ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA

APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® PEMBROLIZUMAB ELISA KIT (CATALOG NO. KBI1084) AS PER FDA/ICH GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline recommended by FDA/ICH.

Document History

First Codification	History	Date
Version#1	**VALIDATION DATA OF KRIBIOLISA® PEMBROLIZUMAB ELISA KIT (CATALOG NO. KBI1084)**	31.12.2025

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

Background

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.

Engineered as an IgG4 isotype to minimize antibody-dependent cellular cytotoxicity (ADCC), Pembrolizumab modulates immune signaling without promoting T-cell depletion, contributing to its durable clinical efficacy and favorable safety profile. Its mechanism is tumor-antigen–agnostic, allowing broad application across cancers that utilize PD-1/PD-L1 interactions to evade immune detection. Pembrolizumab is often administered as monotherapy or in combination with chemotherapy, targeted agents, radiotherapy, or additional immunotherapies to enhance immune infiltration, overcome resistance pathways, and prolong patient survival.

In both clinical and research domains, Pembrolizumab has become a cornerstone molecule in modern immuno-oncology. It is instrumental in studying checkpoint blockade mechanisms, tumor immune escape, T-cell exhaustion, and biomarker-driven treatment designs. Its monitoring and evaluation employ a range of analytical platforms including IHC, ELISA, flow cytometry, cytokine profiling, and multiplex immune assays. Widely available globally, Pembrolizumab continues to be a major focus of ongoing clinical trials exploring combination strategies, precision oncology approaches, and expanded indications across numerous cancer types.

In September 2014, Pembrolizumab was first approved by the U.S. Food and Drug Administration (FDA) under the brand name KEYTRUDA for the treatment of patients with unresectable or metastatic melanoma — a life-threatening skin cancer. Since that initial approval, its clinical use has expanded dramatically: today Pembrolizumab is indicated for a wide range of cancers, including non–small cell lung cancer (NSCLC) whose tumors express PD-L1. It also enjoys tumor-agnostic approvals for metastatic or unresectable solid tumors that are microsatellite instability–high (MSI-H) or mismatch repair deficient (dMMR).

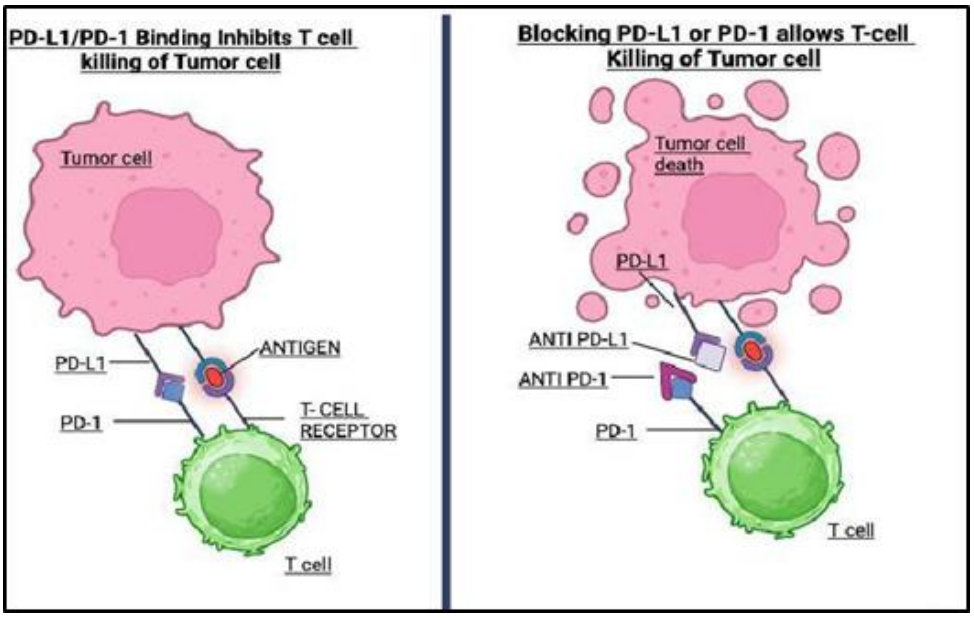


Figure 1: Immune response to Pembrolizumab.

2. Clinical Relevance of Pembrolizumab-Based Monitoring

Therapeutic drug monitoring (TDM) of Pembrolizumab is crucial for optimizing treatment efficacy, particularly in patients demonstrating variable immune responses, aggressive disease progression, or physiological conditions that alter monoclonal antibody clearance or PD-1 pathway dynamics. Monitoring circulating Pembrolizumab levels enables accurate assessment of its pharmacokinetic (PK) profile, the degree of PD-1 receptor occupancy on T-cells, and the restoration of antitumor immune activity—factors essential for predicting clinical benefit and preventing excessive immune activation or immune-related adverse events.

Evaluating drug exposure through TDM also helps establish correlations between Pembrolizumab concentration and key clinical endpoints such as tumor regression, progression-free survival, enhanced T-cell activation, and attenuation of PD-1–mediated immunosuppression within the tumor microenvironment. These insights aid in tailoring dosing intervals, selecting rational combination regimens, and designing patient-specific immunotherapy strategies. Ultimately, Pembrolizumab monitoring supports a personalized medicine approach by aligning therapeutic action with individual variations in tumor biology, immune function, and treatment responsiveness—thereby improving clinical outcomes while minimizing the risk of severe immune-mediated toxicities.

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.
- Sample Handling and Storage Conditions.
- References Pembrolizumab 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 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, enabling precise monitoring of PD-1 immune checkpoint blockade. This assay supports clinical research, therapeutic drug monitoring (TDM), dose individualization, and evaluation of treatment efficacy in oncology applications. It is intended for use in studies assessing pharmacokinetics, immune activation status, and patient response to Pembrolizumab-based immunotherapy.

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.

Experimental Design

- A Sandwich ELISA was performed using Human anti- Pembrolizumab monoclonal antibody as the capture antibody.
- Standards were prepared using purified Pembrolizumab reference material.
- Assay Concentration Range: 0 - 640 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The optimized antibody-coating and detection configuration used in the KRIBIOLISA® Pembrolizumab ELISA enables highly efficient and selective capture of free Pembrolizumab while effectively minimizing nonspecific interactions with endogenous IgG, Fc-binding proteins, or other serum components. This enhances signal-to-noise performance, ensuring superior assay sensitivity and specificity appropriate for both research workflows and clinical drug-monitoring applications.

The KRIBIOLISA® Pembrolizumab ELISA employs a quantitative sandwich immunoassay format utilizing drug-specific monoclonal antibodies for the selective recognition of Pembrolizumab. Human Anti-Pembrolizumab antibodies are pre-coated onto the microwells to act as capture antibodies. When patient samples or Pembrolizumab standards are added, the free drug present in the sample binds to the immobilized capture antibody. An HRP-conjugated Human anti-Pembrolizumab detection antibody is subsequently introduced to form a specific antibody–antigen–antibody sandwich complex. After removing unbound components through washing, TMB substrate is added, producing a measurable color signal directly proportional to the concentration of Pembrolizumab. The reaction is halted using stop solution, and absorbance is read at 450 nm, enabling accurate quantitative determination of Pembrolizumab levels in biological samples.

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	0–500

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 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® 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
(10σ criterion is most common).

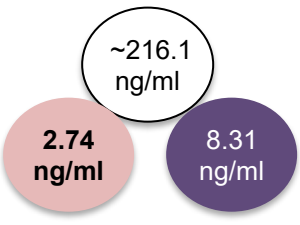
LOQ for KRIBIOLISA® Pembrolizumab ELISA – 8.31 ng/ml

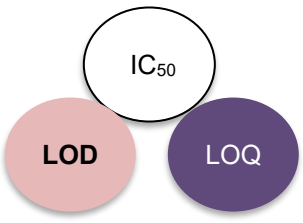
IC_{50} in ELISA (Half Maximal Inhibitory Concentration)
IC_{50} = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

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® 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

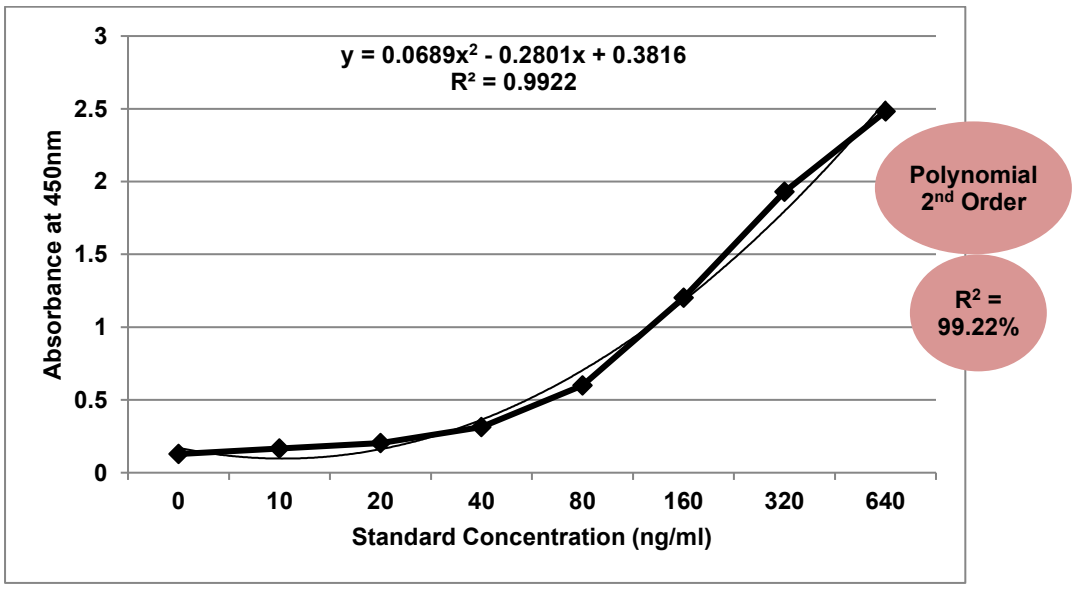


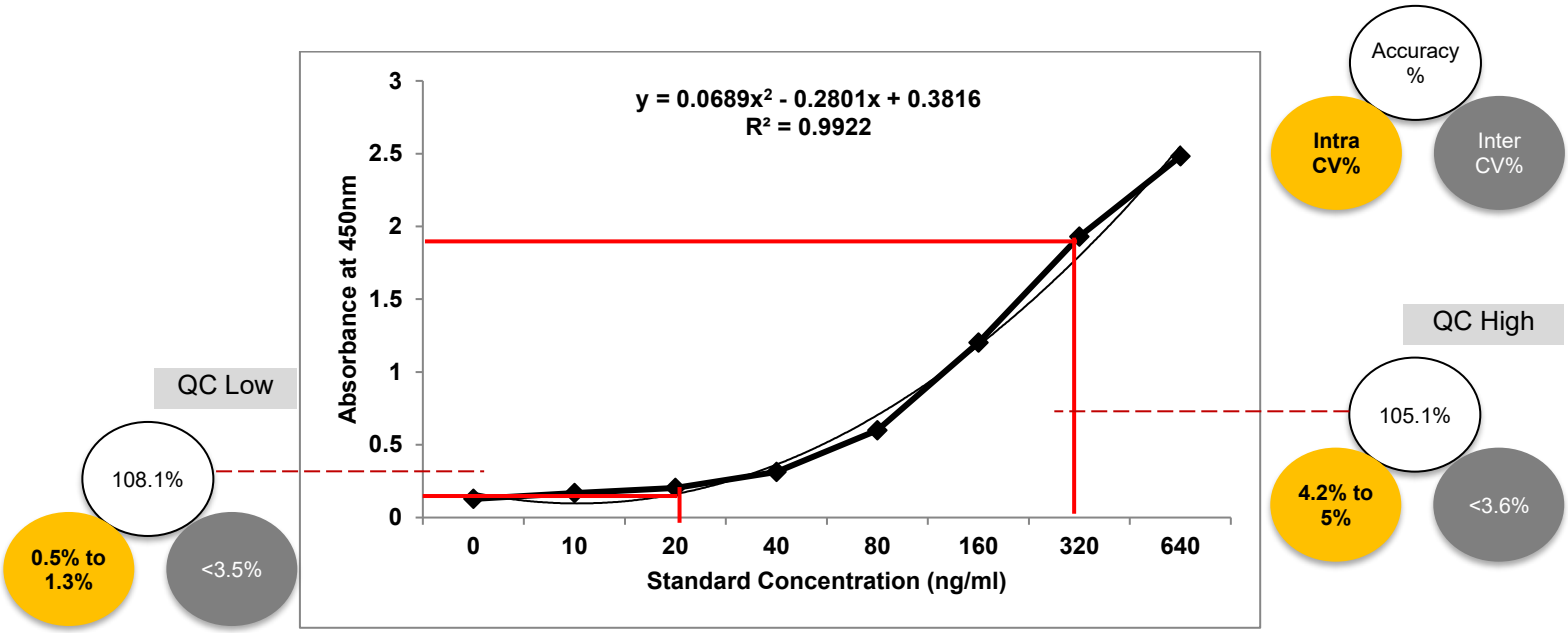


Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% **S/N = Signal / Noise Ratio*

3. Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
0	0.128	--	--
10	0.167	12.5	124.9
20	0.204	21.1	105.3
40	0.314	40	100
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
Positive Control (600 ng/ml)	2.427	538.7	89.8
Low QC Control (20 ng/ml)	0.174	21.6	108.1
High QC Control (320 ng/ml)	2.061	336.4	105.1





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

Precision was assessed by analyzing three standard concentrations (20 ng/ml, 160 ng/ml, and 640 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
20	2.4% to 4.6%	<3.5%
160	3.3% to 4.4%	<4.4%
640	0.8% to 1.8%	<2.2%

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® 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.

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® Pembrolizumab 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	320	1.916	314.3	98.2	101.8
1:4000	160	1.274	172.8	108	92.6
1:8000	80	0.655	86	107.5	93
1:16000	40	0.322	41.2	103	97.1
1:32000	20	0.201	20.4	102.2	97.8
1:64000	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
1:2000	320	2.041	335.1	104.7	95.5
1:4000	160	1.316	173.2	108.2	92.4

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:8000	80	0.631	80.3	100.3	99.7
1:16000	40	0.312	38.9	97.2	102.9
1:32000	20	0.194	19.7	98.5	101.5
1:64000	10	0.145	8.4	84.3	118.7

Results:

i. Parallelism is generally maintained across the 1:2000 to 1:32000 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® 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.

7. 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

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:1000 Human Serum)	% Standard Deviation	% CV
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	1.1
10	0.132	0.161	0	0
20	0.152	0.21	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	1.25	0.07	5.6
320	1.987	1.979	0.07	3.3
640	2.512	2.574	0.08	3.2

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® 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.

8. 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
160	1.187	1.518	1.376	1.513	1.219	11.5
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

9. 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.

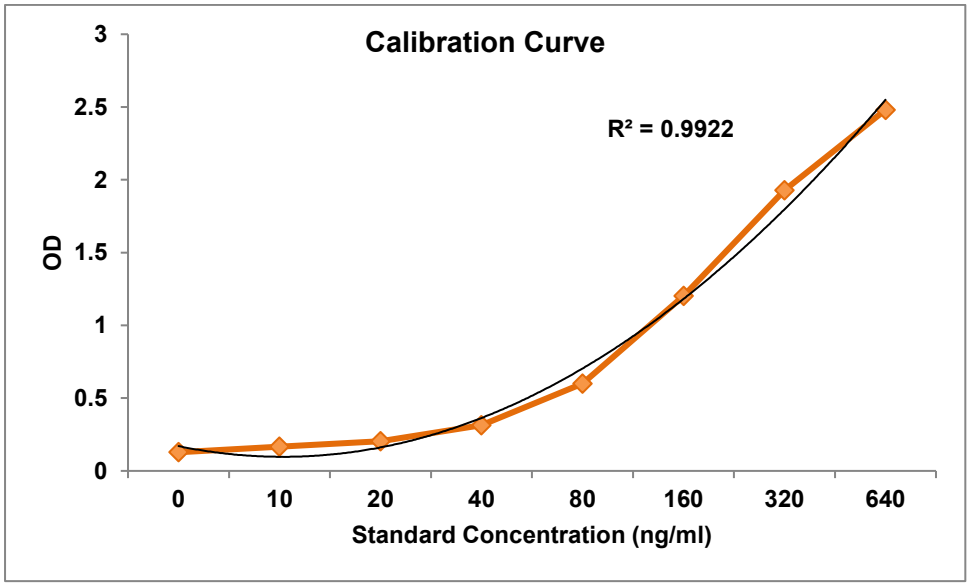
B) Handling / Storage:

- It is advisable to aliquot and stores 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 (18-25ºC). 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%

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