plasma matrices without the need for extensive sample pre-treatment.

The assay is therefore suitable for accurate quantification of Amivantamab in diseased plasma samples across clinically relevant conditions.

Figure 5. Disease-State Sample Recovery — Diseased Plasma (NSCLC, n=4)



6. 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.

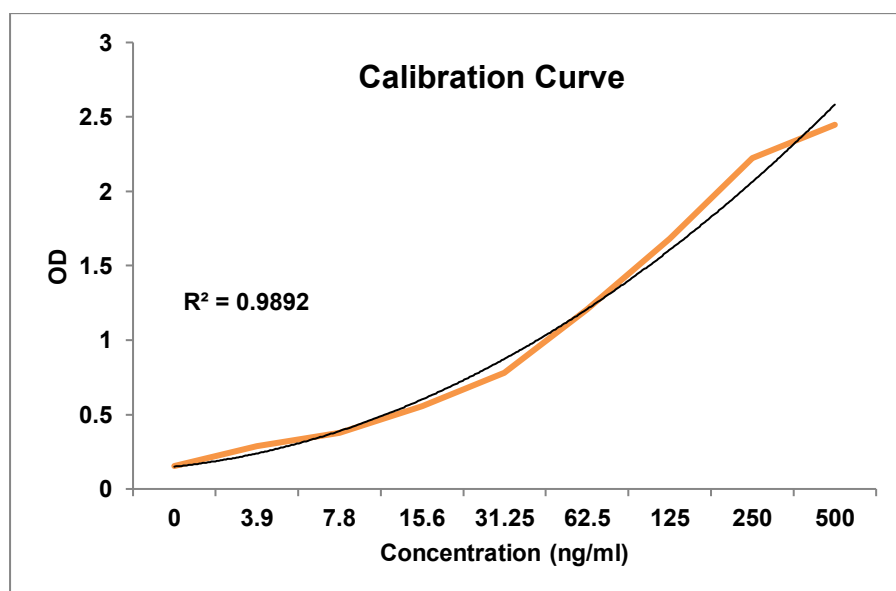
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at $1000 \times g$ within 30 minutes of collection. Assay immediately or store plasma samples at temperature $<-20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

B.) Storage conditions:

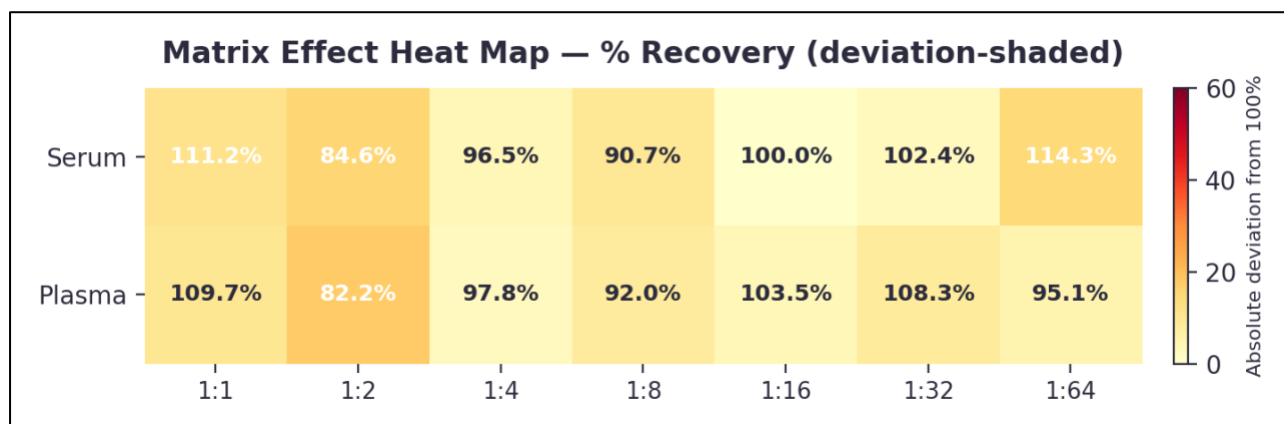
Store main kit components at $2-8^{\circ}\text{C}$.

Store recombinant lyophilized standard at $2-8^{\circ}\text{C}$. Upon reconstitution aliquot standards into polypropylene vials and store at -20°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	1:64
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%	H (null hypothesis) = 100±25 %
Parallelism/Linearity	CV < 30%	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%

References

Park, K., Haura, E. B., Leighl, N. B., et al. (2021). Amivantamab in EGFR Exon 20 Insertion–Mutated Non–Small-Cell Lung Cancer Progressing on Platinum Chemotherapy: Initial Results from the CHRYSALIS Phase I Trial. *Journal of Clinical Oncology*, 39(30), 3391–3402.

Rybrevant™ (Amivantamab-vmjw) Prescribing Information. Janssen Biotech, Inc. Available product information and prescribing information for Amivantamab.

U.S. Food and Drug Administration (FDA). (2022). *Bioanalytical Method Validation: Guidance for Industry*.

U.S. Food and Drug Administration (FDA). (2014). *Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products*.

International Council for Harmonisation (ICH). *ICH M10: Bioanalytical Method Validation and Study Sample Analysis*.

International Council for Harmonisation (ICH). *ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology*.

European Medicines Agency (EMA). *Guideline on the Development, Production, Characterisation and Specifications for Monoclonal Antibodies and Related Products*.

European Medicines Agency (EMA). *Guideline on Immunogenicity Assessment of Therapeutic Proteins*.