

ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN *BioSystems*

OUR REAGENTS, YOUR RESEARCH

Background

1. Introduction to Ipilimumab

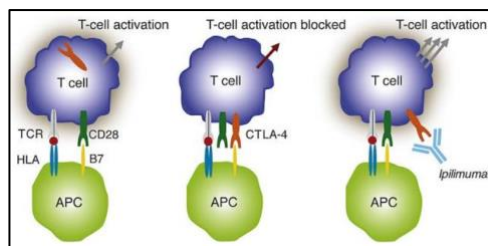
Ipilimumab is a human monoclonal antibody that targets cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an important immune checkpoint receptor involved in the regulation of T-cell activation. By binding to CTLA-4 and blocking its interaction with the B7 ligands, CD80 and CD86, Ipilimumab enhances T-cell activation and promotes antitumor immune responses.

Ipilimumab is used in the treatment of various malignancies, including melanoma and other advanced solid tumors. As an immune checkpoint inhibitor, it has emerged as an important therapeutic agent in modern oncology and immuno-oncology drug discovery and development programs.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- Ipilimumab targets cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an important immune checkpoint receptor involved in the regulation of T-cell activation and immune responses.
- CTLA-4 plays a critical role in maintaining immune homeostasis by interacting with the B7 ligands, CD80 and CD86, on antigen-presenting cells, thereby limiting excessive T-cell activation.
- Blocking the CTLA-4 signaling pathway represents an important therapeutic strategy for enhancing T-cell activation and promoting antitumor immune responses.



2.2 Assay Development

- Ipilimumab can be evaluated during drug discovery and development using various analytical and functional assays, including:
 - ELISA for the quantification of Ipilimumab in biological samples or for the assessment of relevant biomarkers.
 - Cell-based assays for evaluating the biological activity of Ipilimumab and its ability to inhibit CTLA-4-mediated immune checkpoint signaling.
 - Binding assays for assessing the interaction of Ipilimumab with the CTLA-4 receptor.
 - Pharmacokinetic and immunogenicity assays for monitoring drug concentration, exposure, and potential anti-drug antibody responses.

2.3 Biomarkers for Efficacy and Safety

- Ipilimumab-related biomarkers may be used to evaluate target engagement, pharmacodynamic activity, and therapeutic response.
- Evaluation of CTLA-4 pathway-related biomarkers and immune-cell activity may support the assessment of treatment response and immune activation.
- Pharmacokinetic, immunogenicity, and safety assessments are important components of the preclinical and clinical development of Ipilimumab.
- Monitoring immune-related effects and treatment-associated adverse events is important when evaluating the overall safety profile of Ipilimumab.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilar Development

- Ipilimumab is a fully human monoclonal antibody that targets cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an immune checkpoint receptor involved in the regulation of T-cell activation.
- The development and characterization of therapeutic monoclonal antibodies and potential biosimilar products require comprehensive analytical, physicochemical, and functional evaluation, including:
 - Binding assays to assess affinity and interaction with the CTLA-4 receptor.
 - Cell-based potency assays to evaluate the biological activity of the antibody and its ability to block CTLA-4-mediated immune checkpoint signalling.
 - Physicochemical and biological comparability studies to demonstrate consistency between different product batches or candidate products.
 - Pharmacokinetic and immunogenicity assessments as part of product development and regulatory evaluation.

3.2 PK/PD Studies

- Ipilimumab concentrations may be monitored during preclinical and clinical studies to characterize its pharmacokinetic profile.
- Pharmacodynamic assessments may include the evaluation of target engagement and changes in biomarkers associated with CTLA-4 pathway modulation and immune activation.
- PK/PD studies can help establish the relationship between drug exposure, biological activity, therapeutic response, and safety.

3.3 Safety Evaluation

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

4. Importance in Cell and Gene Therapy (CGT)

4.1 Therapeutic Development and Safety Monitoring

- Ipilimumab is an immune checkpoint inhibitor targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and is not a primary cytokine mediator of cytokine release syndrome (CRS).
- However, appropriate safety monitoring is important when evaluating Ipilimumab in combination with cellular or gene-based therapeutic approaches.
- The assessment of treatment-related immune responses, T-cell activation, adverse events, and other relevant safety parameters is important during preclinical and clinical development.

4.2 Target Engagement and Biomarker Evaluation

- Ipilimumab-related studies may be relevant for evaluating CTLA-4 expression and CTLA-4 pathway activity in cellular models used for therapeutic research.
- The assessment of CTLA-4 pathway-related biomarkers, including CD80 and CD86 expression where relevant, may support the evaluation of target engagement, biological activity, and therapeutic response.
- Quantification of Ipilimumab may also support pharmacokinetic and pharmacodynamic studies during therapeutic development.

4.3 Genetic and Cellular Engineering Research

- Genetic and cellular engineering approaches may be used to develop experimental models with altered CTLA-4 expression or related immune checkpoint pathway activity to study the mechanism of action and biological activity of Ipilimumab.
- Such models can support the investigation of immune checkpoint regulation, T-cell activation, treatment response, and potential mechanisms of therapeutic resistance.
- Cellular models may also be used to investigate the effects of CTLA-4 pathway modulation on immune cell activation and antitumor immune responses.
- These studies may contribute to the development of personalized therapeutic strategies and the identification of patient populations that may benefit from CTLA-4-targeted immunotherapies

Scope of Validation

The KRIBIOLISA® Ipilimumab (YERVOY™) ELISA Kit (Catalog No. KBI1025) has been considered by us during the validation of this assay in accordance with applicable FDA/ICH guidelines for bioanalytical method validation. This document is prepared based on tests performed in our laboratory and does not necessarily seek to cover all testing that may be required at the user's end for registration or regulatory submissions. The objective of this validation is to demonstrate that the assay is suitable for its intended purpose—the detection and quantification of Ipilimumab in appropriate biological samples.

The validation characteristics considered by us are listed below:

- Assay Validation.
- Standard Curve.
- Pharmacokinetic Relevance.
- Precision and Reproducibility.
- Parallelism.
- Diseased-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 the user's laboratory. This data has been generated to enable the user to obtain an overview of the assay and its performance characteristics and is generic in nature. We recommend that the user performs, at a minimum, a spike and recovery assay to assure quality results.

For more comprehensive validation, the user may perform additional studies as appropriate to establish quality control parameters for the intended application.

For any queries or support regarding the data and its performance, please contact us at sales1@krishgen.com.

Intended Use of the ELISA

The KRIBIOLISA® Ipilimumab (YERVOY™) ELISA Kit is intended for the quantitative measurement of Ipilimumab in appropriate biological samples, including human serum and plasma, for research, pharmacokinetic and bioanalytical applications.

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. Ipilimumab Pharmacokinetic Relevance and Recommended ELISA Range

The following section summarizes the pharmacokinetic relevance of Ipilimumab and the recommended working range of the KRIBIOLISA® Ipilimumab (YERVOY™) ELISA Kit (Catalog No. KBI1025).

Application	Expected Ipilimumab Concentration	Recommended ELISA Working Range
Bioanalytical and Pharmacokinetic Studies	Concentrations may vary depending on dose level, treatment regimen, combination therapy, and individual patient characteristics.	0–640 ng/ml
Therapeutic Exposure Following 3 mg/kg Intravenous Dosing	Approximately 50–70 µg/ml C _{max} .	Appropriate sample dilution recommended before analysis.
Higher-Dose Therapeutic Exposure	Approximately 150–200 µg/ml C _{max} following a 10 mg/kg intravenous dose.	Appropriate sample dilution recommended before analysis.
Repeated Dosing / Steady-State Exposure	Minimal accumulation may be observed, with peak concentrations generally remaining within a similar exposure range.	Appropriate sample dilution recommended before analysis.
Therapeutic Monitoring and Exposure Assessment	Variable depending on dose, indication, treatment regimen, combination therapy, and patient-specific factors.	Samples should be diluted, where necessary, to fall within the validated assay range.

Note: The KRIBIOLISA® Ipilimumab (YERVOY™) ELISA Kit has an assay concentration range of 0–640 ng/ml. Due to the relatively high circulating concentrations of Ipilimumab following therapeutic administration, clinical serum or plasma samples may require appropriate dilution before analysis to ensure that the measured concentration falls within the linear dynamic range of the assay.

The assay demonstrates measurable sensitivity within the 20–640 ng/ml range, with an approximate LLOQ of 20 ng/ml. The assay is therefore suitable for pharmacokinetic profiling, dose–exposure analysis, and therapeutic monitoring following appropriate sample dilution.

2. Specificity and Selectivity

2.1 Specificity

The KRIBIOLISA® Ipilimumab (YERVOY™) ELISA employs an Anti-Ipilimumab Monoclonal Antibody as the capture protein to facilitate the selective detection of Ipilimumab.

Ipilimumab is specifically recognized and captured by the Anti-Ipilimumab Monoclonal Antibody pre-coated onto the microwell surface through high-affinity interactions with epitopes present on the Ipilimumab molecule.

2.2 Selectivity

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

2.3 LOD, LOQ and IC₅₀

LOD (Limit of Detection)

The lowest analyte concentration that can be reliably distinguished from blank/background noise but not necessarily quantified precisely.

Statistically:

LOD = Mean of Blank + 3X SD of Blank
(3σ criterion is most common).

The approximate LOD for the KRIBIOLISA® Ipilimumab (YERVOY™) ELISA is 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).

The approximate LOQ/LLOQ for the KRIBIOLISA® Ipilimumab (YERVOY™) ELISA is 20 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.

For the KRIBIOLISA® Ipilimumab (YERVOY™) ELISA validation, the observed IC₅₀ value is 191.1 ng/ml

Summary:

Parameter	Value (ng/ml)
LOD	~6.32 ng/ml
LOQ	~20 ng/ml
IC ₅₀	~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 (ng/ml)	% Recovery
0	0.110	--	--
20	0.308	18.8	94.0
40	0.615	42.1	105.2
80	1.055	79.2	99.0

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
160	1.695	157.3	98.3
320	2.340	330.3	103.2
640	2.715	624.9	97.6
Low QC (20 ng/ml)	0.303	18.3	91.7
Mid QC (500 ng/ml)	2.524	433.3	86.7
High QC (600 ng/ml)	2.601	535.5	89.3

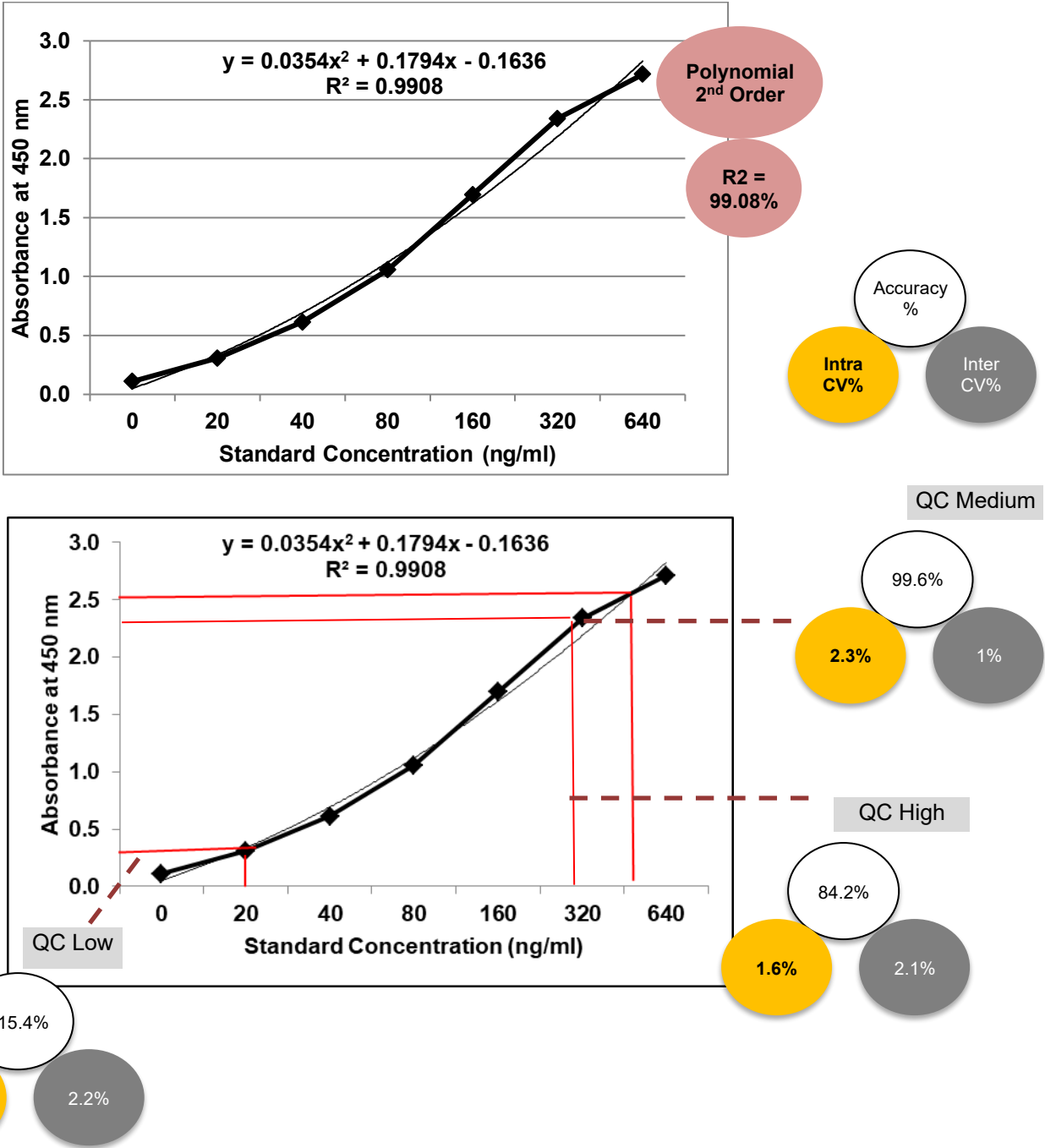


Figure 1. Standard Curve Accuracy — % Recovery Across the Assay Range

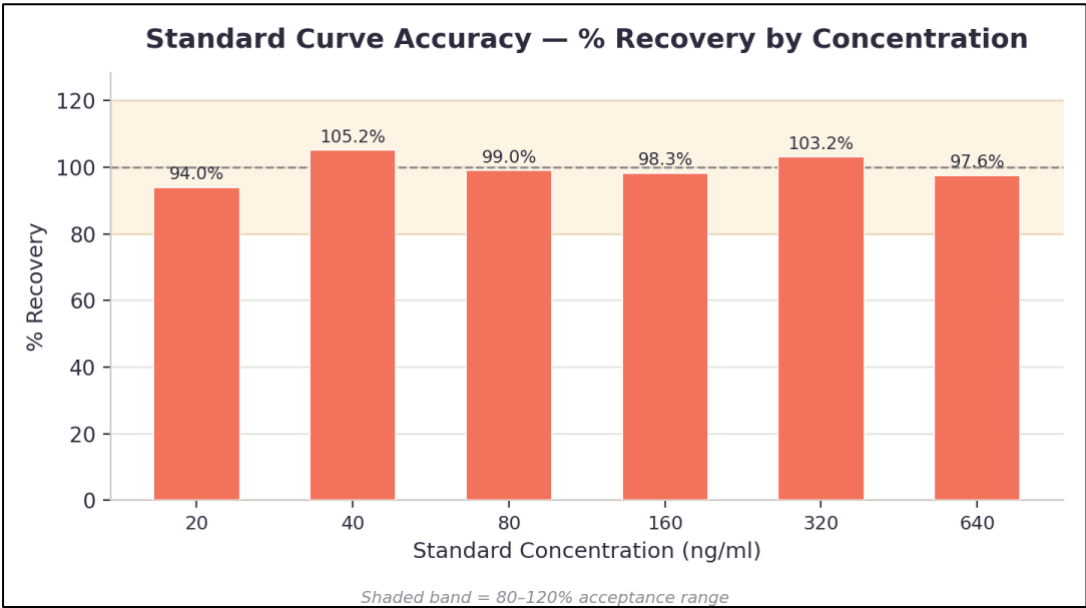
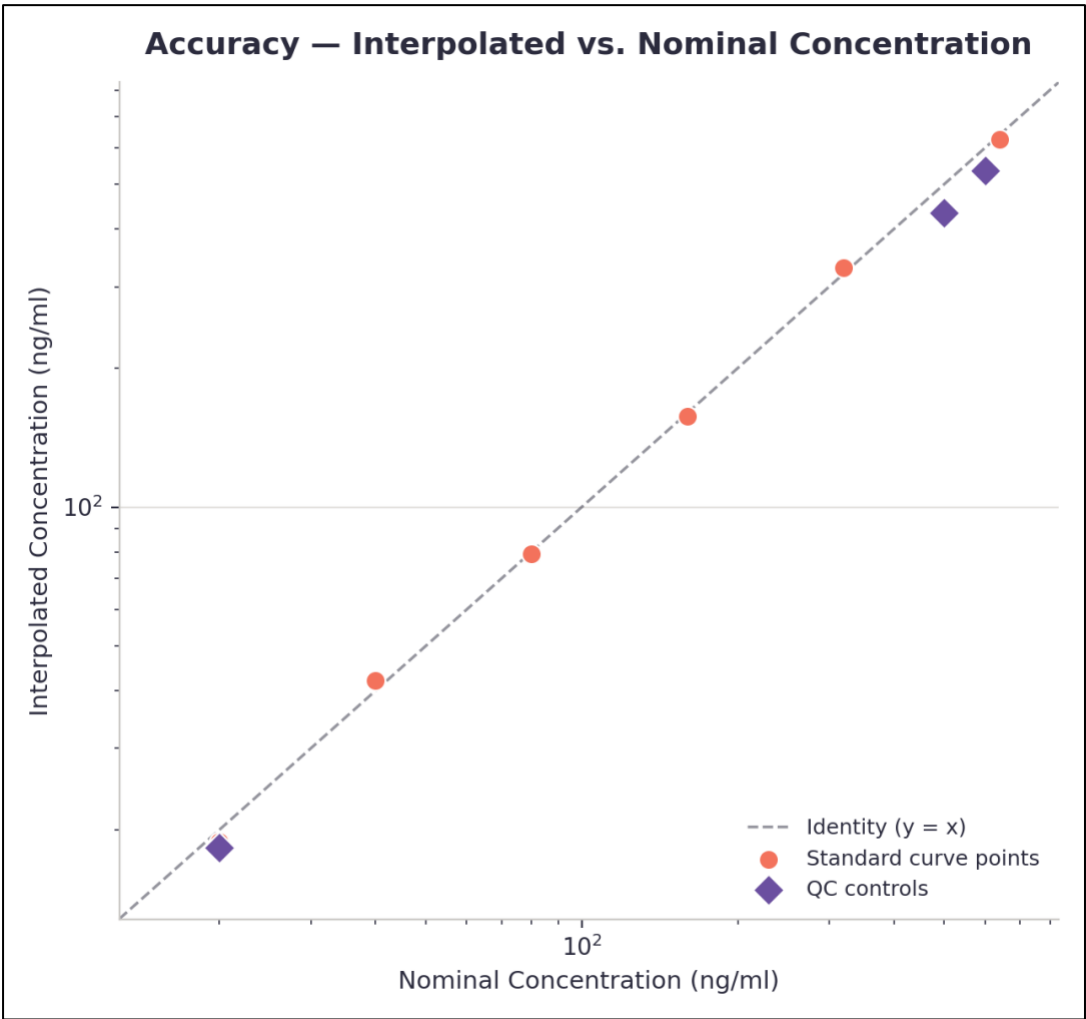


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

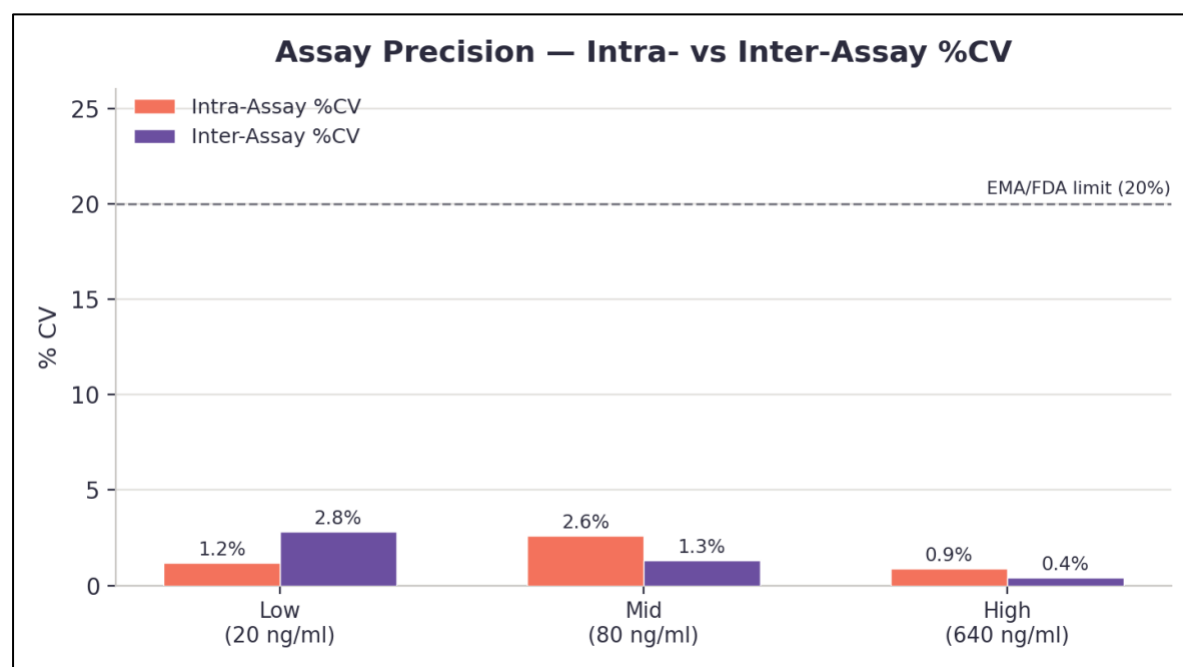
A) Intra-Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
20	0.311	0.00	1.2
80	1.083	0.03	2.6
640	2.702	0.02	0.9

B) Inter Assay:

Standard Concentration (ng/ml)	Mean OD450	SD	%CV
20	0.324	0.02	2.8
80	1.079	0.01	1.3
640	2.709	0.01	0.4

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



4. Parallelism and Matrix Effect

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

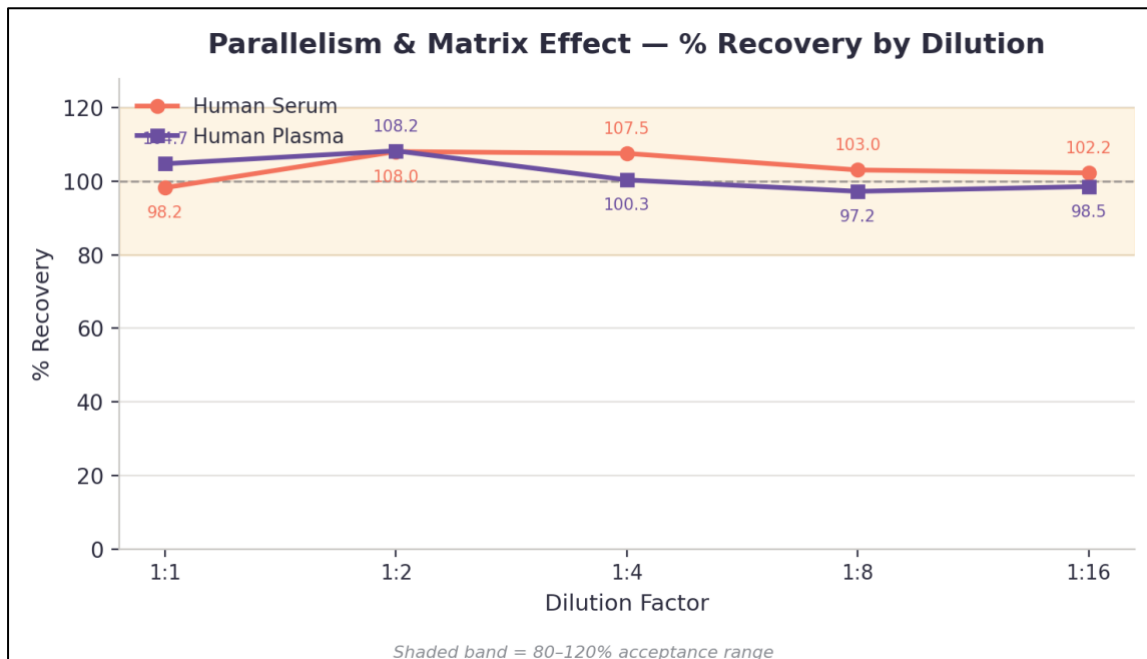
A) Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	2.354	314.3	98.2	101.8
1:2	160	1.589	172.8	108.0	92.6
1:4	80	1.054	86.0	107.5	93.0
1:8	40	0.605	41.2	103.0	97.1
1:16	20	0.302	20.4	102.2	97.8

B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	320	2.365	335.1	104.7	95.5
1:2	160	1.625	173.2	108.2	92.4
1:4	80	1.068	80.3	100.3	99.7
1:8	40	0.598	38.9	97.2	102.9
1:16	20	0.295	19.7	98.5	101.5

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



Results:

- i) Parallelism is maintained across the 1:1 to 1:16 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® Ipilimumab ELISA kit was tested for matrix effect on human serum and human plasma samples.

5. Disease State Sample Recovery

Disease Samples:

Diseased-state sample validation was performed using three clinically relevant human biological matrix samples (recovered plasma and off-the-clot serum) from patients with non-small cell lung cancer (NSCLC, Stage 4) and chronic myelogenous leukemia (CML). The samples included both male and female subjects, with ages ranging from 51 to 74 years, and all samples were of Caucasian demographic. 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	Human Plasma, Non-Small Lung Cancer, Stage 4	Caucasian 74/ Female	Yes/No	Negative	Nil	Nil /34.3	K2 EDTA
NSCLC 170427 B	Human Plasma, Non-Small Lung Cancer, Stage 4	Caucasian 51/ Male	No/No	Negative	Nil	Nil /25.4	K2 EDTA
DS-03	Human Serum, Disease Chronic Myelogenous Leukemia (CML)	Caucasian 60/Male	No/No	Negative	Nil	Nil	Nil

Acceptance Criteria:

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

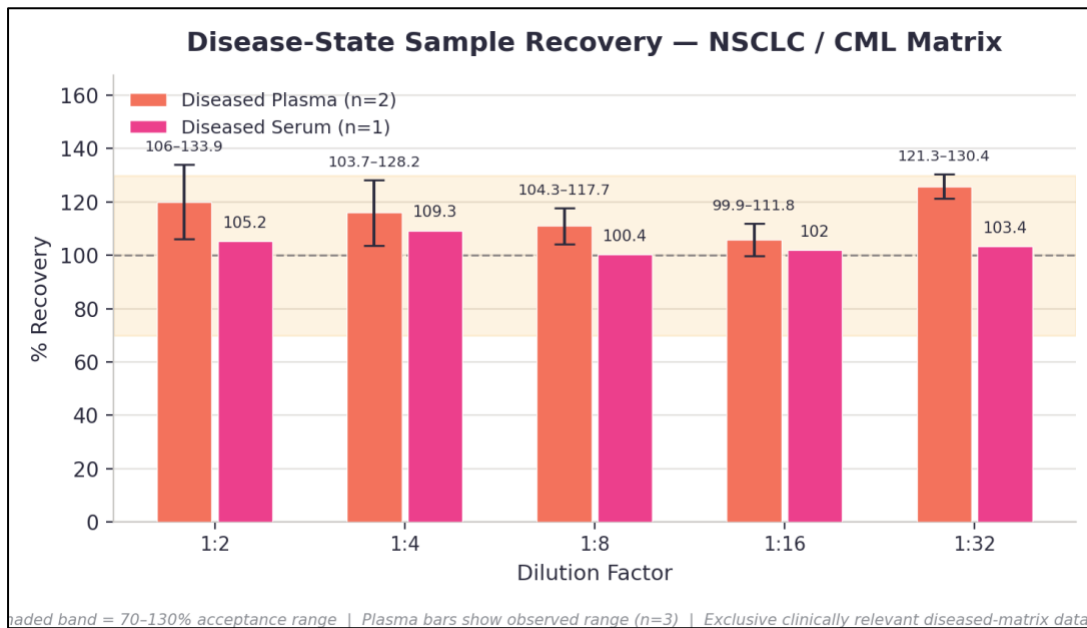
Diseased State Sample Impact on Ipilimumab Recovery

Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Serum (n=1)	105.2	109.3	100.4	102	103.4
Diseased Plasma (n=2)	106 –133.9	103.7 –128.2	104.3 - 117.7	99.9 – 111.8	121.3 -130.4

Observations:

- Recovery in diseased serum remained within the typical acceptance range across all dilutions.
- Recovery in diseased plasma remained within the typical acceptance range across all dilutions.
- The observed trend suggests that no matrix effects are observed for analyte detection in diseased samples.

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



6. 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.

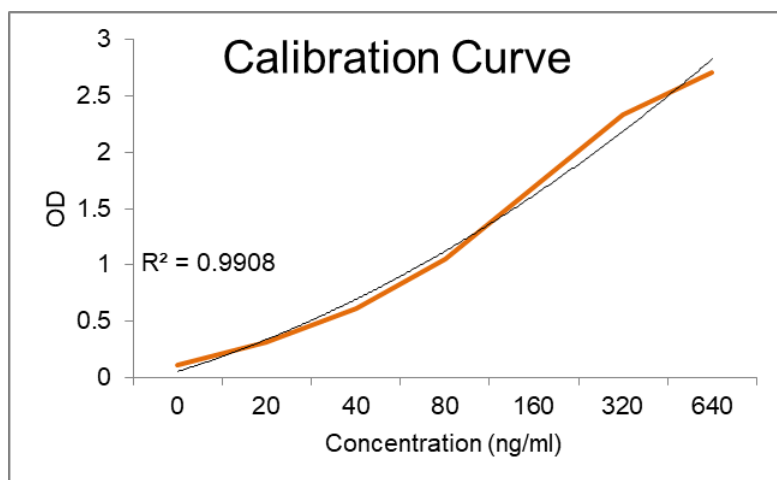
Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at $1000 \times g$. Remove serum layer and assay immediately or store serum samples at temperature $<-20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

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.

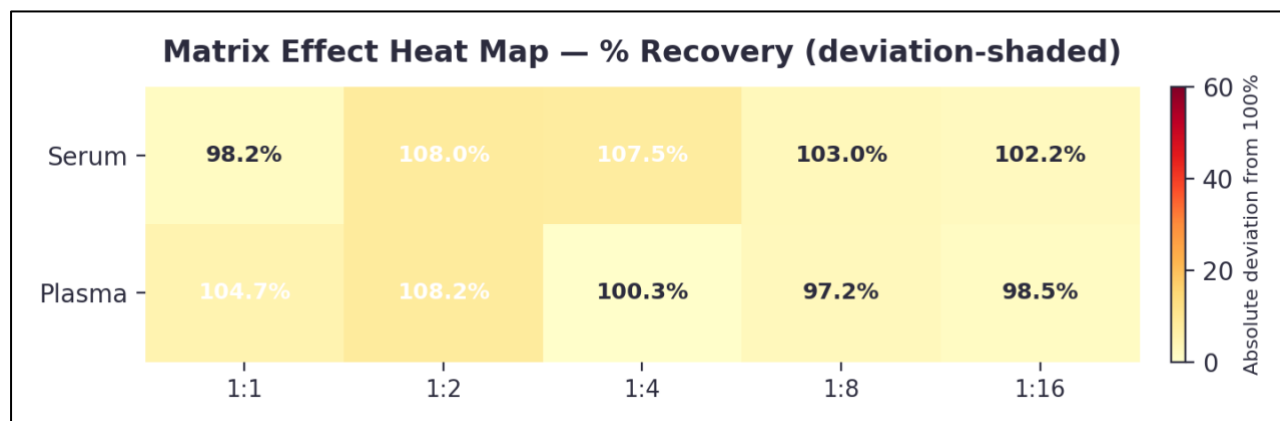
B.) Storage conditions:

- 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
Serum					
Plasma					

Determined Limits for Acceptance according to EMA/FDA and CLSI regulations

	Limits for Acceptance (EMA/FDA)	Determined Limits for Acceptance (CLSI)
Intra Precision	CV < 20% (25% at LLOQ)	-
Inter Precision	CV < 20 % (25% at LLOQ)	-
Accuracy at LLOQ	Recovery 100 ± 20%	-
Total Error (TE)	TE < 30% (40% at LLOQ and ULOQ)	-
Specificity/Interference	Recovery 100 ± 25%	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30%	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%

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