ELISA VALIDATION GUIDE

KRIBIOLISA® ATEZOLIZUMAB (TECENTRIQ™) ELISA

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.

The antibody was engineered to precisely block immune-suppressive signalling pathways without depleting activated T-cells, allowing sustained antitumor activity with a favourable safety profile. Its mechanism functions independently of tumor antigen specificity, making it a versatile immunotherapeutic option across a broad spectrum of cancers exhibiting PD-L1–mediated immune evasion. Atezolizumab can also be combined with chemotherapy, targeted agents, and radiotherapy to enhance therapeutic outcome, improve immune infiltration, and overcome tumor-induced resistance mechanisms.

Atezolizumab plays a crucial role in modern immuno-oncology research as well as clinical therapeutics, with applications in drug-responsiveness studies, biomarker evaluation, and immune microenvironment profiling. Its clinical performance is monitored using assays such as IHC, ELISA, flow cytometry, and multiplex immune profiling platforms. In research settings, it is frequently used to study T-cell activation, tumor immune escape, and PD-L1–based therapeutic interventions. Commercially available worldwide, Atezolizumab continues to be widely investigated in combination trials for expanding its therapeutic scope and improving patient survival outcomes.

In May 2016, Atezolizumab was granted its first regulatory approval by the Food and Drug Administration (FDA) under the brand name TECENTRIQ, for the treatment of patients with locally advanced or metastatic urothelial carcinoma whose disease had progressed after platinum-containing chemotherapy. Over time, its approved indications expanded to include other solid tumors — such as metastatic non–small cell lung cancer (NSCLC), small-cell lung cancer, and more — based on evidence of anti-tumor activity and acceptable safety profile. Because of the risks inherent to immune-checkpoint therapies — including immune-related adverse events and clinically significant toxicities — Atezolizumab's use is reserved for defined cancer types and clinical settings, generally after evaluation of risk-benefit profile, PD-L1 expression, and in some cases prior therapy history.

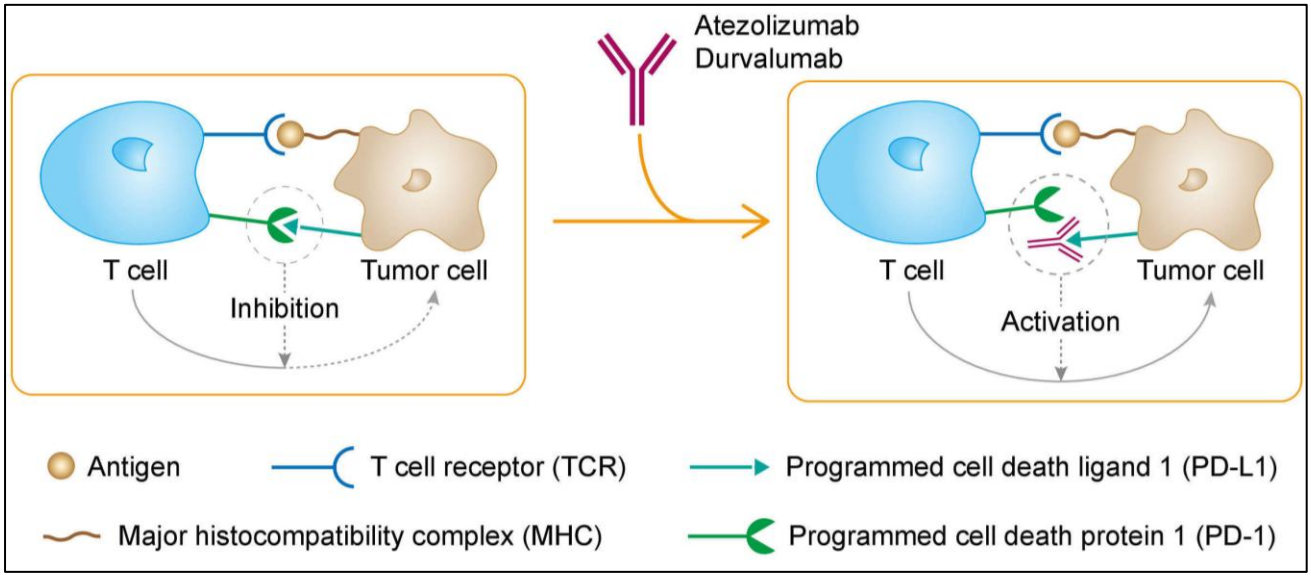


Figure 1: Immune response to Atezolizumab.

2. Clinical Relevance of Atezolizumab-Based Monitoring

Therapeutic drug monitoring (TDM) of Atezolizumab is essential for optimizing dosage regimens, particularly in patients exhibiting heterogeneous immune response, rapid disease progression, or comorbid physiological conditions that influence antibody clearance or immune checkpoint activity. Regular monitoring of circulating Atezolizumab levels allows precise evaluation of pharmacokinetic (PK) behavior, therapeutic saturation of PD-L1 binding sites, and the extent of T-cell reactivation, which are critical indicators for predicting therapeutic success and avoiding unnecessary immune-related toxicity.

Assessing drug exposure through TDM also helps correlate Atezolizumab concentrations with measurable clinical outcomes such as tumor shrinkage, progression-free survival, enhanced T-cell infiltration, and reduction of immunosuppressive signaling within the tumor microenvironment. This data supports informed adjustments in dosing schedules, combination therapy selection, and patient-specific immunotherapy planning. Ultimately, Atezolizumab monitoring facilitates a personalized treatment approach by aligning drug activity with individual tumor biology, immune competence, and therapeutic responsiveness — thereby improving overall treatment outcomes and minimizing adverse immune-mediated reactions.

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).
- Matrix Effect (serum, plasma).
- Sample Handling and Storage Conditions.
- References Atezolizumab Values and Recommended ELISA Range).

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 based on a sandwich immunoassay format. Anti-Atezolizumab capture antibodies are immobilized on 96-well microplate wells. Atezolizumab present in the standards and test samples specifically binds to the coated antibodies during incubation. Following a wash step to remove unbound components, an HRP-conjugated detection antibody is added, which binds to the captured Atezolizumab, forming an antibody–antigen–antibody complex. After additional washing to eliminate excess conjugate, TMB substrate is added, allowing HRP to catalyze a colorimetric reaction. The reaction is then stopped by adding stop solution. The resulting yellow color is measured at 450 nm, where the optical density (OD) is directly proportional to the concentration of Atezolizumab present in the samples or standards.

Experimental Design

- A Sandwich ELISA was performed using anti-Atezolizumab monoclonal antibody as the capture antibody.
- Standards were prepared using purified Atezolizumab reference material.
- Assay Concentration Range: 0 - 500 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The optimized antibody-coating and detection strategy employed in the KRIBIOLISA® Atezolizumab ELISA ensures efficient capture of free drug molecules while minimizing background reactivity from endogenous IgG or other serum proteins, enabling high assay sensitivity and specificity suitable for research and clinical monitoring applications.

The KRIBIOLISA® Atezolizumab ELISA utilizes a quantitative sandwich immunoassay format based on the interaction between drug-specific monoclonal antibodies for selective recognition of Atezolizumab. Anti-Atezolizumab antibodies are pre-coated onto microwells to function as capture molecules. Patient samples and Atezolizumab standards are added, allowing the drug to bind to the immobilized capture antibody. An HRP-conjugated anti-Atezolizumab detection antibody is then introduced to form the antibody–antigen–antibody complex. Following washing to remove unbound material, TMB substrate is added, generating a color signal proportional to the Atezolizumab concentration in the sample. The reaction is terminated with stop solution, and absorbance is measured at 450 nm, providing a quantitative assessment of Atezolizumab levels.

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
Standard therapeutic dose (routine clinical treatment cycles)	30–100	0–150

Application	Expected Atezolizumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
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. The kit has also been validated upto 64000 fold dilution and the values are within the acceptable range.

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. The specificity profile enables selective detection of Atezolizumab in complex biological matrices—such as serum, plasma, or cell-culture supernatants—while demonstrating minimal interference from structurally related therapeutic antibodies, soluble PD-L1, or unrelated immunoglobulins. This high level of molecular discrimination ensures accurate measurement of Atezolizumab irrespective of patient background, provided the epitope integrity is preserved.

2.2 Selectivity

The ELISA exhibits minimal to no cross-reactivity with endogenous human IgG subclasses, recombinant antibodies, or structurally unrelated therapeutic monoclonal antibodies. It effectively excludes molecules that do not share the unique idiotype or antigenic determinants of Atezolizumab, including soluble PD-L1, PD-1, or other immune checkpoint proteins present in circulation. The assay also maintains high selectivity in complex biological matrices (e.g., serum, plasma, or cell-culture supernatants), displaying negligible interference from matrix proteins, cytokines, heterophilic antibodies, or Fc-binding serum factors. This ensures reliable and accurate quantification of Atezolizumab without false positives arising from structurally similar or immunologically active components.

2.3LOD, LOQ and IC_{50}

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

IC_{50} in ELISA (Half Maximal Inhibitory Concentration)

IC_{50} = 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:

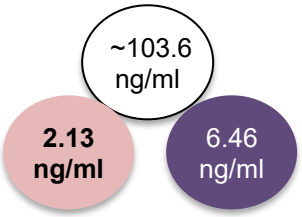
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.

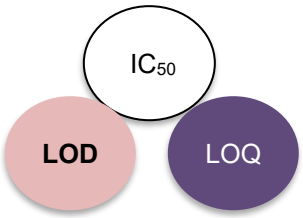
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC_{50} 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



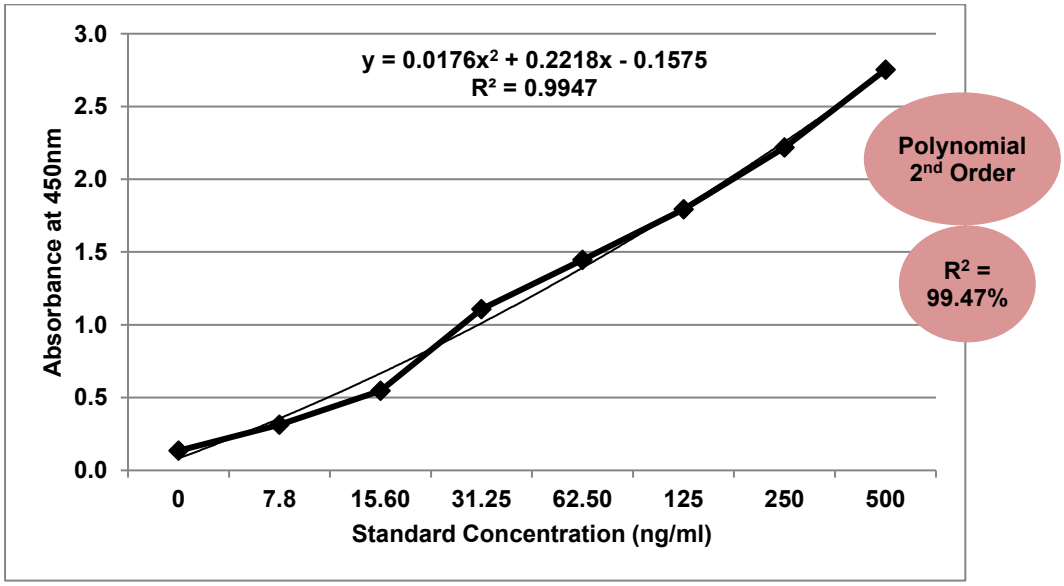


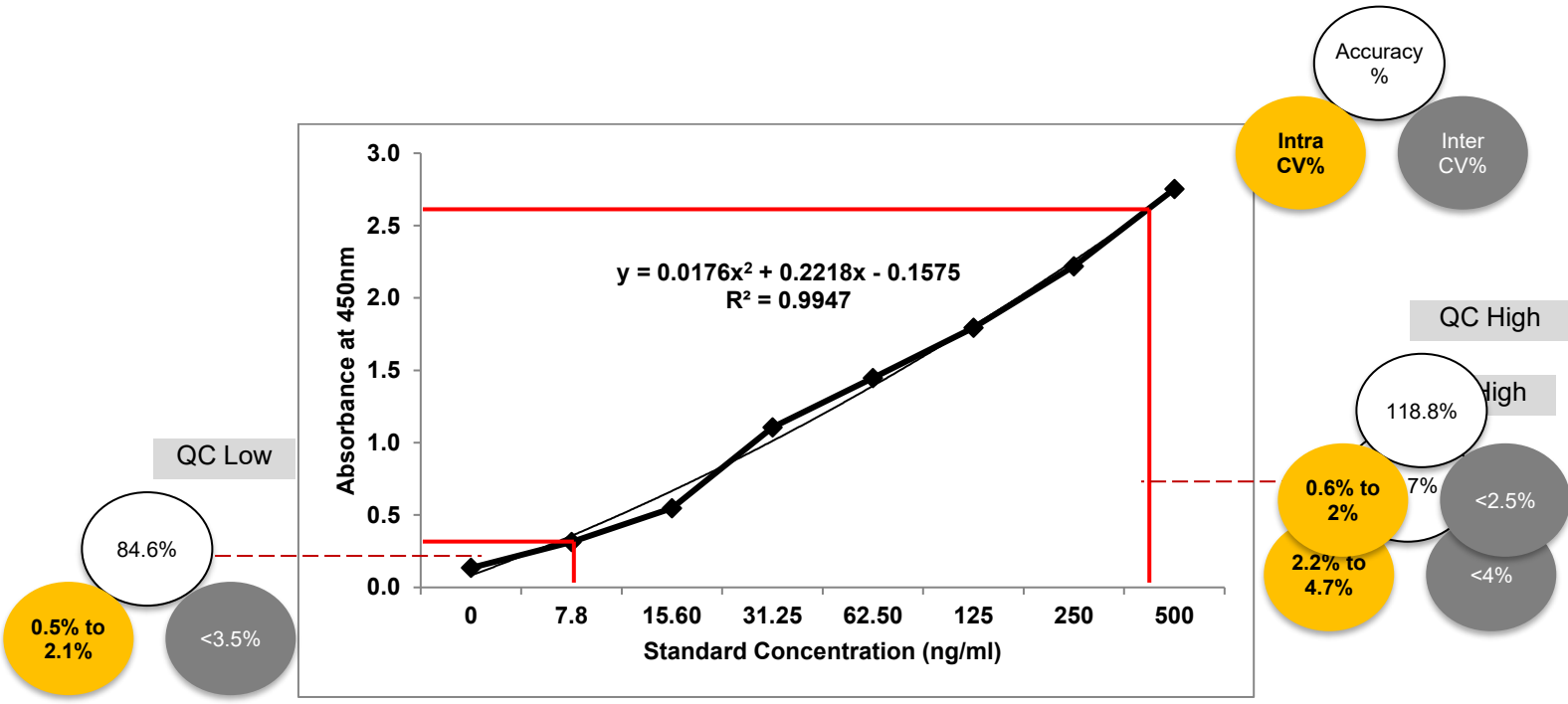
Regulatory Note:

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
15.6	0.547	12.8	82.1
31.25	1.107	37.7	120.6
62.5	1.447	69.2	110.7
125	1.795	114.5	91.6
250	2.219	215.1	86
500	2.753	584.1	116.8
Positive Control (450 ng/ml)	2.673	488	108.4
Low QC Control (7.8 ng/ml)	0.358	6.6	84.6
High QC Control (400 ng/ml)	2.576	475.2	118.8





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 ≤15% for QC samples.
- Inter-assay %CV should be ≤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.

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 ±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
1:2000	250	2.21	216.2	86.5	115.6
1:4000	125	1.834	121.1	96.9	103.2
1:8000	62.5	1.44	68.5	109.6	91.2
1:16000	31.25	1.084	39.2	125.4	79.7
1:32000	15.6	0.523	13.8	88.5	113.2
1:64000	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
1:2000	250	2.099	230.2	92.1	108.6
1:4000	125	1.614	117.1	93.7	106.7

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:8000	62.5	1.295	73	116.8	85.6
1:16000	31.25	1.001	44.4	142.1	70.4
1:32000	15.6	0.451	13.1	84	119.3
1:64000	7.8	0.3	7.1	91	110

Results:

i. Parallelism is generally maintained across the 1:2000 to 1:64000 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® Atezolizumab ELISA kit was tested for matrix effect on human serum, plasma and physiological buffer 7.4.

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 + 1:1000 human serum)
- Assay buffer spiked with human plasma (buffer + 1:1000 human plasma)

Samples were tested across the standard curve range (0–500 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 + 1:1000 Human Serum)	% Standard Deviation	% CV
0	0.112	0.135	1.6	13
7.8	0.258	0.324	4.66	16

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:1000 Human Serum)	% Standard Deviation	% CV
15.6	0.432	0.532	7.07	14.7
31.25	1.089	1.107	1.24	1.1
62.5	1.412	1.447	2.44	1.7
125	1.687	1.795	7.6	4.4
250	2.119	2.219	7.07	3.3
500	2.859	2.753	7.5	2.7

Standard (ng/ml)	Mean Absorbance (Buffer + 1:1000 Human Serum)	Mean Absorbance (Buffer + 1:1000 Human Plasma)	% Standard Deviation	% CV
0	0.135	0.149	1.02	7.2
7.8	0.324	0.289	2.47	8.1
15.6	0.532	0.444	6.22	12.7
31.25	1.107	0.989	8.32	7.9
62.5	1.447	1.258	13.33	9.9
125	1.795	1.589	14.53	8.6
250	2.219	2.111	7.64	3.5
500	2.753	2.658	6.72	2.5

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 analyzing biological samples.

7. Sample Handling and Storage Conditions

A) Specimen Collection and Handling:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

For Cell Culture Supernatant – If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80ºC. Avoid repeated freeze-thaw cycles.

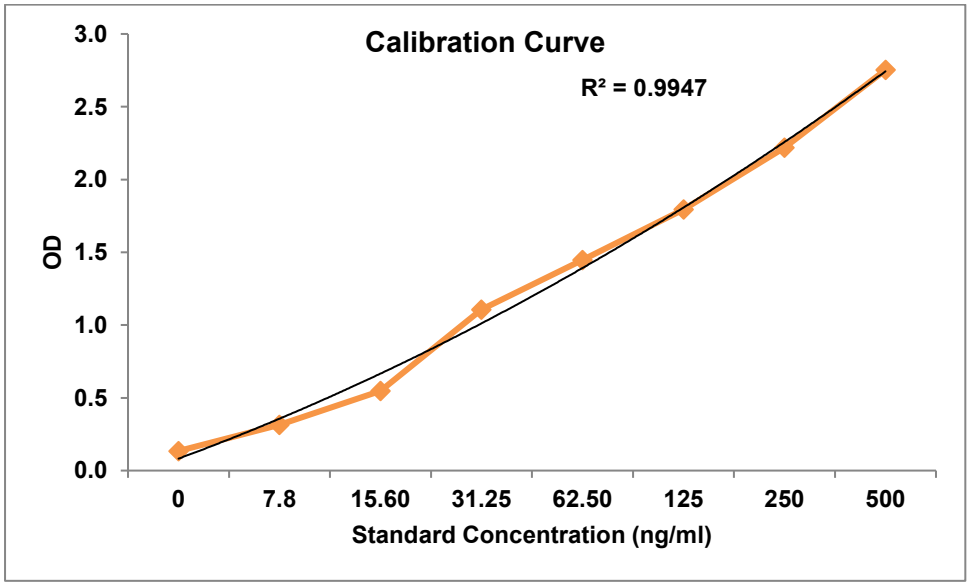
B) Handling / Storage:

- It is advisable to aliquot and store the Anti-Atezolizumab Antibody:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess working Anti-Atezolizumab Antibody:HRP Conjugate after running your assay.
- All the reagents and wash solutions should be used within 12 months from manufacturing date.
- Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
- The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

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% (100 ± 25%	-
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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