

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA
APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

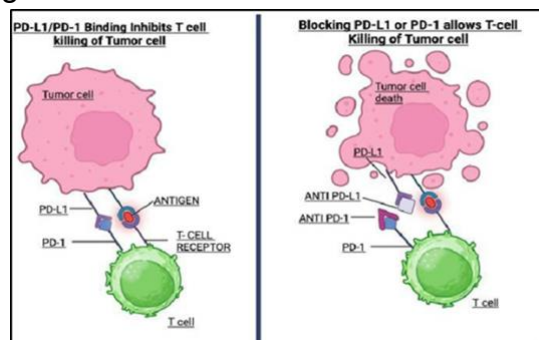
1. Introduction to Pembrolizumab (KEYTRUDA™)

Pembrolizumab is a highly selective, humanized monoclonal IgG4 antibody that targets programmed death-1 (PD-1), a key inhibitory receptor expressed on activated T-cells, B-cells, and natural killer (NK) cells. By binding to PD-1 with high affinity, Pembrolizumab blocks its interaction with the ligands PD-L1 and PD-L2—molecules often overexpressed on tumor cells and antigen-presenting cells within the tumor microenvironment. This blockade reverses tumor-induced immune suppression, restoring cytotoxic T-cell activity and enabling robust antitumor immune responses. Clinically, Pembrolizumab is widely used in immunotherapy for various solid and hematological malignancies, including melanoma, non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), urothelial carcinoma, Hodgkin lymphoma, colorectal cancer with MSI-H/dMMR, and other PD-1/PD-L1–driven cancers.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- PD-1 is an immune checkpoint receptor that negatively regulates T-cell activation and can contribute to tumor immune evasion through interaction with PD-L1 and PD-L2.
- Pembrolizumab specifically binds to PD-1 and blocks its interaction with PD-L1 and PD-L2, thereby releasing PD-1-mediated inhibition of the anti-tumor immune response.
- PD-L1 expression is an important biomarker used for patient selection and evaluation of response to pembrolizumab in several cancer indications.



2.2 Assay Development

- Pembrolizumab serves as an important model for evaluating immune-checkpoint blockade and PD-1 target engagement through:
- ELISA – quantitative detection of pembrolizumab or assessment of PD-1/ligand interactions.
- Cell-based assays – evaluation of T-cell activation and functional immune responses following PD-1 blockade.
- Immunoassays / multiplex biomarker panels – monitoring immune-response biomarkers such as cytokines and immune-cell activation markers.

2.3 Biomarker for Efficacy and Safety

- PD-L1 expression, immune-cell populations, cytokine changes, and PD-1 target engagement can be evaluated as biomarkers of pharmacodynamic response to pembrolizumab.
- Monitoring immune biomarkers can help characterize treatment response, immune activation, and potential immune-related adverse effects during preclinical and clinical development.
- Pharmacodynamic studies of pembrolizumab have evaluated PD-1 receptor modulation/target engagement and immune activation to support dose and exposure–response assessment.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilar Development

- Pembrolizumab is a humanized IgG4 monoclonal antibody targeting the PD-1 immune checkpoint receptor and is an important therapeutic antibody in oncology.
- Development and characterization of pembrolizumab and potential biosimilars require:
 - PD-1 binding assays
 - PD-1/PD-L1 blocking assays
 - Cell-based functional assays
 - Comparability studies

3.2 PK/PD Studies

- Pembrolizumab serum concentrations are evaluated for PK parameters such as C_{max}, C_{min}, AUC, and half-life, while PD assessment focuses on PD-1 receptor occupancy and target engagement.

3.2 Safety Evaluation

- Safety evaluation focuses on monitoring immune-related adverse events and immune activation associated with PD-1 blockade, including effects on organs such as the lungs, liver, colon, and endocrine system.

4. Importance in Cell and Gene Therapy (CGT)

4.1 PD-1-Targeted Cell Therapy

- Pembrolizumab targets the PD-1 receptor on T cells and can be used to investigate immune-checkpoint blockade in engineered cell therapies.
- In CAR-T, CAR-NK, and TCR-engineered cell therapies, PD-1 blockade may help overcome T-cell exhaustion and enhance T-cell persistence, activation, and anti-tumor activity.

4.2 Biomarker and Target Validation

- PD-L1 expression and receptor occupancy are important biomarkers for evaluating checkpoint activity and target engagement in CGT studies.
- Pembrolizumab can be evaluated in binding, target-engagement, and functional assays to characterize PD-1 pathway inhibition and immune-cell activation.

4.3 Genetic Modulation

- Gene-editing approaches target TNF- α suppression to treat inflammatory conditions or reduce immune rejection in cell-based therapies.

Scope of Validation

This document presents a discussion of the characteristics of our KRIBIOLISA® Pembrolizumab ELISA KIT (CATALOG NO. KBI1084) 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 Pembrolizumab.

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).
- Accelerated stability study.
- Disease state sample recovery

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 evaluate the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Pembrolizumab ELISA KIT, developed to quantitatively measure free, active Pembrolizumab with high sensitivity and specificity.

Principle of the Assay

This ELISA is based on a sandwich immunoassay format. Human Anti- Pembrolizumab capture antibodies are immobilized on 96-well microplate wells. Pembrolizumab present in the standards and test samples specifically binds to the coated antibodies during incubation. Following a wash step to remove unbound components, an Human Anti- Pembrolizumab HRP-conjugated detection antibody is added, which binds to the captured Pembrolizumab, 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 Pembrolizumab present in the samples or standards.

Validation Parameters and Acceptance Criteria

1. Pembrolizumab Values and Recommended ELISA Range

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

Application	Expected Pembrolizumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Post low-dose administration (early-phase immunotherapy dosing))	10–40	0–50
Standard therapeutic dose (routine clinical treatment cycles)	50–150	0–150
High-dose or combination therapy regimens (oncology escalation protocols)	120–300	0–300
Pharmacokinetic monitoring / drug exposure–response evaluation	200–600	0–640

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

The KRIBIOLISA® Pembrolizumab ELISA kit is developed using an assay range of 0 - 640 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 64,000 fold dilution and the values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity

The capture and detection antibodies employed in the Pembrolizumab ELISA are monoclonal antibodies that specifically recognize the intact humanized IgG4 anti-PD-1 therapeutic antibody without cross-reacting with endogenous human immunoglobulins. These assay antibodies are engineered to target unique idiotype or framework-region epitopes of Pembrolizumab, ensuring high-affinity binding to the drug molecule in its native conformation. The specificity profile allows selective detection of Pembrolizumab in complex biological matrices—such as serum, plasma, or cell-culture supernatants—while exhibiting minimal interference from structurally related therapeutic antibodies, soluble PD-1/PD-L1 ligands, or unrelated immunoglobulins. This high degree of molecular discrimination ensures accurate quantification of Pembrolizumab regardless of patient background, provided that the structural integrity of the target epitope is maintained.

2.2 Selectivity

The ELISA demonstrates 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 Pembrolizumab, including soluble PD-1, PD-L1, PD-L2, or other immune checkpoint proteins circulating in biological samples. The assay maintains high selectivity in complex biological matrices (e.g., serum, plasma, or cell-culture supernatants), with negligible interference from matrix proteins, cytokines, heterophilic antibodies, Fc-binding serum factors, or immune-modulating molecules. This ensures reliable and accurate quantification of Pembrolizumab without false positives arising from structurally related antibodies or immunologically active components.

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® Pembrolizumab ELISA = 2.74 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).

LOQ for KRIBIOLISA® Pembrolizumab ELISA – 8.31 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:

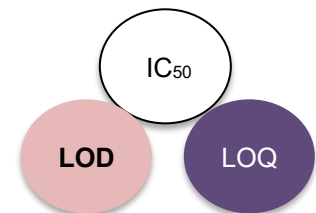
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.

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

IC₅₀ for KRIBIOLISA® Pembrolizumab ELISA = ~216.1 ng/ml

Summary:

Parameter	Value (ng/ml)
LOD	2.74 ng/ml
LOQ	8.31 ng/ml
IC ₅₀	216.1 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	0.128	--	--
10	0.167	12.5	124.9
20	0.204	21.1	105.3
40	0.314	40.0	100.0
80	0.599	78.8	98.5
160	1.202	161.4	100.9
320	1.929	318.4	99.5
640	2.481	641.7	100.3

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
Low QC Control (20 ng/ml)	0.194	21.6	108.1
High QC Control (320 ng/ml)	2.061	336.4	105.1
Mid QC Control (600 ng/ml)	2.427	538.7	89.8

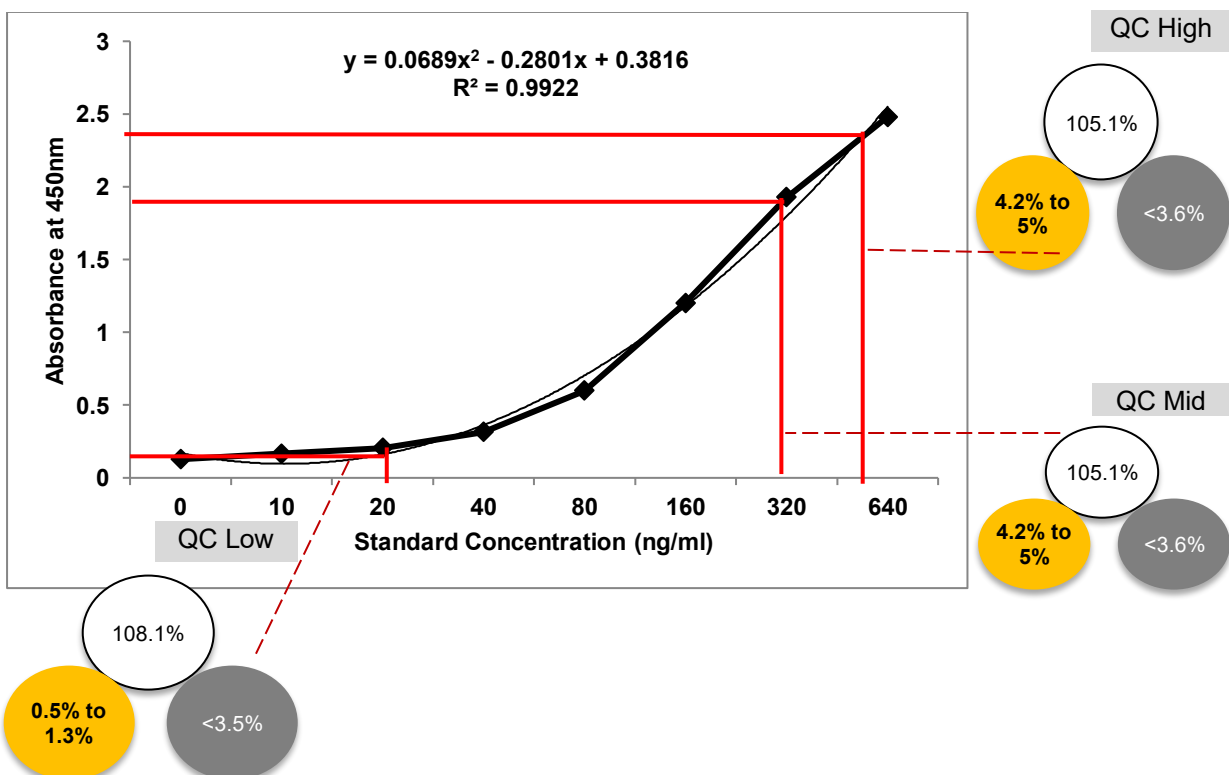
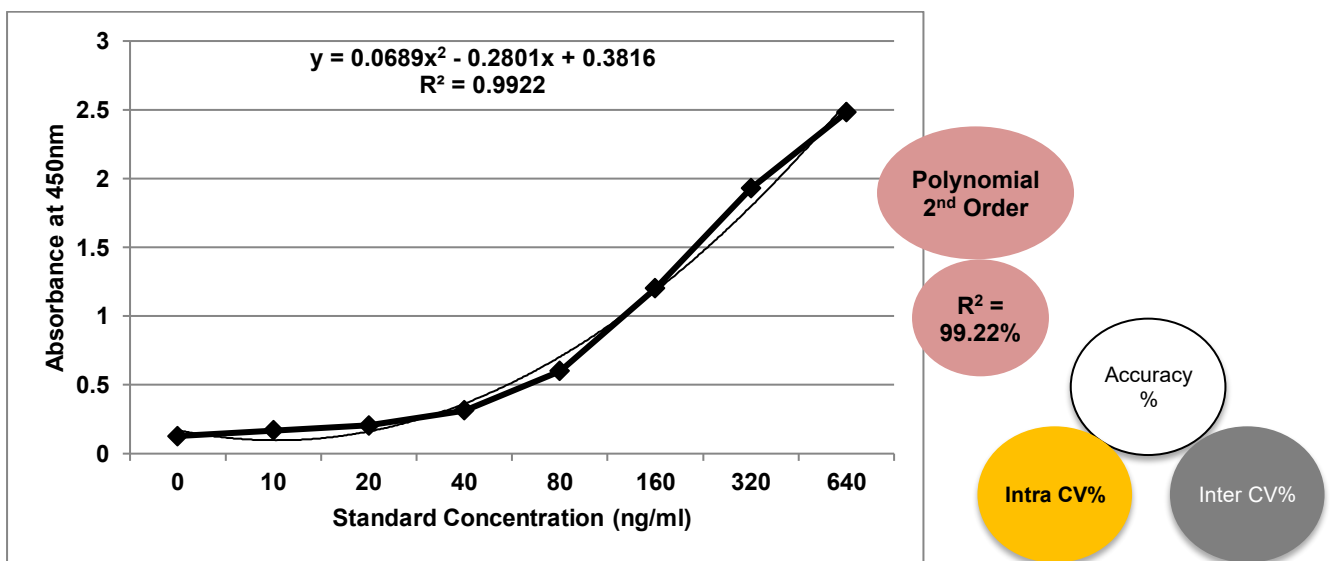


Figure 1. Standard Curve Accuracy — % Recovery Across the Assay Range

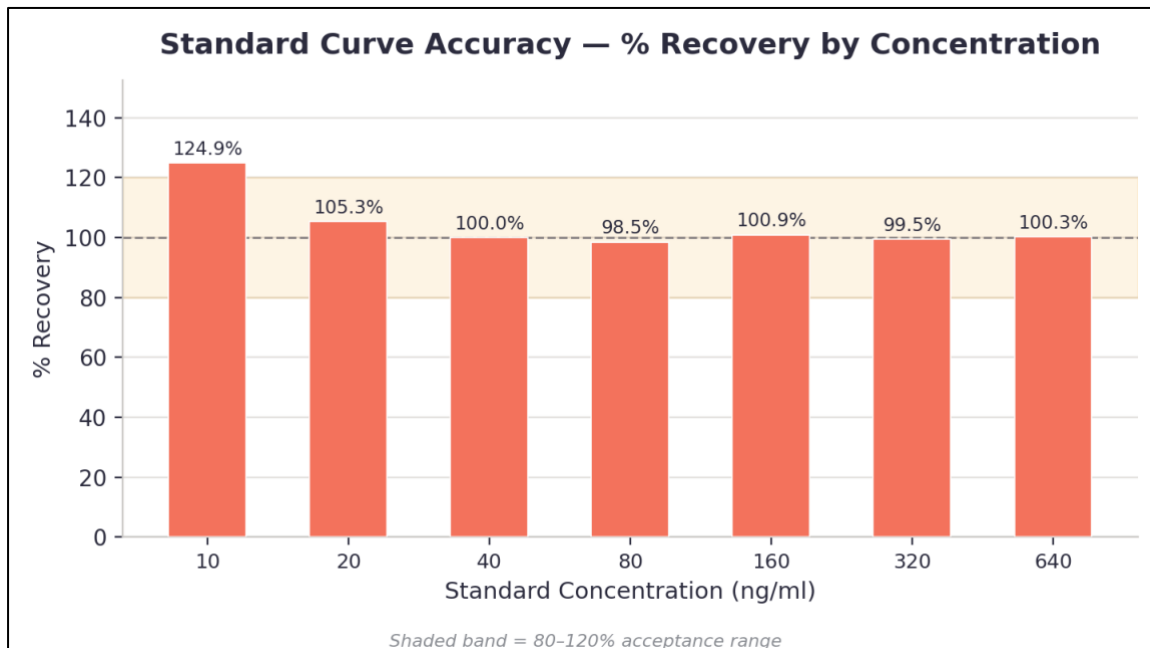
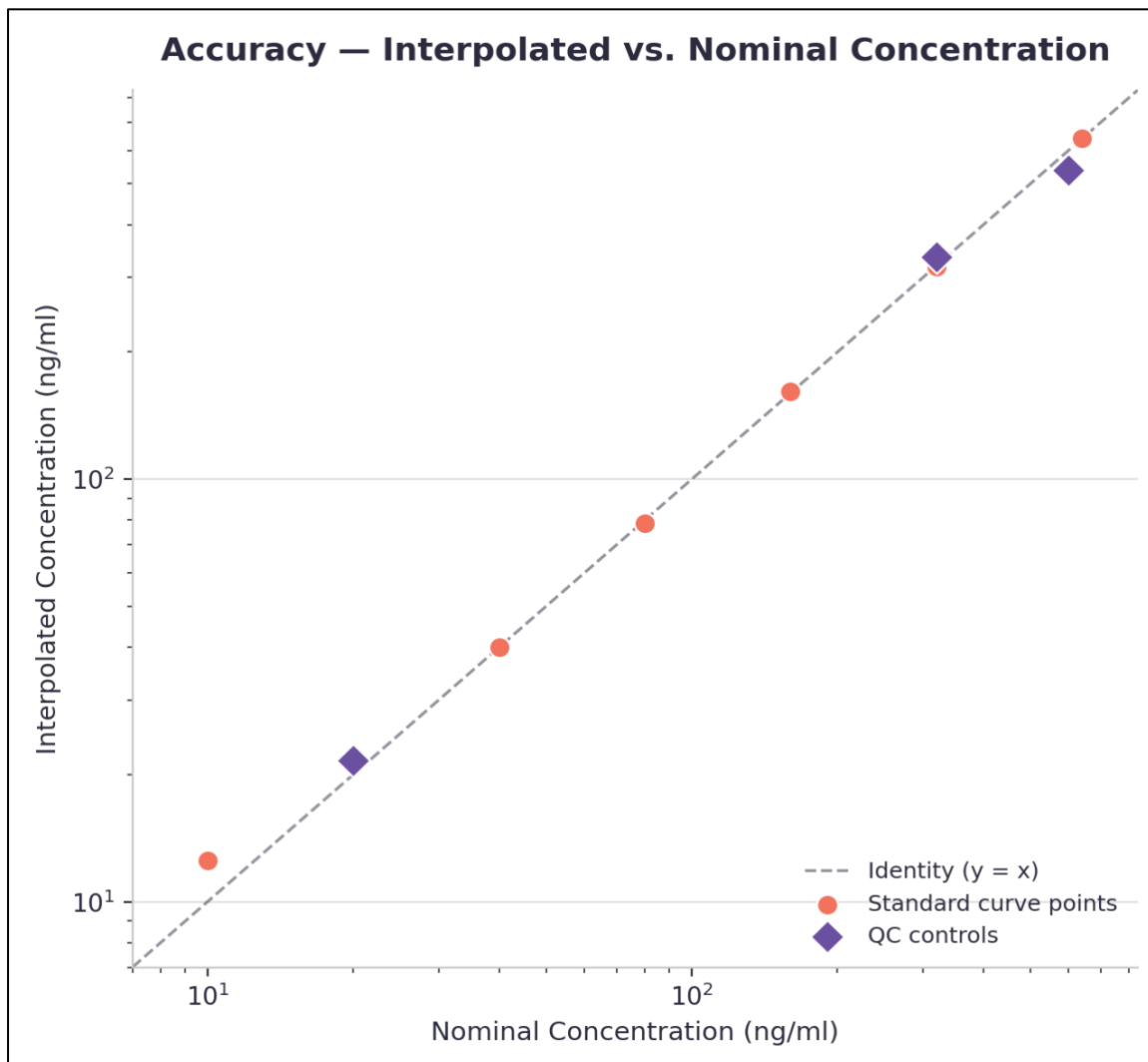


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

A) Intra-Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
10	0.162	0.04	4.5
160	1.195	0.08	4.3
640	2.384	0.04	1.9

B) Inter Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
10	0.166	0.05	3.2
160	1.201	0.02	4.2
640	2.425	0.04	2.1

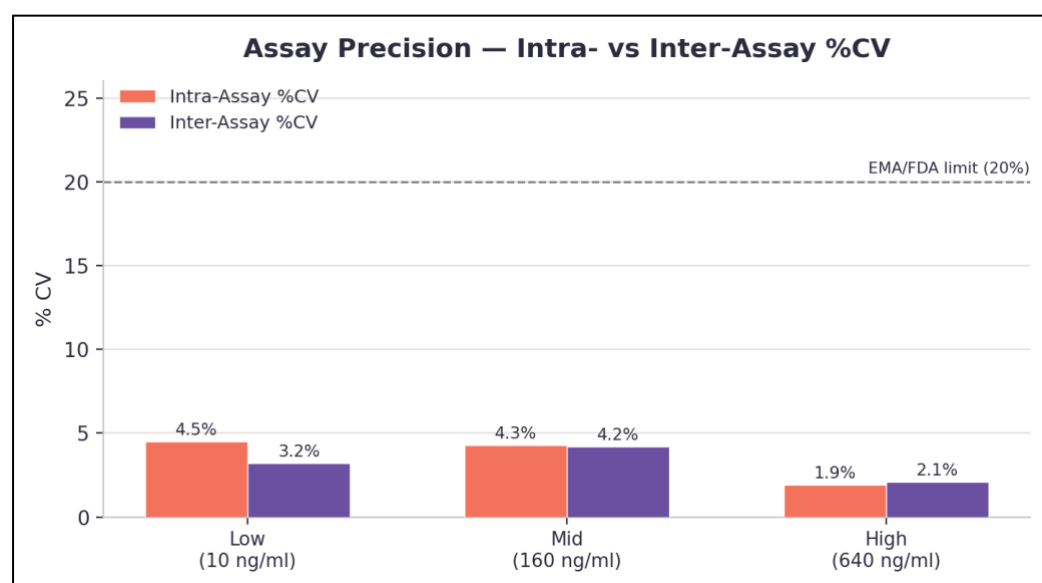
Observations:

- Intra-assay precision was consistently less than 4.6% across all levels tested.
- Inter-assay precision was consistently less than 4.5%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:

The KRIBIOLISA® Pembrolizumab 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.

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



5. Parallelism

Serial dilutions of a high-concentration sample were prepared at dilutions of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 for both human serum and human plasma. Each dilution was assayed using the KRIBIOLISA® Pembrolizumab 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.

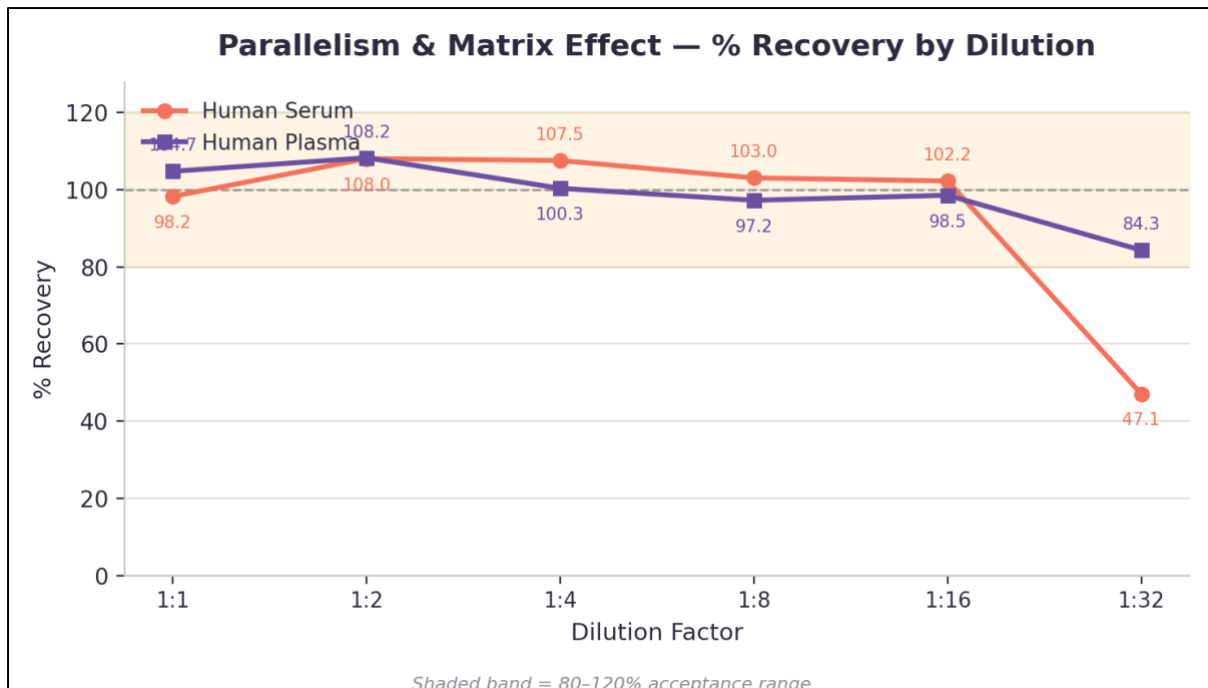
A) Human Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	1.916	314.3	98.2	101.8
1:2	160	1.274	172.8	108.0	92.6
1:4	80	0.655	86.0	107.5	93.0
1:8	40	0.322	41.2	103.0	97.1
1:16	20	0.201	20.4	102.2	97.8
1:32	10	0.144	4.7	47.1	212.1

B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	2.041	335.1	104.7	95.5
1:2	160	1.316	173.2	108.2	92.4
1:4	80	0.631	80.3	100.3	99.7
1:8	40	0.312	38.9	97.2	102.9
1:16	20	0.194	19.7	98.5	101.5
1:32	10	0.145	8.4	84.3	118.7

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® Pembrolizumab ELISA kit was tested for matrix effect on human serum and plasma.

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 Pembrolizumab 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 serum (buffer + 1:1000 human plasma)

Samples were tested across the standard curve range (0–640 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	0.114	0.124	0.68	5.7
10	0.132	0.145	0.93	6.7
20	0.152	0.171	1.38	8.5
40	0.255	0.253	0.12	0.5
80	0.489	0.547	4.07	7.9
160	1.008	1.105	6.89	6.5
320	1.987	2.052	4.58	2.3
640	2.512	2.494	1.27	0.5

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:1000 Human Plasma)	% Standard Deviation	% CV
0	0.114	0.118	0.00	1.1
10	0.132	0.161	0.00	0.0
20	0.152	0.210	0.01	7.1
40	0.255	0.302	0.02	7.8
80	0.489	0.621	0.03	4.8
160	1.008	1.250	0.07	5.6
320	1.987	1.979	0.07	3.3
640	2.512	2.574	0.08	3.2

Results:

- Very low %CV across all concentrations.
- Minimal shift in absorbance values between buffer-only and buffer + serum and buffer + plasma conditions.
- No significant matrix effect observed

Conclusion:

The KRIBIOLISA® Pembrolizumab 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. Accelerated Stability Study:

Accelerated stability studies in ELISA are performed to predict the shelf life and long-term stability of an ELISA kit or its individual components by exposing them to elevated stress conditions (typically higher temperatures) for a defined period.

The following table demonstrates the relation of temperature with time point and number of days:

Accelerated Study Day (37 degrees)	Real-Time Equivalent Age (2-8 degree)	Interpretation
Day 0	Present day (0 months)	Initial / release testing
Day 1	26 days (Approx. 1 month)	Early stability checkpoint
Day 4	104 days (Approx. 3.5 months)	Short-term stability trend
Day 7	182 days (Approx. 6 months)	Mid-term shelf-life prediction
Day 14	364 days (Approx. 1 year)	One-year shelf-life equivalence

Accelerated Stability Study data:

Standard Concentration (ng/ml)	Absorbance (Day 0)	Absorbance (Day 1)	Absorbance (Day 4)	Absorbance (Day 7)	Absorbance (Day 14)	%CV
0	0.142	0.123	0.156	0.114	0.118	13.6
10	0.181	0.172	0.214	0.161	0.153	13.4
20	0.216	0.232	0.248	0.213	0.199	8.5
80	0.485	0.465	0.478	0.456	0.455	7.6
160	1.187	1.518	1.376	1.513	1.219	11.5
320	1.958	1.952	1.934	1.925	1.920	3.6
640	2.438	2.638	2.547	2.692	2.844	5.8

Results:

- I. %CV is less than 15% across all days.
- II. Based on the accelerated stability study results, the Pembrolizumab ELISA kit demonstrates satisfactory stability and robustness, supporting its viability with an extended shelf life and an assigned expiry of 1 year under recommended storage conditions

8. Disease State sample recovery:

Recovery was assessed by analysing diseased serum (n=1) and diseased plasma (n=5) samples spiked and evaluated across multiple dilution factors (1:2 to 1:32) against the calibration standards. The study was performed to evaluate matrix effects and the ability of the assay to accurately recover analyte in clinically relevant diseased sample matrices.

Sample Details:

Diseased-state sample validation was performed using six clinically relevant human serum and plasma samples from patients with non-small cell lung cancer (NSCLC) and chronic myelogenous leukemia (CML). The samples included both male and female subjects, with ages ranging from 56 to 68 years, and all samples were of Hispanic and Caucasian demographics. 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
042115 D	Chronic Myelogenous Leukemia (CML) Human Serum	Caucasian 60/ Male	No/No	Negative	Nil	Nil/ 23.5	Nil
042115 D	Chronic Myelogenous Leukemia (CML) Human Plasma	Caucasian 60/ Male	No/No	Negative	Nil	Nil/ 23.5	Sodium Heparin

Acceptance

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

Criteria:

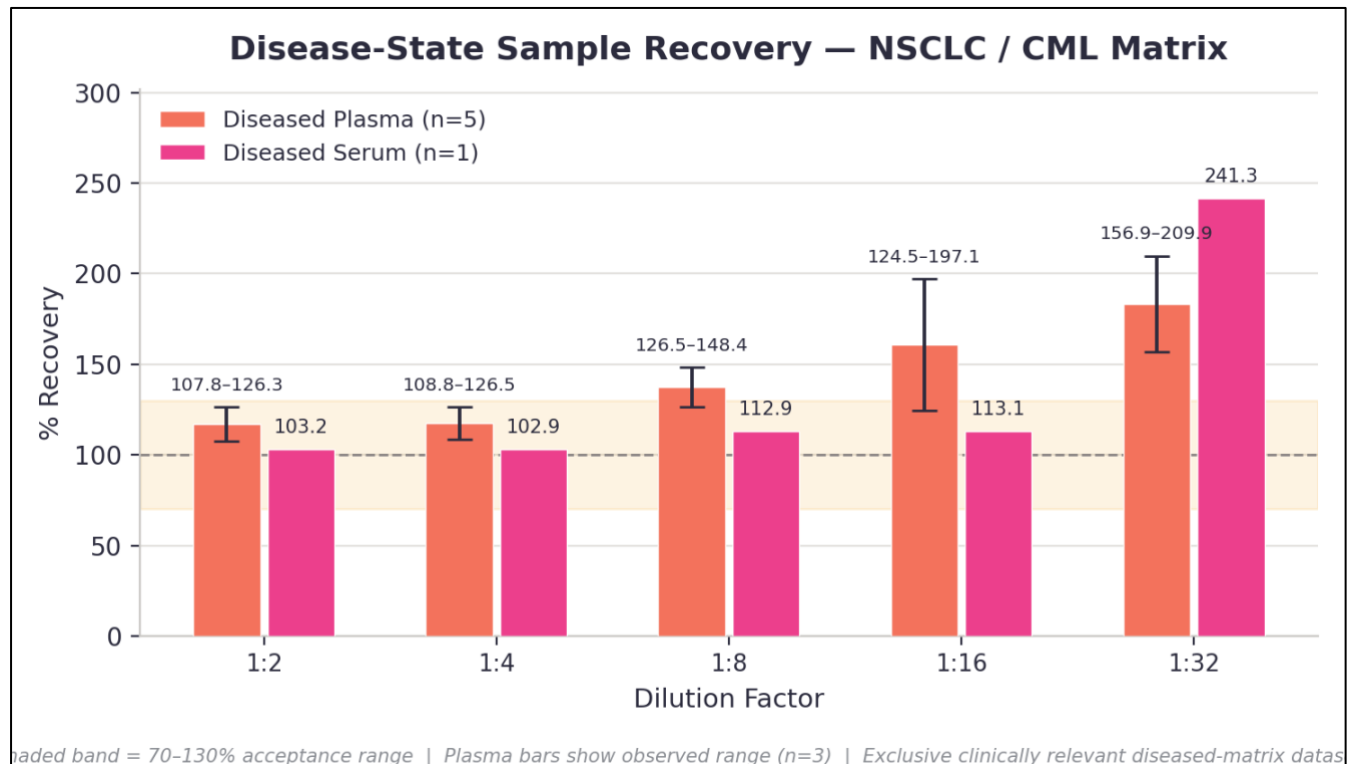
Diseased State Sample Impact on Pembrolizumab Recovery

Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Serum (n=1)	103.2	102.9	112.9	113.1	241.3
Diseased Plasma (n=5)	107.8- 126.3	108.8 – 126.5	126.5- 148.4	124.5 – 197.1	156.9 – 209.9

Observations:

- Recovery of Pembrolizumab in diseased serum remained within the acceptance range from 1:2 to 1:16 dilution, demonstrating good assay performance and minimal matrix interference across these dilution levels.
- Diseased plasma samples exhibited acceptable recovery at 1:2 and 1:4 dilutions, with recovery values ranging from 107.8% to 126.5%.
- Recovery in diseased plasma increased beyond the acceptance criterion at higher dilutions (1:8 to 1:32), reaching up to 209.9%, indicating dilution-dependent over-recovery.
- The observed increase in recovery with dilution suggests potential matrix-related effects, assay non-linearity, or altered antibody–analyte interactions at higher dilution factors.
- The assay demonstrates reliable recovery of Pembrolizumab in diseased serum up to 1:16 dilution and in diseased plasma primarily at lower dilution factors (1:2–1:4).
- Based on these results, lower dilution ranges are recommended for routine analysis to ensure accurate quantification and minimize dilution-related bias.

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



9. 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.

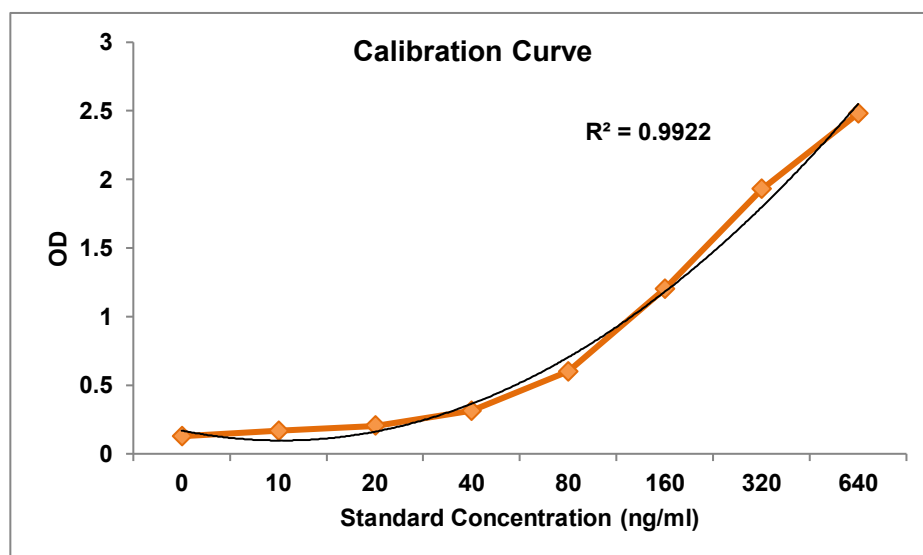
Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at $1000 \times g$. Remove serum layer and assay immediately or store serum samples at temperature $<-20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

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.

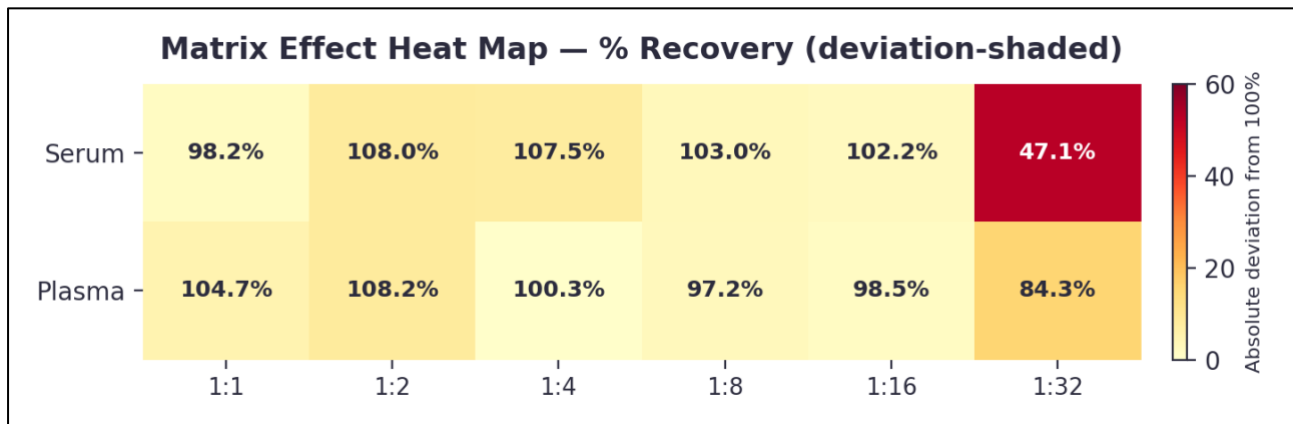
B) Storage conditions:

- It is advisable to aliquot and store the Anti-Pembrolizumab: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-Pembrolizumab: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 (18-25°C). 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 source.

Graphs, Maps and Appendices:



Matrix Effect Heat Map



	1:1	1:2	1:4	1:8	1:16	1:32
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%

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