

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

Background

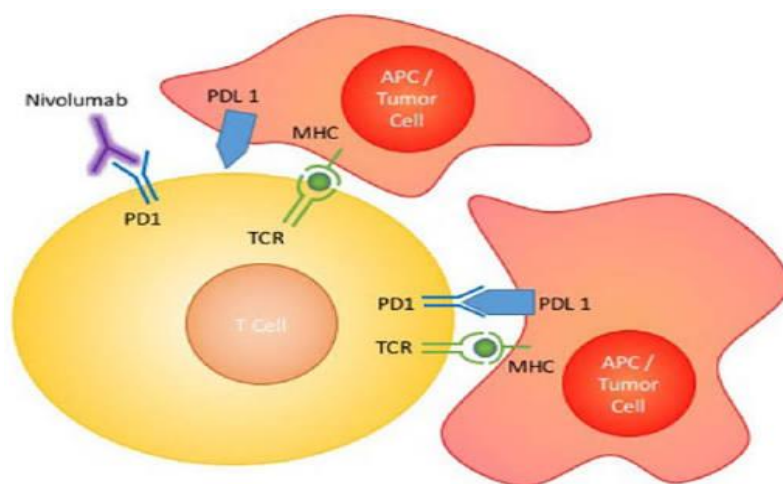
1. Introduction to Nivolumab

Nivolumab is a fully human monoclonal antibody that targets programmed cell death protein-1 (PD-1), an immune checkpoint receptor expressed primarily on activated T cells. By binding to PD-1, nivolumab blocks its interaction with PD-L1 and PD-L2, thereby preventing inhibitory signaling and restoring T-cell activity against tumor cells. Nivolumab promotes T-cell proliferation, cytokine production, and cytotoxic immune responses, enhancing immune-mediated tumor cell elimination. It is widely used in the treatment of various cancers, including melanoma, non-small cell lung cancer, renal cell carcinoma, and several other malignancies. Due to its role in immune checkpoint blockade, nivolumab has become an important therapeutic agent in cancer immunotherapy and immuno-oncology research, and measurement of nivolumab levels can be relevant for pharmacokinetic, pharmacodynamic, and therapeutic monitoring studies.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- Nivolumab targets programmed cell death protein-1 (PD-1), an immune checkpoint receptor that regulates T-cell activation and prevents excessive immune responses.
- Binding of nivolumab to PD-1 blocks its interaction with PD-L1 and PD-L2, thereby relieving inhibitory signaling and restoring T-cell-mediated antitumor activity.
- The PD-1/PD-L1 pathway is a validated therapeutic target in cancer immunotherapy, with its modulation demonstrating significant clinical benefit across multiple malignancies, including melanoma, non-small cell lung cancer, renal cell carcinoma, and other cancers.
- Investigation of PD-1/PD-L1 signaling is important for identifying mechanisms of tumor immune evasion, treatment resistance, and response to immune checkpoint inhibitors.



2.2 Assay Development

- Nivolumab is evaluated in the development and screening of immune checkpoint inhibitors through:
 - ELISA or ligand-binding assays for quantitative measurement of nivolumab concentrations in biological samples and assessment of pharmacokinetic and immunogenicity profiles.

- Cell-based bioassays to evaluate the functional activity of nivolumab by measuring restoration of T-cell activation, cytokine production, or other immune effector responses following blockade of the PD-1/PD-L1 pathway.
- PD-1/PD-L1 reporter assays to assess the ability of nivolumab to block PD-1-mediated inhibitory signaling and restore reporter activity, providing a functional measure of checkpoint inhibition.
- Flow cytometry-based assays for evaluating PD-1/PD-L1 expression and changes in immune-cell activation following nivolumab treatment.

2.3 Biomarker for Efficacy and Safety

- Nivolumab treatment can be evaluated by monitoring immune-related biomarkers, including PD-1/PD-L1 expression, T-cell activation, and inflammatory cytokine responses, which may correlate with therapeutic response.
- PD-1/PD-L1 expression and immune activation status can serve as biomarkers for predicting treatment response, immune activation, or resistance to nivolumab-based immunotherapy, particularly in cancer clinical trials.
- Monitoring nivolumab concentrations and anti-drug antibodies (ADAs) can provide important information regarding pharmacokinetics, immunogenicity, and variability in therapeutic response.
- Monitoring immune activation and inflammatory biomarkers can also support the assessment of immune-related adverse events (irAEs) associated with nivolumab and other immune checkpoint inhibitors.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Nivolumab is a fully human monoclonal antibody targeting PD-1 and serves as an important therapeutic platform for the development and evaluation of immune checkpoint inhibitors for cancer treatment.
- Development of nivolumab and nivolumab biosimilars involves comprehensive characterization of structural, binding, functional, and physicochemical properties to demonstrate similarity to the reference product.
- Biopharmaceutical development involves:
 - PD-1 binding assays to evaluate binding affinity and specificity of nivolumab to its target receptor.
 - PD-1/PD-L1 blocking assays to assess the ability of nivolumab to inhibit the interaction between PD-1 and PD-L1.
 - Cell-based bioactivity assays to evaluate functional activity through restoration of T-cell signaling, cytokine production, or other immune responses following PD-1 blockade.
- Stability, forced degradation, and comparability studies are performed to evaluate product integrity, potency, and consistency throughout development and to support preclinical and regulatory submissions.

3.2 PK/PD Studies

- Nivolumab exposure, together with pharmacodynamic markers such as PD-1/PD-L1 pathway inhibition, T-cell activation, CD8⁺ T-cell proliferation, and cytokine responses, can be evaluated as pharmacodynamic (PD) readouts in clinical trials.
- These studies support dose optimization and evaluation of the magnitude and durability of immune activation and antitumor response following nivolumab treatment.

3.3 Safety Evaluation

- Nivolumab enhances antitumor immune responses by blocking PD-1 signaling; however, excessive or uncontrolled immune activation may result in immune-related adverse events (irAEs), including inflammatory and autoimmune-like toxicities.
- Monitoring immune activation biomarkers and inflammatory cytokines is important for evaluating the safety profile of nivolumab, particularly when used alone or in combination with other immunotherapies.
- Safety profiling may include cytokine panel monitoring, immune cell subset analysis, liver and renal function assessment, and evaluation of autoimmune/inflammatory markers to identify and characterize immune-related toxicities during treatment.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- Nivolumab promotes T-cell activation by blocking PD-1-mediated inhibitory signaling. Therefore, its use in combination with cell-based immunotherapies such as CAR-T or TCR-engineered therapies may contribute to enhanced immune activation.
- Enhanced T-cell activity following PD-1 blockade may increase inflammatory cytokine production and should be considered when evaluating the risk of immune-mediated toxicities, including CRS, particularly in combination therapies.
- Monitoring inflammatory cytokines such as IL-6, IFN- γ , and TNF- α , together with immune-cell activation markers, can support risk assessment and management of CRS and other immune-related adverse events.

4.2 Immune Modulation Biomarker

- Nivolumab-mediated PD-1 blockade can be evaluated through changes in T-cell activation, proliferation, cytotoxicity, and immune-cell function during CGT studies.
- Assessment of PD-1/PD-L1 pathway activity and immune activation markers can help evaluate the therapeutic efficacy, persistence, and functional activity of engineered immune cells.
- Nivolumab can also serve as an important immune checkpoint-modulating tool for investigating mechanisms of immune exhaustion and improving antitumor responses in cancer-focused CGT platforms.

4.3 Genetic Modulation

- Genetic engineering strategies may investigate modification of the PD-1/PD-L1 pathway in CAR-T, TCR-engineered T cells, and other immune-cell therapies to enhance T-cell persistence, proliferation, and tumor-killing activity.
- Approaches may include PD-1 gene disruption or modification of checkpoint signaling pathways to reduce T-cell exhaustion and improve the functionality of engineered immune cells.
- Alternatively, combining PD-1 checkpoint blockade with genetically engineered immune cells can be explored to enhance antitumor activity and overcome immunosuppressive mechanisms within the tumor microenvironment.
- PD-1/PD-L1 pathway modulation is therefore an important strategy for optimizing immune-cell activation and persistence in advanced CGT platforms.

Scope of Validation

The KRIBIOLISA® Nivolumab (OPDIVO™) ELISA (Catalog No KBI1050) kit is 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 Nivolumab.

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 and plasma).
- Disease-State Sample Recovery.
- Matrix Effect.

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 a preview of the assay and the characteristics of the kit and is generic in nature. We 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

The KRIBIOLISA® Nivolumab (Opdivo™) ELISA kit is intended to measure the Nivolumab in serum, plasma, cell culture supernatant and other biological fluids.

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. Nivolumab Cmax Values and Recommended ELISA Range

This table summarizes Nivolumab Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected Nivolumab Range (ng/ml)	Recommended ELISA Range (ng/ml)
Healthy Baseline	Not expected / below assay detection limit	0 - 640
Cancer Patients Receiving Nivolumab	Variable; dependent on dose and sampling time	0 - 640
Pharmacokinetic (PK) Studies	Variable; generally higher than the ng/mL assay range after dosing	0 - 640
Therapeutic Drug Monitoring (TDM)	Variable depending on treatment regimen and sampling time	0 - 640

Note: Nivolumab is a therapeutic monoclonal antibody and is not expected to be present in untreated individuals. Following administration, circulating Nivolumab concentrations vary according to dose, dosing interval, sampling time, and patient-specific pharmacokinetic factors. The Nivolumab ELISA has a standard range of 0–640 ng/mL. Samples with concentrations above 640 ng/mL should be appropriately diluted and re-assayed.

The KRIBIOLISA® Nivolumab (Opdivo™) ELISA kit is developed using an assay range of 0–640 ng/mL with dilutional linearity accuracy to measure Nivolumab concentrations in relevant clinical and pharmacokinetic applications. The kit has also been validated up to 1600-fold dilution, and the measured values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity

The capture antibody and detection antibody are both specific to Nivolumab and are monoclonal antibodies. They show high affinity and specificity for binding to Nivolumab, enabling accurate and selective quantification of Nivolumab in biological samples.

2.2 Selectivity

The Nivolumab ELISA demonstrates no or low cross-reactivity with other therapeutic monoclonal antibodies and unrelated proteins, indicating high specificity for Nivolumab.

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® Nivolumab (Opdivo™) ELISA = 6.32 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® Nivolumab (Opdivo™) ELISA – 10 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:

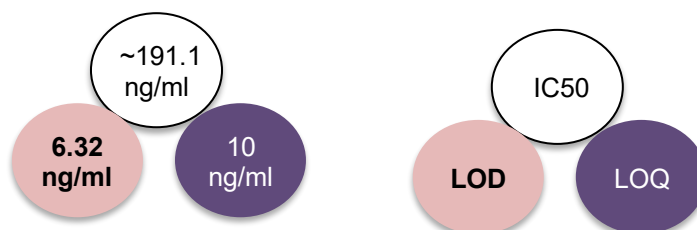
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.

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

IC50 for KRIBIOLISA® Nivolumab (Opdivo™) ELISA = ~191.1 ng/ml

Summary:

Parameter	Value (ng/mL)
LOD	6.32 ng/ml
LOQ	10 ng/ml
IC50	~191.1 ng/ml



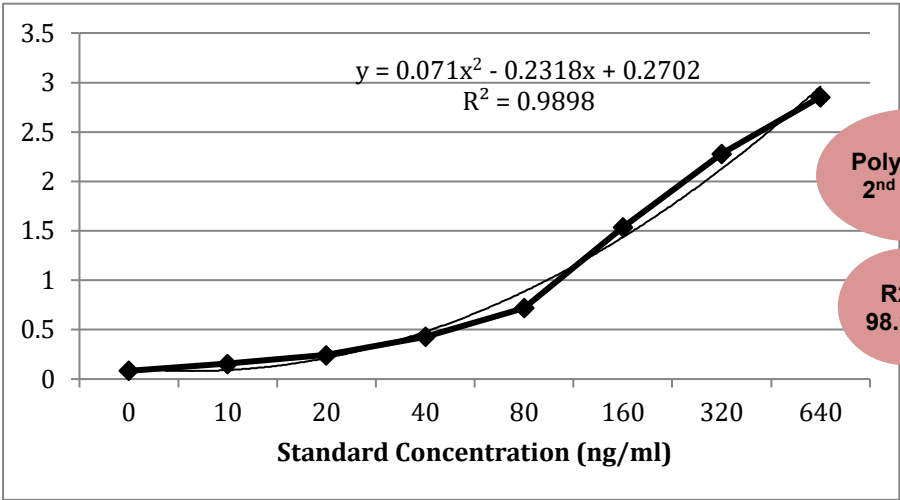
Regulatory Note:

LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20%

*S/N = Signal / Noise Ratio

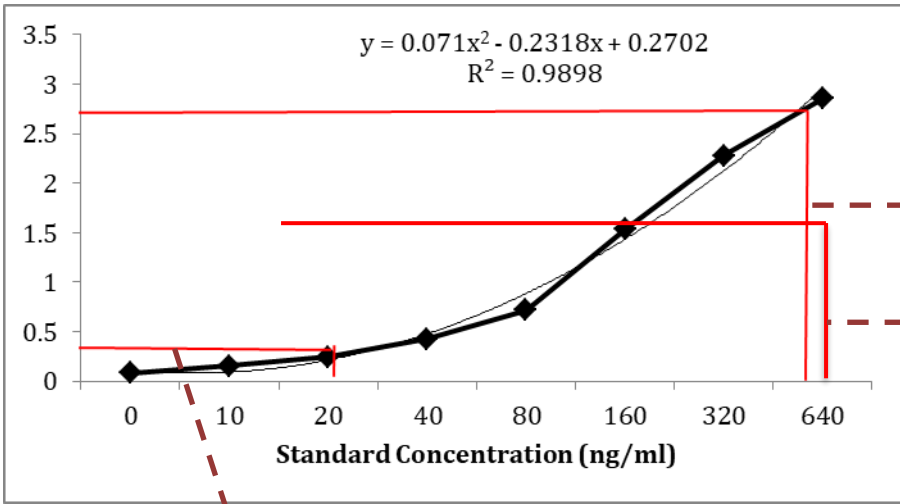
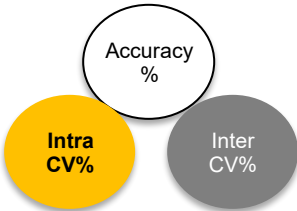
3. Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Recovery
0	0.083	--	---
10	0.154	10.6	106.3
20	0.244	23.6	118.0
40	0.428	44.1	110.3
80	0.719	73.1	91.4
160	1.534	165.8	103.6
320	2.279	315.8	98.7
640	2.854	642.2	100.3
Low QC control (20 ng/ml)	0.232	18.5	92.5
Mid QC Control (160 ng/ml)	1.485	157.8	98.6
High QC control (600 ng/ml)	2.695	598.1	99.6

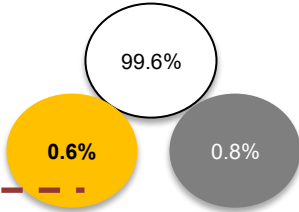


Polynomial
2nd Order

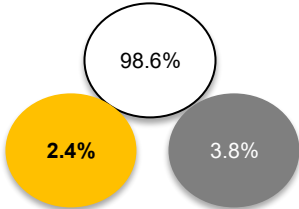
R² =
98.98%



QC High



QC Mid



QC Low

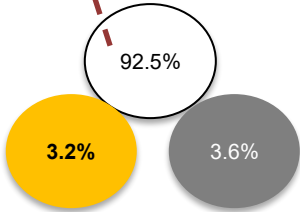


Figure 1. Standard Curve Accuracy — % Recovery Across the Assay Range

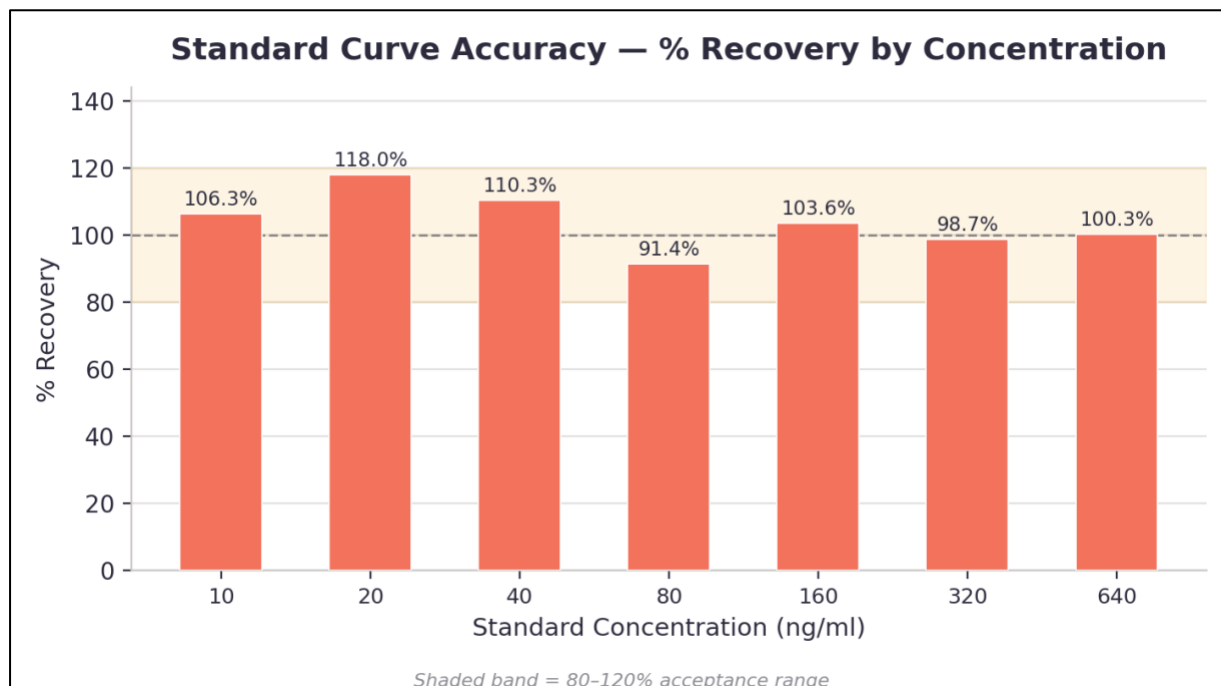
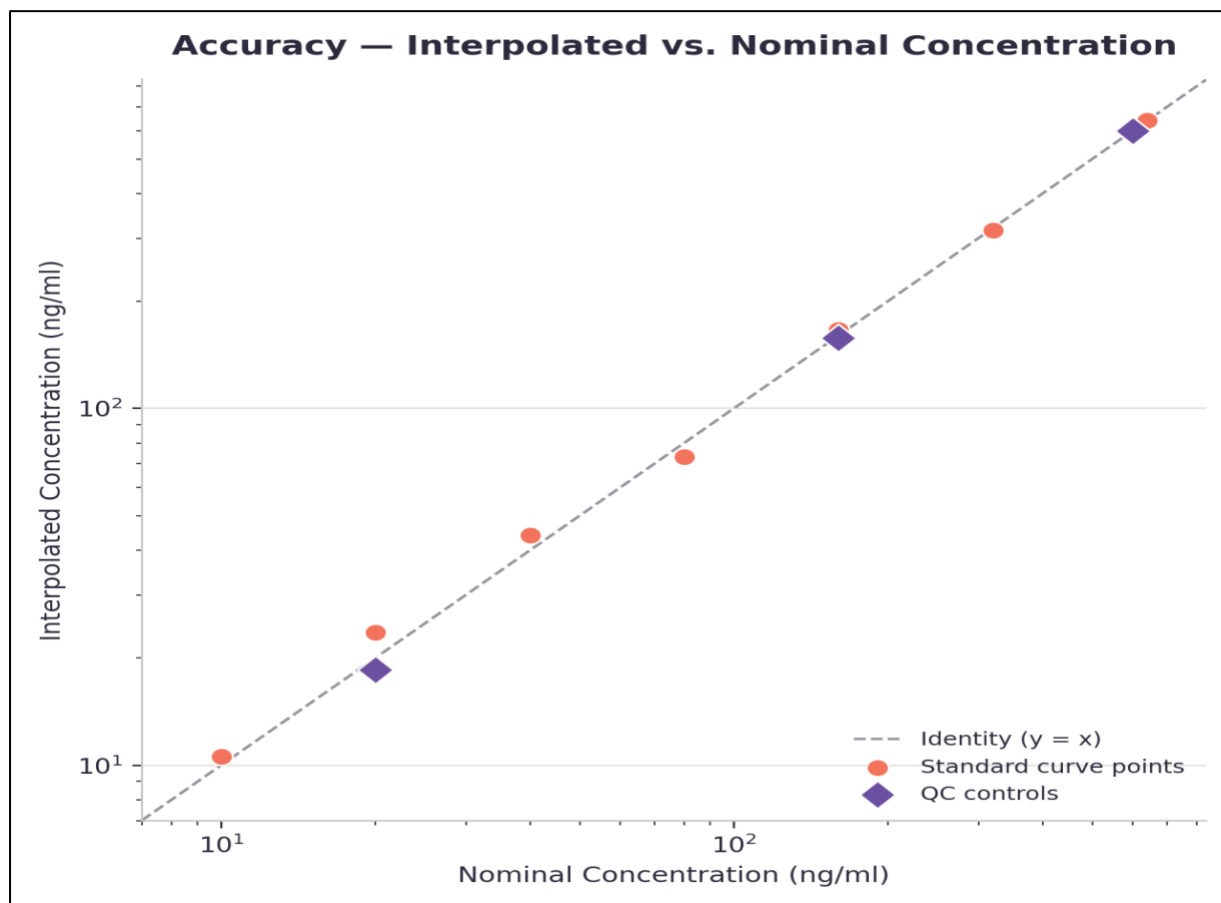


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

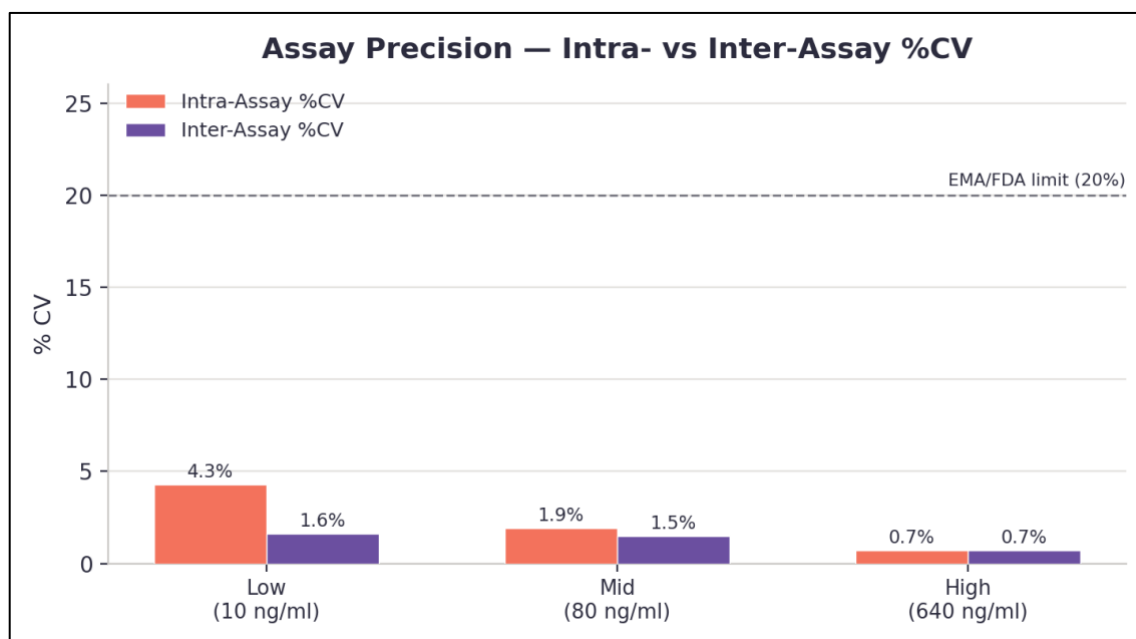
A) Intra-Assay:

Standard (ng/ml)	Mean OD450	SD	%CV
10	0.159	0.1	4.3
80	1.548	0.3	1.9
640	2.854	0.2	0.7

B) Inter assay:

Standard (ng/ml)	Mean OD450	SD	%CV
10	0.155	0.25	1.6
80	1.553	2.36	1.5
640	2.863	1.92	0.7

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



5. Parallelism and Matrix Effect

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

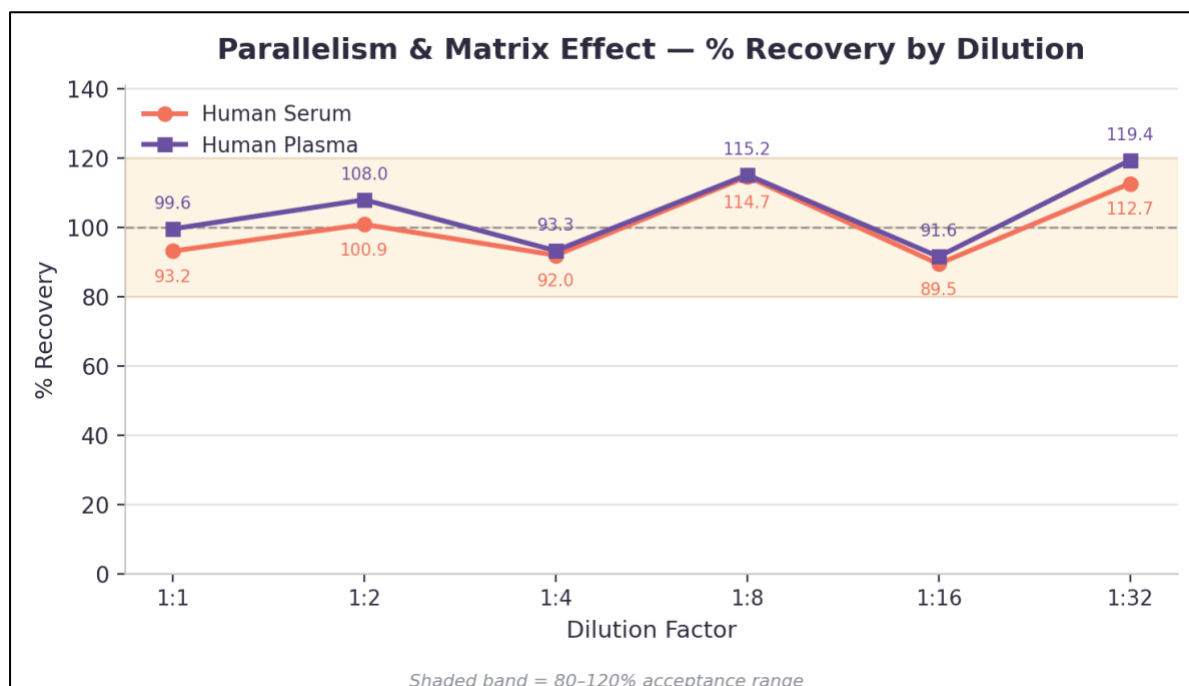
A) Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	2.219	298.31	93.2	100.7
1:2	160	1.503	161.47	100.9	94.5
1:4	80	0.723	73.56	92.0	107.3
1:8	40	0.445	45.87	114.7	109.6
1:16	20	0.278	17.90	89.5	102.7
1:32	10	0.128	11.27	112.7	56.0

B) Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	2.289	318.8	99.6	76.4
1:2	160	1.584	172.9	108.0	85.4
1:4	80	0.734	74.7	93.3	96.6
1:8	40	0.447	46.1	115.2	74.6
1:16	20	0.281	18.3	91.6	101.3
1:32	10	0.179	12.9	119.4	108.9

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



Results:

- i) Parallelism is maintained across the 1:1 to 1:32 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® Nivolumab (Opdivo™) ELISA kit was tested for matrix effect on human serum and human plasma samples.

6. Disease State Sample Recovery

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

Sample Details:

Diseased-state sample validation was performed using two clinically relevant human plasma samples from patients with lung cancer and Stage 4 non-small cell lung cancer. The samples included both male and female subjects, with ages ranging from 56 to 74 years, and were of Hispanic and Caucasian demographics. Samples included both diabetic and non-diabetic profiles, and all samples were reported as 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	Medi- cation	Blood Type/ BMI	Anticoa- gulant
NSCLC 170427 A	Lung Cancer Human Plasma (NSCLC)	Caucasian 74/ Female	Yes/No	Negative	Nil	Nil/34.3	K2 EDTA
PL1022 19N	Lung Cancer Human Plasma (NSCLC)	Caucasian 56/ Male	No/No	Negative	Nil	Nil	K2 EDTA

Acceptance

Criteria:

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

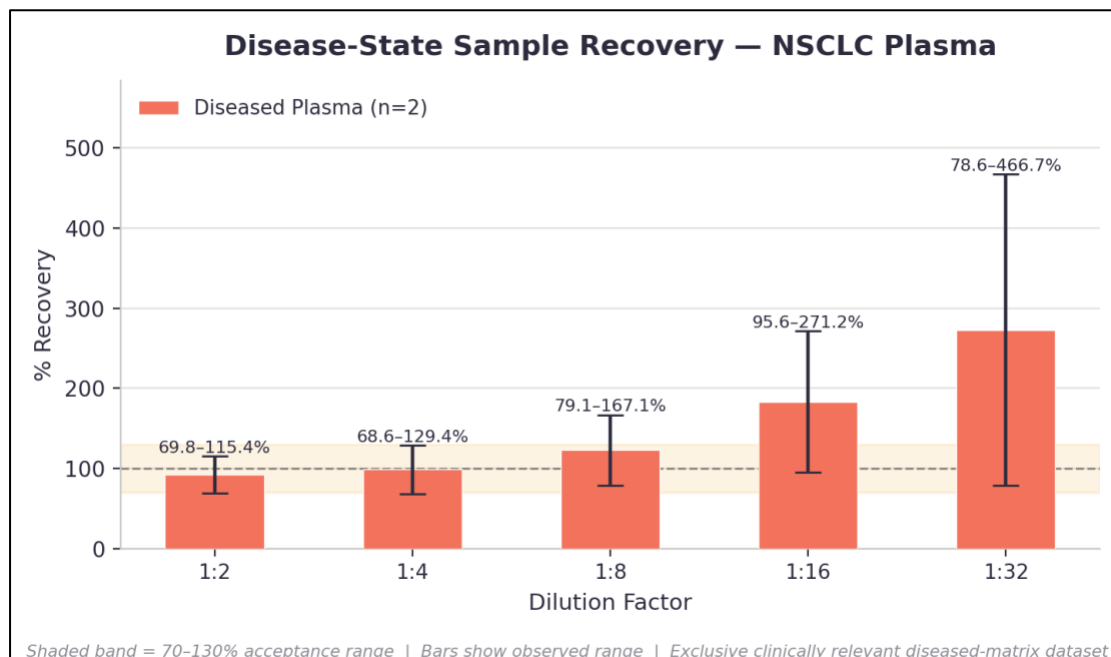
Diseased State Sample Impact on Nivolumab Recovery

Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Plasma (n=2)	69.8 - 115.4	68.6 - 129.4	79.1 - 167.1	95.6 - 271.2	78.6 - 466.7

Observations:

- Diseased plasma samples demonstrated variable recovery across the evaluated dilution range, indicating the presence of matrix-related effects influencing assay performance.
- Recovery values at lower dilution levels (1:2 and 1:4) were closer to the acceptable range, suggesting comparatively improved assay performance at these dilutions.
- Increased variability and elevated recovery values were observed at higher dilution levels (1:8 to 1:32), with recoveries extending beyond the acceptance criteria, indicating possible dilution-related non-linearity and matrix-associated interference in diseased plasma samples.
- The broad recovery ranges observed across dilutions suggest inter-sample variability within the diseased plasma matrix, which may affect accurate quantification at higher dilution factors.
- Overall, the results indicate that the assay demonstrates better reliability at lower plasma dilution ranges, while additional optimization may be required for higher dilutions to ensure consistent and accurate recovery of Pertuzumab in diseased plasma samples.

Figure 5. Disease-State Sample Recovery — Diseased Plasma (NSCLC)



7. Sample Handling and Storage Conditions

A) Sample collection and handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at temperature $<-20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

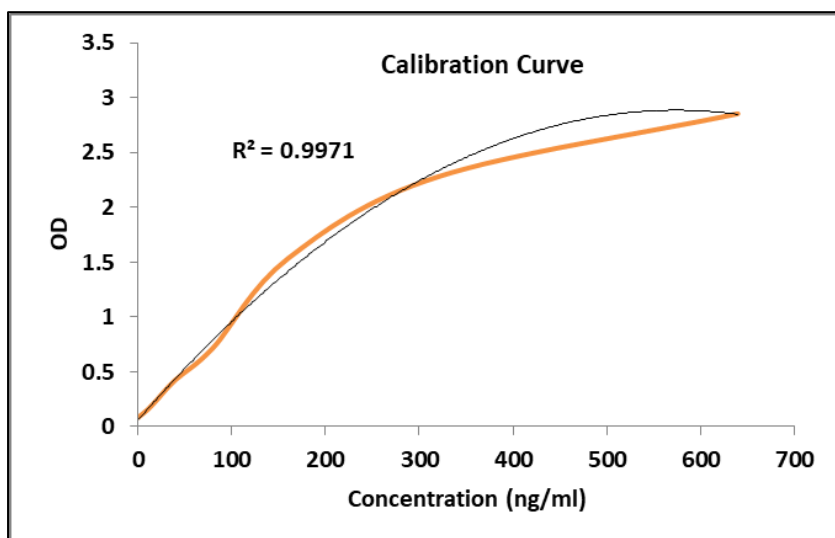
Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at $1000 \times g$. Remove serum layer and assay immediately or store serum samples at temperature $<-20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

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

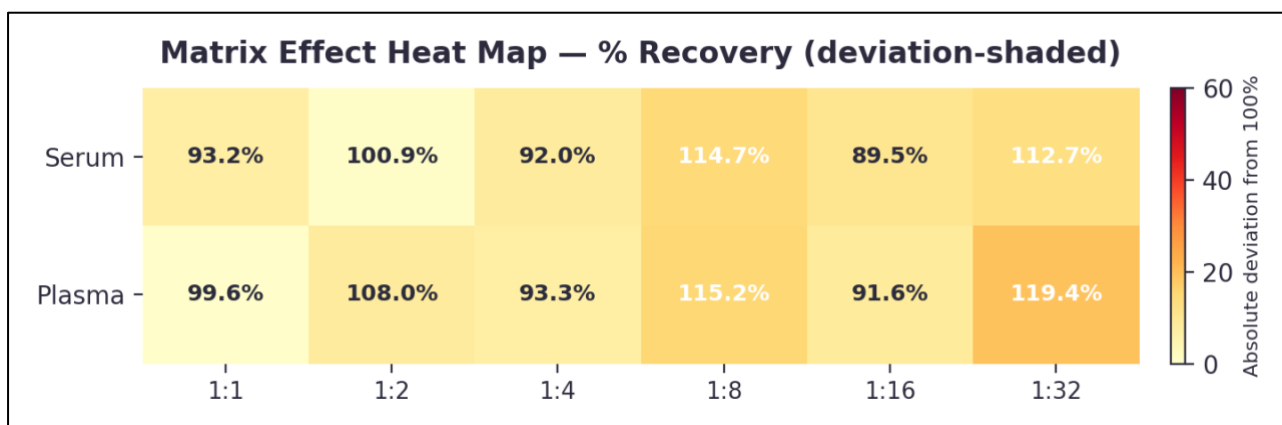
B) Storage conditions:

- Store main kit components at $2-8^{\circ}\text{C}$.
- Store recombinant lyophilized standard at $2-8^{\circ}\text{C}$. Upon reconstitution aliquot standards into polypropylene vials and store at -20°C as per assay requirements. Do not freeze thaw for more than two times.
- Before using, bring all components to room temperature ($18-25^{\circ}\text{C}$). Upon assay completion return all components to appropriate storage conditions.

Graphs, Maps and Appendices:



Matrix Effect Heat Map



	1:1	1:2	1:4	1:8	1:16	1:32
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% ²	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30% ²	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%

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