

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA
APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

Background

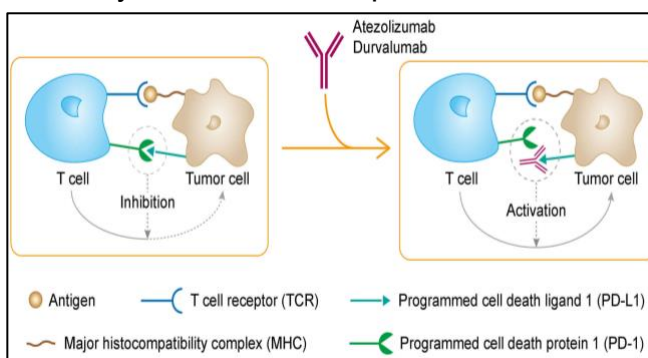
1. Introduction to Atezolizumab (TECENTRIQ™)

Atezolizumab is a fully humanized monoclonal IgG1 antibody that selectively binds to programmed death-ligand 1 (PD-L1), a key immune checkpoint regulator expressed on tumor cells and tumor-infiltrating immune cells. By inhibiting the interaction of PD-L1 with its receptors PD-1 and B7.1, Atezolizumab reactivates suppressed cytotoxic T-cell responses, enabling the immune system to recognize and eliminate cancer cells more effectively. It is clinically utilized in cancer immunotherapy, particularly for the treatment of multiple solid tumors such as non-small cell lung cancer (NSCLC), urothelial carcinoma, triple-negative breast cancer, hepatocellular carcinoma, and other PD-L1–expressing malignancies.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- Atezolizumab targets programmed death-ligand 1 (PD-L1), an immune checkpoint ligand involved in the regulation of T-cell activity and immune responses.
- PD-L1 interacts with PD-1 on T cells and B7.1 (CD80) on immune cells, contributing to inhibition of T-cell activation and allowing tumor cells to evade immune surveillance.
- Blocking the PD-L1/PD-1 signaling pathway represents an important therapeutic strategy for restoring T-cell activity and enhancing antitumor immune responses.



2.2 Assay Development

- Atezolizumab can be evaluated during drug discovery and development using various analytical and functional assays, including:
 - ELISA for the quantification of Atezolizumab in biological samples or for the assessment of relevant biomarkers.
 - Cell-based assays for evaluating the biological activity of Atezolizumab and its ability to block PD-L1-mediated immune checkpoint signaling.
 - Binding assays for assessing the interaction of Atezolizumab with the PD-L1 target.
 - Pharmacokinetic and immunogenicity assays for monitoring drug concentration, exposure, and potential anti-drug antibody responses.

2.3 Biomarkers for Efficacy and Safety

- Atezolizumab-related biomarkers may be used to evaluate PD-L1 target engagement, pharmacodynamic activity, and therapeutic response.
- Evaluation of PD-L1 expression and immune-cell activity may support the assessment of treatment response and immune activation.

- Pharmacokinetic, immunogenicity, and safety assessments are important components of the preclinical and clinical development of Atezolizumab.
- Monitoring immune-related effects and treatment-associated adverse events is important when evaluating the overall safety profile of Atezolizumab.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilar Development

- Atezolizumab is a humanized monoclonal antibody that targets programmed death-ligand 1 (PD-L1), an immune checkpoint ligand involved in the regulation of T-cell activity.
- The development and characterization of therapeutic monoclonal antibodies and potential biosimilar products require comprehensive analytical, physicochemical, and functional evaluation, including:
 - Binding assays to assess affinity and interaction with the PD-L1 target.
 - Cell-based potency assays to evaluate the biological activity of the antibody and its ability to block CTLA-4-mediated immune checkpoint signalling.
 - 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

- Atezolizumab concentrations may be monitored during preclinical and clinical studies to characterize its pharmacokinetic profile.
- Pharmacodynamic assessments may include evaluation of PD-L1 target engagement and changes in biomarkers associated with PD-L1/PD-1 pathway modulation and immune activation.
- PK/PD studies can help establish the relationship between drug exposure, biological activity, therapeutic response, and safety.

3.3 Safety Evaluation

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

4. Importance in Cell and Gene Therapy (CGT)

4.1 Therapeutic Development and Safety Monitoring

- Atezolizumab is an immune checkpoint inhibitor targeting PD-L1 and is not a primary cytokine mediator of cytokine release syndrome (CRS).

- However, appropriate safety monitoring is important when evaluating Atezolizumab in combination with cellular or gene-based therapeutic approaches.
- The assessment of treatment-related immune responses, T-cell activation, adverse events, and other relevant safety parameters is important during preclinical and clinical development.

4.2 Target Engagement and Biomarker Evaluation

- Atezolizumab-related studies may be relevant for evaluating PD-L1 expression and PD-L1/PD-1 pathway activity in cellular models used for therapeutic research.
- Assessment of PD-L1 expression and downstream immune-cell activity may support the evaluation of target engagement, biological activity, and therapeutic response.
- Quantification of Atezolizumab 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 PD-L1 expression or related immune checkpoint pathway activity to study the mechanism of action and biological activity of Atezolizumab.
- Such models can support the investigation of immune checkpoint regulation, T-cell activation, treatment response, and potential mechanisms of therapeutic resistance.
- Cellular models may also be used to investigate the effects of PD-L1 pathway modulation on immune-cell activation and antitumor immune responses.
- These studies may contribute to the development of personalized therapeutic strategies and identification of patient populations that may benefit from PD-L1-targeted immunotherapies.

Scope of Validation

This document presents a discussion of the characteristics of our KRIBIOLISA® Atezolizumab ELISA KIT (CATALOG NO. KBI1027) 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 Atezolizumab.

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.
- Diseased-State Sample Recovery
- Matrix Effect (serum, plasma).

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 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

To assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Atezolizumab ELISA KIT designed specifically to measure free, active Atezolizumab molecules with high sensitivity and specificity, enabling accurate monitoring of immune checkpoint inhibition status. This assay supports clinical research, therapeutic drug monitoring (TDM), dose optimization, and assessment of treatment efficacy in oncology settings.

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. Atezolizumab Values and Recommended ELISA Range

This table summarizes Atezolizumab levels across different therapies and suggested corresponding ELISA working ranges.

Application	Expected Atezolizumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Post low-dose administration (early-phase immunotherapy dosing)	5–25	0–50

Application	Expected Atezolizumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Standard therapeutic dose (routine clinical treatment cycles)	30–100	0–150
High-dose or combination therapy regimens (oncology escalation protocols)	90–250	0–300
Pharmacokinetic monitoring / drug exposure-response evaluation	150–400	0–500

Note: Assay sensitivity <5 ng/mL is recommended for baseline Atezolizumab detection; an upper quantification limit of ≥250 ng/mL is advised for therapeutic monitoring and high-exposure assessment in oncology settings.

The KRIBIOLISA® Atezolizumab ELISA kit is developed using an assay range of 0 - 500 ng/ml with the dilutional linearity accuracy to measure responses as per the application table above on patient C_{max} values.

2. Specificity and Selectivity

2.1 Specificity

The capture and detection antibodies utilized in the Atezolizumab ELISA are monoclonal antibodies that specifically recognize the intact humanized IgG1 anti-PD-L1 therapeutic antibody without cross-reacting with endogenous human immunoglobulins. These assay antibodies are designed to target unique idiotype or Fc/hinge-region epitopes of Atezolizumab, ensuring high affinity binding to the drug molecule in its native conformation.

2.2 Selectivity

The ELISA shows no or low cross-reactivity with other immune checkpoint inhibitors, such as nivolumab and pembrolizumab.

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

(3σ criterion is most common).

LOD for KRIBIOLISA® Atezolizumab ELISA = 2.13 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® Atezolizumab ELISA – 6.46 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® Atezolizumab ELISA = ~103.6 ng/ml

Summary:

Parameter	Value (ng/ml)
LOD	2.13 ng/ml
LOQ	6.46 ng/ml
IC ₅₀	103.6 ng/ml



Regulatory Note:

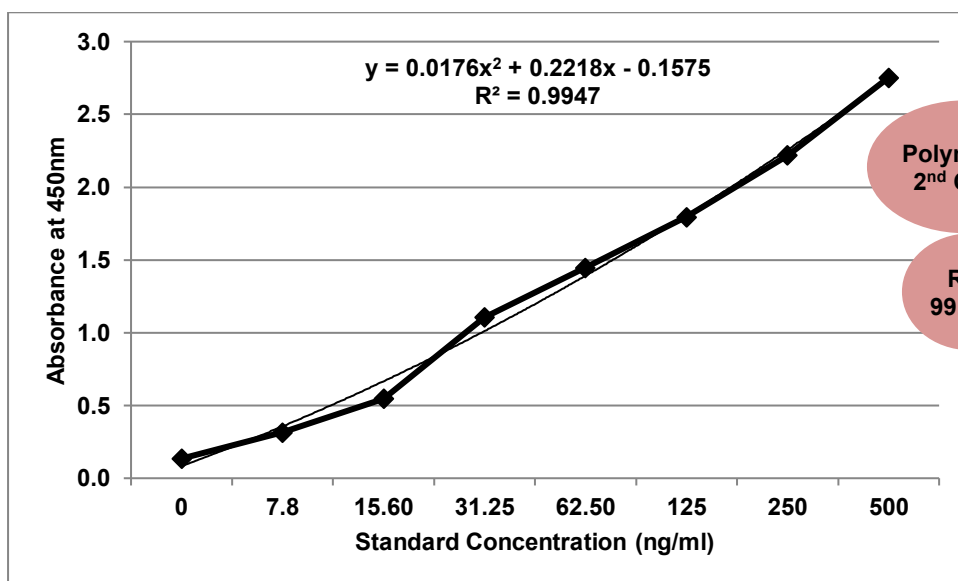
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20%

*S/N = Signal / Noise Ratio

3. Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
0	0.135	--	--
7.8	0.314	6.3	80.8
15.60	0.547	12.8	82.1
31.25	1.107	37.7	120.6
62.50	1.447	69.2	110.7
125	1.795	114.5	91.6
250	2.219	215.1	86.0
500	2.753	584.1	116.8

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
Low Control (7.8 ng/ml)	0.304	7.2	92.3
Mid QC Control (125 ng/ml)	1.785	124.2	99.3
High QC Control (500 ng/ml)	2.684	498.5	99.7



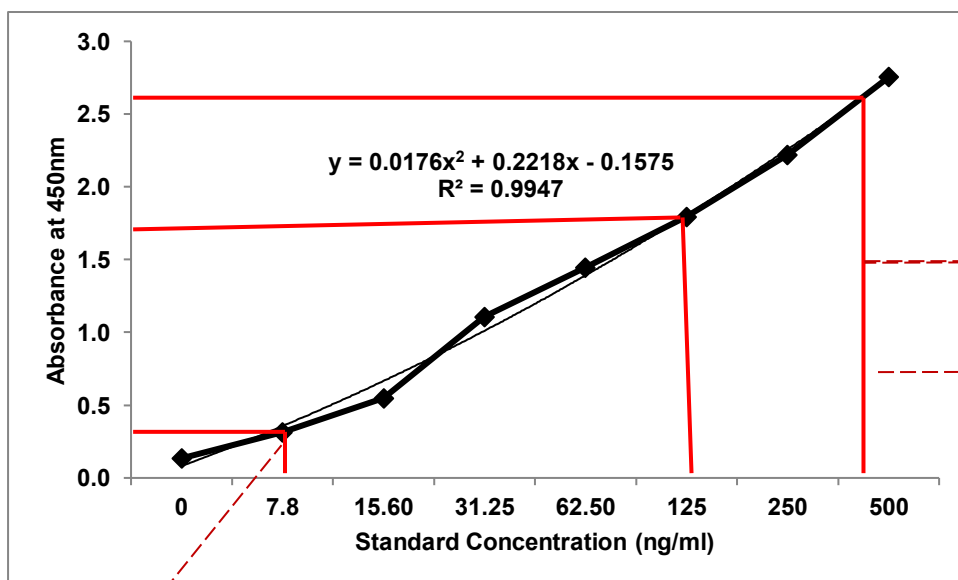
Polynomial
2nd Order

$R^2 =$
99.47%

Accuracy
%

Intra
CV%

Inter
CV%



QC High

99.7%

2.2% to
4.7%

<4%

QC Mid

99.3%

0.6% to
2%

<2.5%

QC Low

92.3%

0.5% to
2.1%

<3.5%

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

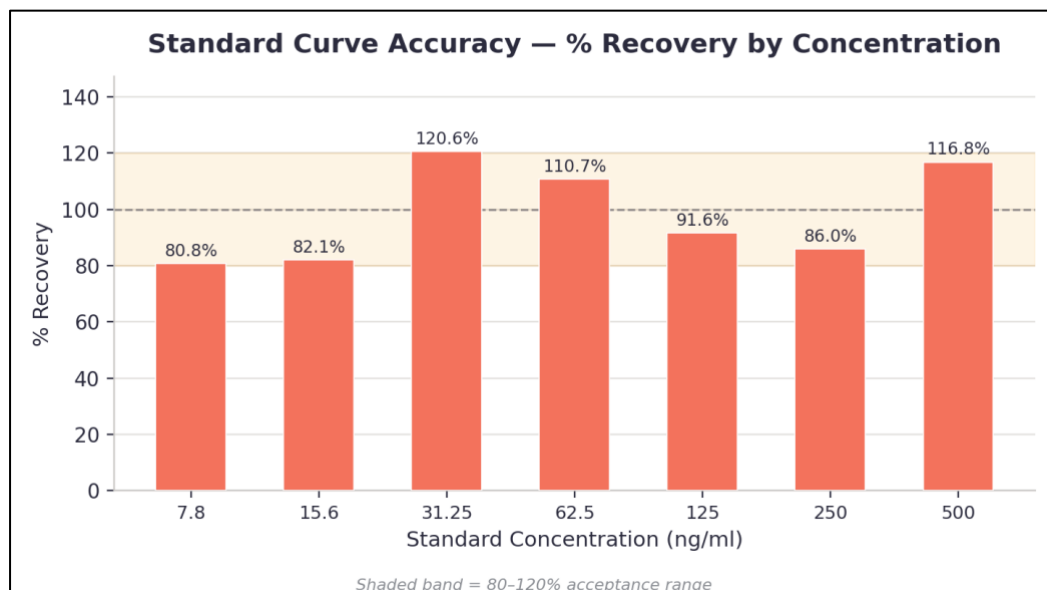
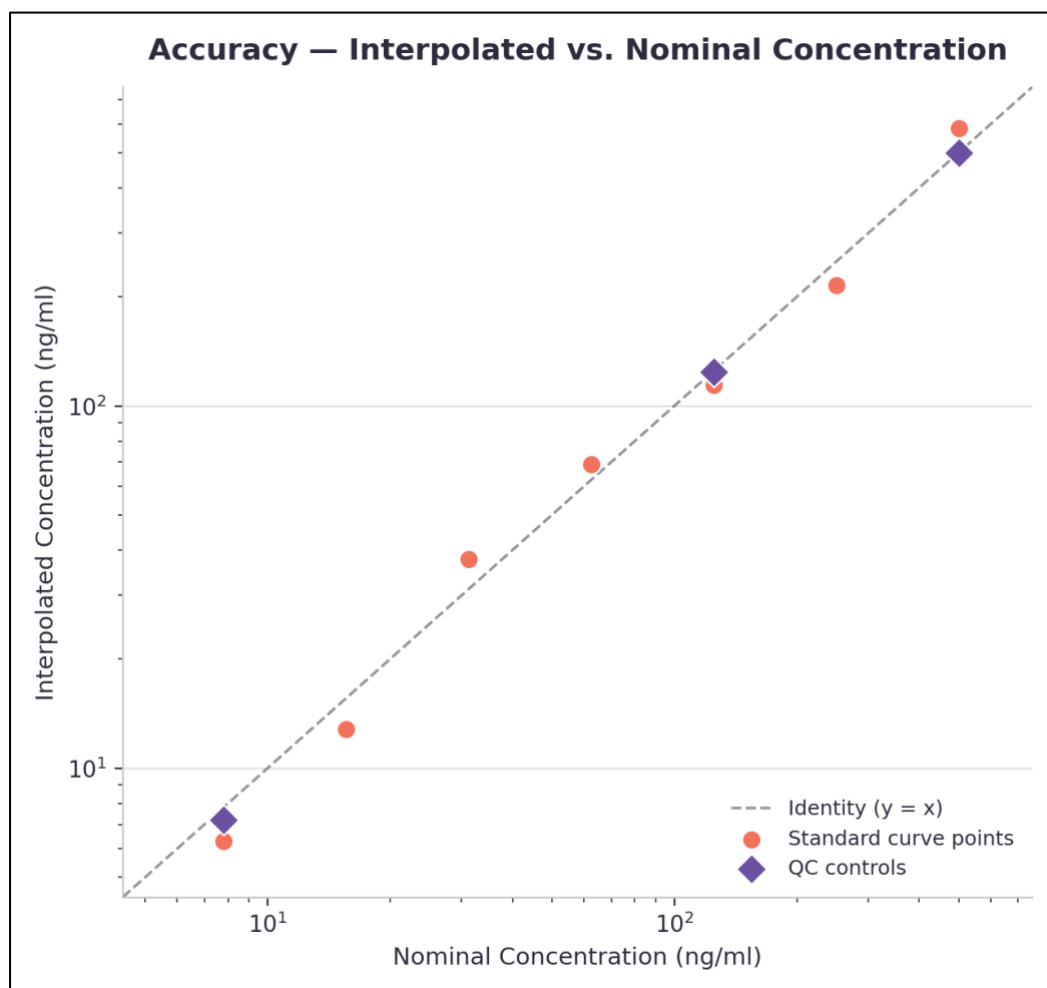


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

Precision was assessed by analyzing three standard concentrations (7.8 ng/ml, 62.5 ng/ml, and 500 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 $\leq 15\%$ for QC samples.
- Inter-assay %CV should be $\leq 15\%$ for QC 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
7.8	0.5% to 2.1%	<6%
62.5	1.9% to 2.6%	<5.5%
500	0.6% to 3.5%	<5%

Observations:

- Intra-assay precision was consistently less than 7% across all levels tested.
- Inter-assay precision was consistently less than 7%.
- All precision values met the acceptance criteria for ELISA validation.

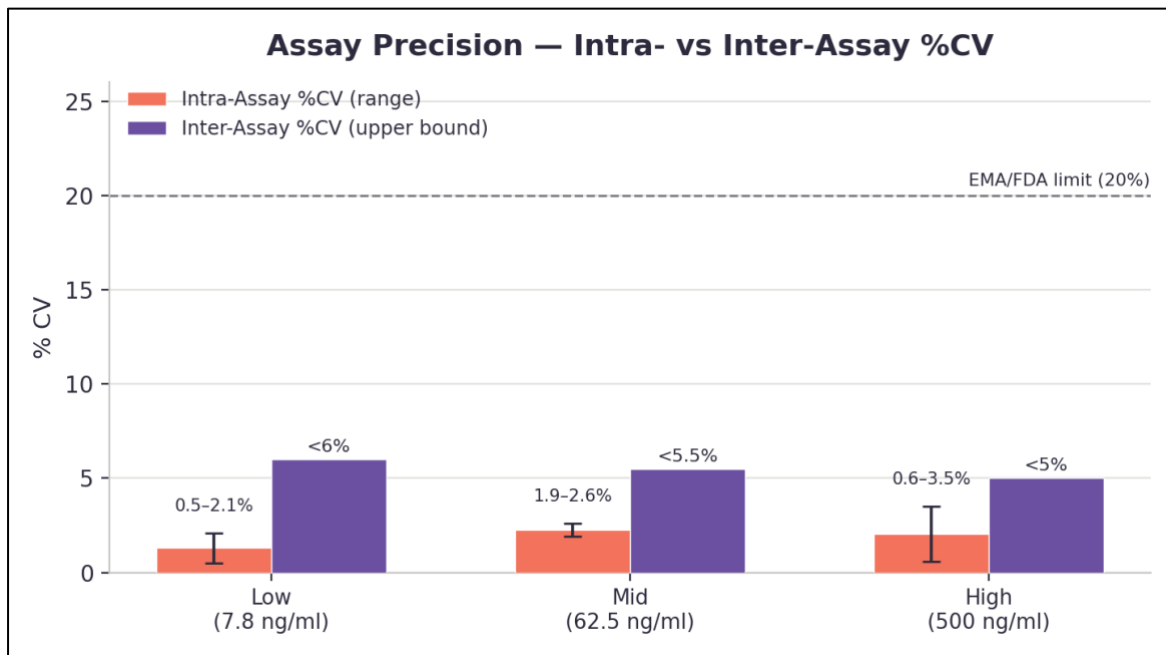
Conclusion:

The KRIBIOLISA® Atezolizumab 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.

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



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® Atezolizumab ELISA and compared to the standard curve.

Acceptance Criteria:

- The back-calculated concentration (interpolated) should fall within $\pm 20\%$ of the expected concentration across the tested range.
- % Recovery should be between 80% and 120% for most samples.

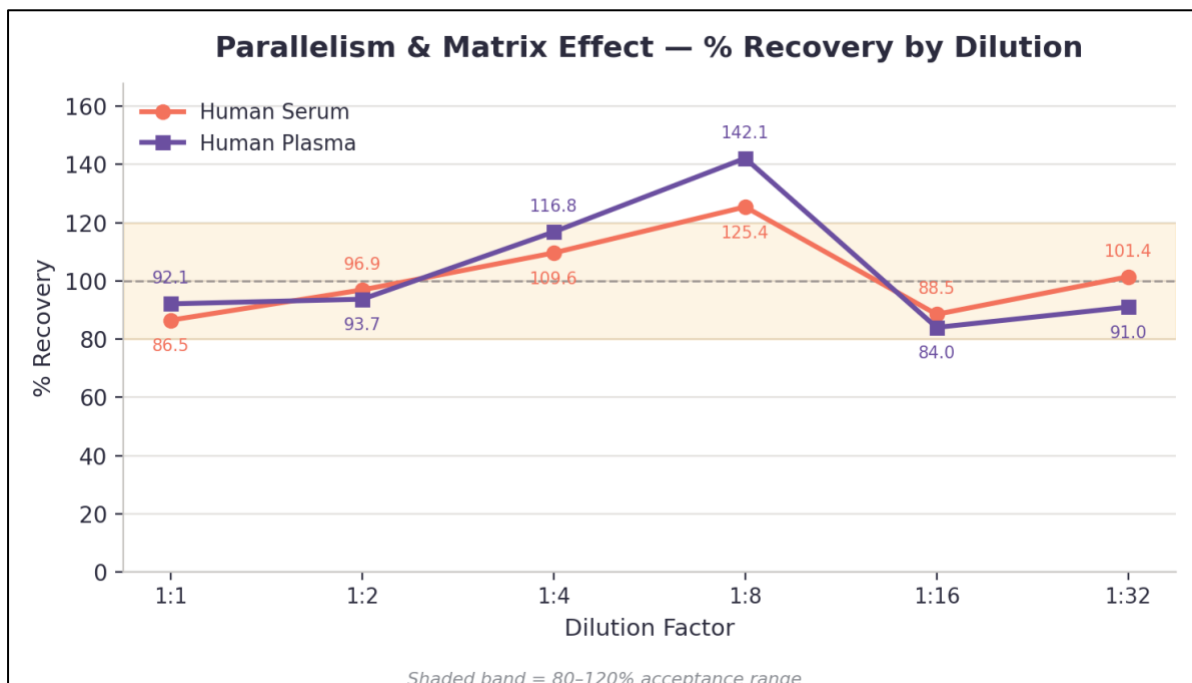
A) Human Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	250	2.210	216.2	86.5	115.6
1:2	125	1.834	121.1	96.9	103.2
1:4	62.5	1.440	68.5	109.6	91.2
1:8	31.25	1.084	39.2	125.4	79.7
1:16	15.6	0.523	13.8	88.5	113.2
1:32	7.8	0.331	7.9	101.4	98.8

B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	250	2.099	230.2	92.1	108.6
1:2	125	1.614	117.1	93.7	106.7
1:4	62.5	1.295	73.0	116.8	85.6
1:8	31.25	1.001	44.4	142.1	70.4
1:16	15.6	0.451	13.1	84.0	119.3
1:32	7.8	0.300	7.1	91.0	110.0

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



Results:

- Parallelism is generally maintained across the 1:1 to 1:32 dilutions.
- % Recovery for most dilutions falls within the acceptable range of 80–120%.
- No significant matrix effect observed at higher dilutions.
- The KRIBIOLISA® Atezolizumab ELISA kit was tested for matrix effect on human serum and human plasma 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 Atezolizumab 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 + 1:100 human serum)
- Assay buffer spiked with human serum (buffer + 1:100 human plasma)

Samples were tested across the standard curve range (0–100 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 + 1:100 Human Serum)	% Standard Deviation	% CV
0	0.112	0.135	1.60	13.0
7.8	0.258	0.324	4.66	16.0
15.60	0.432	0.532	7.07	14.7
31.25	1.089	1.107	1.24	1.1
62.50	1.412	1.447	2.44	1.7
125	1.687	1.795	7.60	4.4
250	2.119	2.219	7.07	3.3
500	2.859	2.753	7.50	2.7

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:100 Human Plasma)	% Standard Deviation	% CV
0	0.155	0.189	2.40	14.0
0.781	0.578	0.658	5.65	9.1
1.560	0.733	0.898	11.64	14.3
3.125	1.120	1.229	7.74	6.6
6.25	1.475	1.612	9.70	6.3
12.5	1.853	2.008	10.97	5.7
25	2.138	2.298	11.35	5.1
50	2.295	2.512	15.37	6.4
100	2.430	2.698	18.98	7.4

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.

Conclusion:

The KRIBIOLISA® Atezolizumab 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 analysing biological samples.

7. Disease State Sample Recovery

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

Sample Details:

Diseased-state sample validation was performed using four clinically relevant human serum/plasma samples representing different disease conditions. The sample panel included three breast cancer serum samples (Stage II–IV) and one non-small cell lung cancer (NSCLC) plasma sample. The samples represented both male and female subjects, with ages ranging from 45 to 56 years, and included diabetic and non-diabetic individuals. All samples were confirmed negative for the tested pathogen, minimizing potential pathogen-related interference. This sample panel was used to evaluate the assay performance and suitability in clinically relevant diseased matrices.

Sample ID	Disease Type	Patient details Ethnicity Age/Sex	Diabetic/ Smoker	Pathogen	Medi- cation	Blood Type/ BMI	Anticoa- gulant
PL10221 9N	Lung Cancer Human Plasma (NSCLC)	Hispanic 56 /Male	No/No	Negative	Nil	Nil	K2 EDTA
CS- BRS-001	Breast Cancer Stage 4 Human Serum.	Caucasian 55 /Female	Yes /Not reported	Negative	Nil	Nil /31.6	Nil
CS- BRS-005	Breast Cancer Human Serum.	Caucasian 45 /Female	No/No	Negative	Nil	Nil /31.6	Nil

Sample ID	Disease Type	Patient details Ethnicity Age/Sex	Diabetic/ Smoker	Pathogen	Medi- cation	Blood Type/ BMI	Anticoa- gulant
CS-BRS-007	Breast Cancer, STAGE II A, Human Serum.	Caucasian 55 /Female	Yes /Not reported	Negative	Nil	Nil /40.2	Nil

Acceptance Criteria:

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

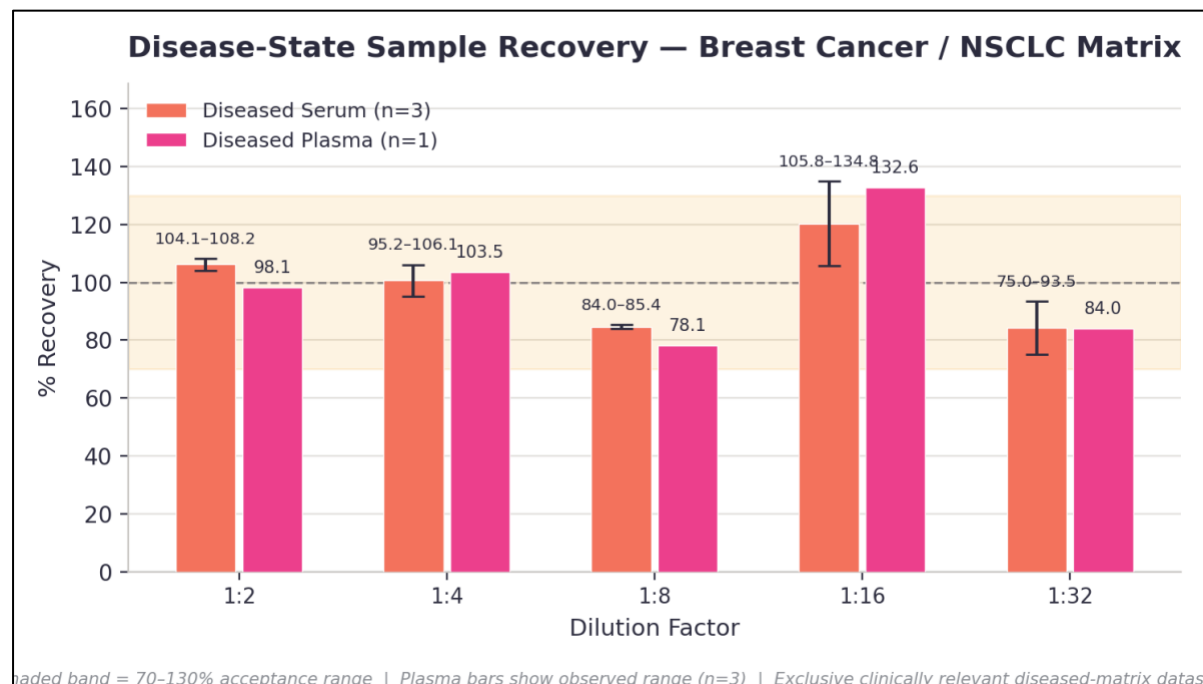
Diseased State Sample Impact on Atezolizumab Recovery

Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Serum (n=3)	104.1 - 108.2	95.2 - 106.1	84.0 - 85.4	105.8 - 134.8	75.0 - 93.5
Diseased Plasma (n=1)	98.1	103.5	78.1	132.6	84.0

Observations:

- Diseased serum samples demonstrated recovery largely within the acceptable range (80–130%) across most dilutions, indicating minimal matrix interference.
- Slightly elevated recovery was observed at 1:16 dilution (up to ~134.8%), suggesting potential over-recovery or matrix-enhanced signal at this dilution.
- Diseased plasma showed acceptable recovery at lower dilutions (1:2 and 1:4), with variability at higher dilutions, including elevated recovery at 1:16 dilution.
- Overall, recovery trends indicate that the assay performs reliably in diseased matrices, with minor variability at specific dilutions.
- The assay is suitable for accurate quantification of Atezolizumab in diseased serum and plasma, with recommended evaluation of dilution factors to ensure results remain within the optimal assay range.

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



8. Sample Handling and Storage Conditions

A) Specimen Collection and Handling:

For prepared solutions: Dilute to expected concentration within the kit assay range using Sample Diluent provided in the kit.

For lyophilized preparations including vaccines: Reconstitute using the Sample Diluent. Keep for 5 mins and mix well. Use the Sample Diluent for further dilutions to bring the sample within the expected assay range of the kit.

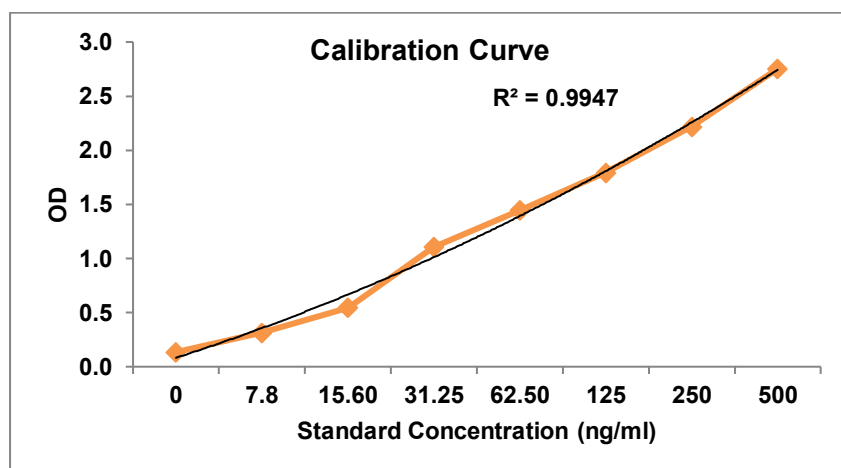
B) Handling / Storage:

- All reagents should be stored at temperature indicated on the labels.
- Upon receiving the kit, prepare small aliquots of dsRNA-specific K1 Detection HRP Conjugate and store it at -20°C. Avoid repeated freeze and thaw.
- All the reagents and wash solutions should be used within 12 months from manufacturing date.
- Before using, bring all components to room temperature (18-25°C). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.

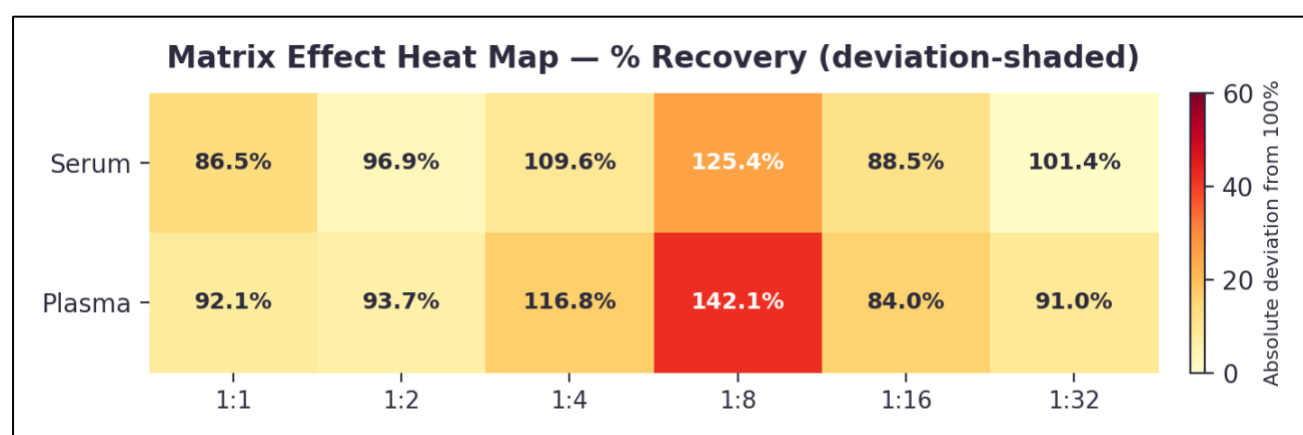
C) Health Hazard Warnings:

- 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%	-
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

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