ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH,
BIOPHARMA APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

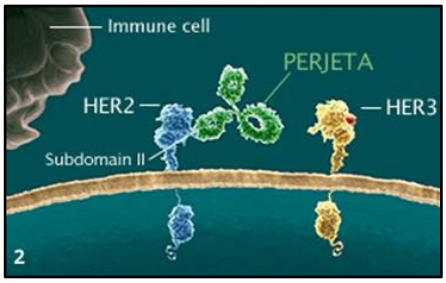
Background

1. Introduction to Pertuzumab

Pertuzumab is a recombinant humanized monoclonal antibody primarily used for the treatment of HER2-positive cancers, particularly breast cancer. It specifically targets the extracellular domain II of the human epidermal growth factor receptor 2 (HER2), thereby inhibiting HER2 dimerization with other members of the HER receptor family, especially HER3. Pertuzumab interferes with HER2-mediated signaling pathways involved in tumor cell proliferation, survival, and growth. In addition to its direct signaling-inhibitory effects, Pertuzumab can promote antibody-dependent cellular cytotoxicity (ADCC) through interaction with immune effector cells. Due to its targeted mechanism of action, Pertuzumab is widely used in combination with other anti-HER2 therapies for the management of HER2-positive breast cancer and has become an important therapeutic agent in oncology research, cancer treatment, and HER2-targeted drug development.

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation



- Pertuzumab specifically targets HER2 (Human Epidermal Growth Factor Receptor 2) and binds to extracellular domain II, blocking HER2 dimerization and downstream signaling involved in tumor cell proliferation and survival.
- HER2 overexpression or amplification is associated with several cancers, particularly HER2-positive breast cancer, as well as gastric, gastroesophageal, and other solid tumors, making HER2 an important therapeutic target and biomarker.
- Targeting HER2 with Pertuzumab has been clinically validated through inhibition of HER2-mediated signaling and enhancement of anti-tumor immune responses, supporting its application in HER2-targeted oncology and therapeutic development.

2.2 Assay Development

- Pertuzumab can be evaluated for target binding and functional activity through:
  - ELISA or ligand-binding assays for quantification of Pertuzumab binding to recombinant HER2/ErbB2, particularly its extracellular domain II.
  - Cell-based bioassays using HER2-positive cells to assess inhibition of HER2 signaling, proliferation, or survival following Pertuzumab treatment.
  - Phosphorylation assays such as pHER2, pAKT, and pERK measurement to evaluate inhibition of downstream PI3K/AKT and MAPK/ERK signaling pathways.
  - Cell-based proliferation or viability assays to determine the anti-proliferative activity of Pertuzumab in HER2-dependent tumor cells.

2.3 Biomarker for Efficacy and Safety

- HER2 expression/amplification is strongly associated with response to Pertuzumab, with HER2-positive tumors being the primary population in which its therapeutic efficacy is evaluated.
- HER2 receptor occupancy, inhibition of HER2 dimerization, and reduction in downstream signaling markers such as pHER2, pAKT, and pERK can serve as pharmacodynamic indicators of Pertuzumab activity.
- Monitoring tumor-cell proliferation, viability, and tumor burden can help assess the therapeutic efficacy and response to Pertuzumab, particularly in HER2-positive cancers.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Pertuzumab is a recombinant humanized monoclonal antibody targeting HER2 (ErbB2), primarily used in HER2-positive cancers.
- HER2–Pertuzumab binding assays such as ELISA or ligand-binding assays are used to evaluate target specificity and binding affinity.
- Cell-based bioactivity assays using HER2-positive cell lines assess inhibition of HER2 signaling and tumor-cell proliferation.
- Comparability and stability studies evaluate potency, purity, structural integrity, and consistency of Pertuzumab and its biosimilars for preclinical and regulatory submissions.

3.2 PK/PD Studies

- Pharmacokinetic (PK) and pharmacodynamic (PD) studies evaluate Pertuzumab serum concentrations and exposure parameters such as Cmax, AUC, half-life, and clearance, along with HER2 target engagement and inhibition of downstream signaling markers such as pHER2, pAKT, and pERK.
- These studies support dose and dosing-interval optimization by establishing the relationship between Pertuzumab exposure, target inhibition, and therapeutic response.

3.3 Safety Evaluation

- Pertuzumab may cause infusion-related reactions, diarrhea, and cardiac toxicity, particularly through effects on HER2 signaling in cardiac tissue.
- Monitoring of cardiac function and clinical safety parameters is critical, especially when pertuzumab is used in combination with trastuzumab or chemotherapy.
- Safety profiling includes cardiac function assessment (LVEF), liver function tests, hematological parameters, infusion-reaction monitoring, and evaluation of adverse events.

4. Importance in Cell and Gene Therapy (CGT)

4.1 HER2-Targeted Cell Therapy

- Pertuzumab targets the HER2 receptor and can be used as a reference molecule for evaluating HER2-directed immune and cell-based therapies.
- In HER2-targeted CAR-T, CAR-NK, or TCR-based approaches, pertuzumab binding and HER2 expression can support the assessment of target-specific recognition and therapeutic activity.

4.2 Biomarker and Target Validation

- Quantification of HER2 expression is important for evaluating target availability and selecting suitable cell models or patients for HER2-directed CGT approaches.
- Pertuzumab can be used in binding, competition, and target-engagement studies to characterize HER2-targeted therapeutic strategies.

4.3 Combination and Immune-Modulation Studies

- Pertuzumab may be investigated in combination with cell-based therapies to enhance HER2-directed antitumor activity through complementary mechanisms.
- Monitoring HER2 expression, immune-cell activation, cytokine release, and tumor cell killing can help evaluate efficacy and potential immune-related effects.

Scope of Validation

The KRIBIOLISA® Pertuzumab (PERJETA™) ELISA (Catalog No KBI1086) 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 Pertuzumab.
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

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® Pertuzumab (PERJETA™) ELISA kit is intended to measure Pertuzumab in serum, plasma, cell culture supernatant and other biological fluids.

Principle of the Assay

This KRIBIOLISA® Pertuzumab (PERJETA™) 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. Pertuzumab Cmax Values and Recommended ELISA Range

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

Application	Expected Pertuzumab Cmax (ng/ml)	Recommended ELISA Range (ng/ml)
Healthy Baseline	Below quantifiable level	0 – 2560
HER2-Positive Breast Cancer-Loading Dose	180,000 – 300,000	Appropriate sample dilution recommended before analysis.
HER2-Positive Breast Cancer-Maintenance Dose	180,000 – 230,000	Appropriate sample dilution recommended before analysis.
Therapeutic Drug Monitoring	60000 - 300,000	Samples should be diluted, where necessary, to fall within the validated assay range.

The KRIBIOLISA® Pertuzumab (PERJETA™) kit is developed using an assay range of 40 - 2560 ng/ml with the dilutional linearity accuracy to measure responses as per the application table above on patient Cmax values. The kit has also been validated upto 1600 fold dilution and the values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity

The capture antibody and detection antibody are both specific to Pertuzumab and are monoclonal antibodies. They exhibit high affinity and specificity for native as well as recombinant Pertuzumab, enabling its accurate and quantitative detection in biological samples.

2.2 Selectivity

The ELISA is designed to specifically detect Pertuzumab with minimal or no cross-reactivity toward structurally unrelated therapeutic monoclonal antibodies and other proteins that may be present in biological samples.

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® Pertuzumab ELISA = 5.45 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® Pertuzumab (PERJETA™) ELISA – 40 ng/ml

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

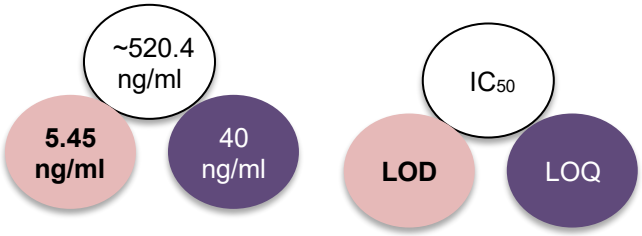
IC_{50} = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

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® Pertuzumab (PERJETA™) ELISA = ~520.4 ng/ml

Summary:

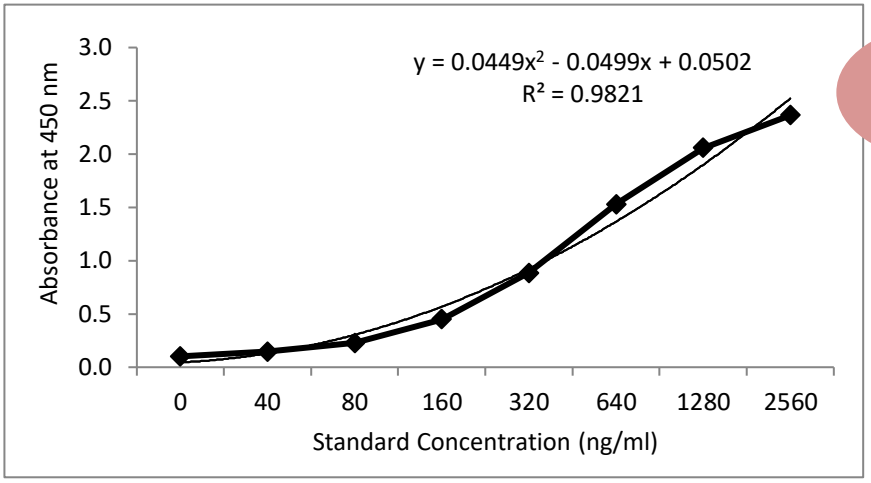
Parameter	Value (ng/ml)
LOD	5.45 ng/ml
LOQ	40 ng/ml
IC_{50}	~520.4 ng/ml



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	0.102	--	--
40	0.146	39.5	98.8
80	0.228	79.0	98.7
160	0.448	161.5	100.9
320	0.883	317.8	99.3
640	1.529	644.9	100.8
1280	2.058	1266.2	98.9
2560	2.364	2586.1	101.0
Low QC control (100 ng/ml)	0.295	105.9	105.9
Mid QC control (600 ng/ml)	1.422	574.9	95.8
High QC control (2500 ng/ml)	2.367	2615.0	104.6



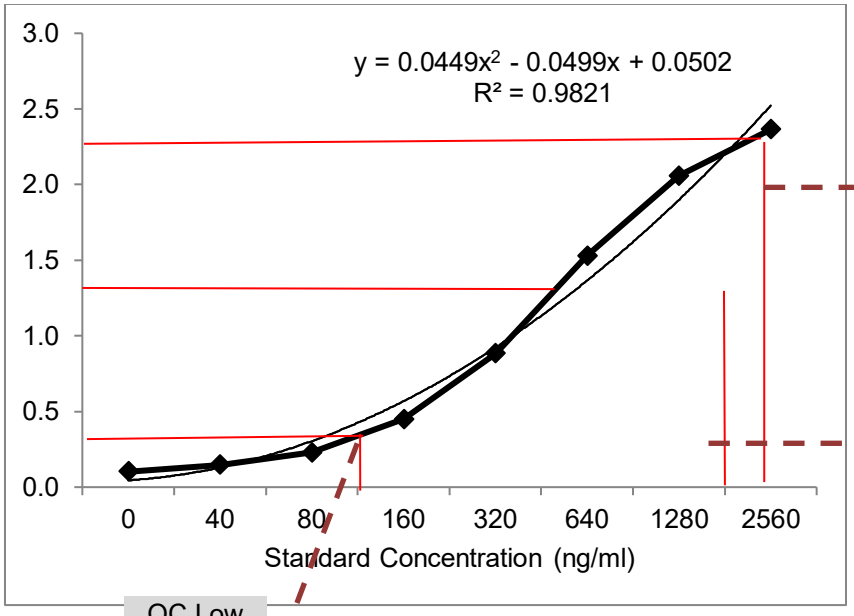
Polynomial 2nd Order

R2 = 98.21%

Accuracy %

Intra CV%

Inter CV%



QC High

104.6%

0.5%

0.4%

QC Medium

95.8%

1.0%

1.6%

QC Low

105.9%

1%

0.8%

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

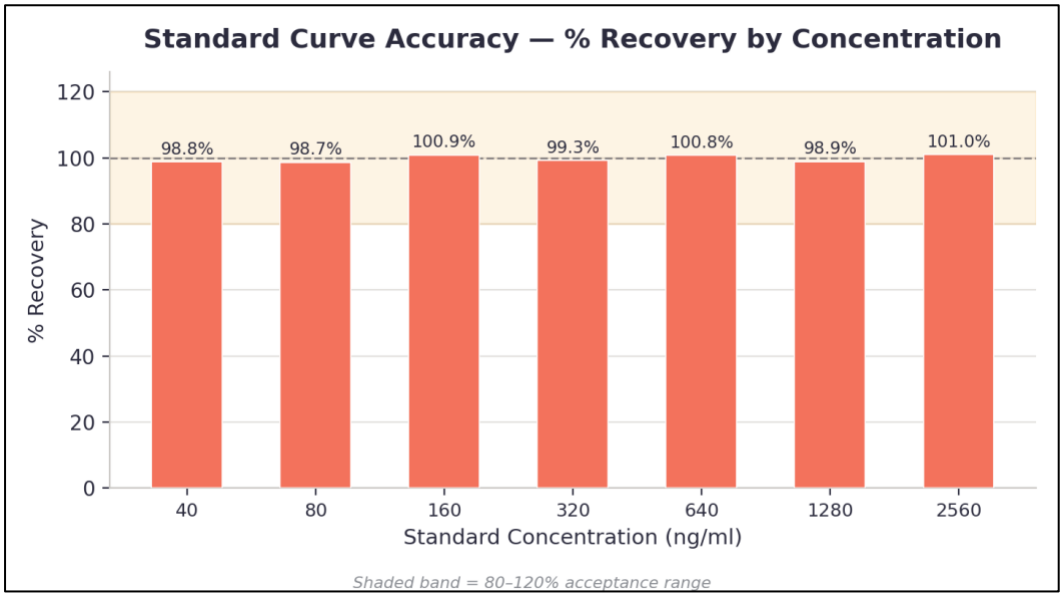
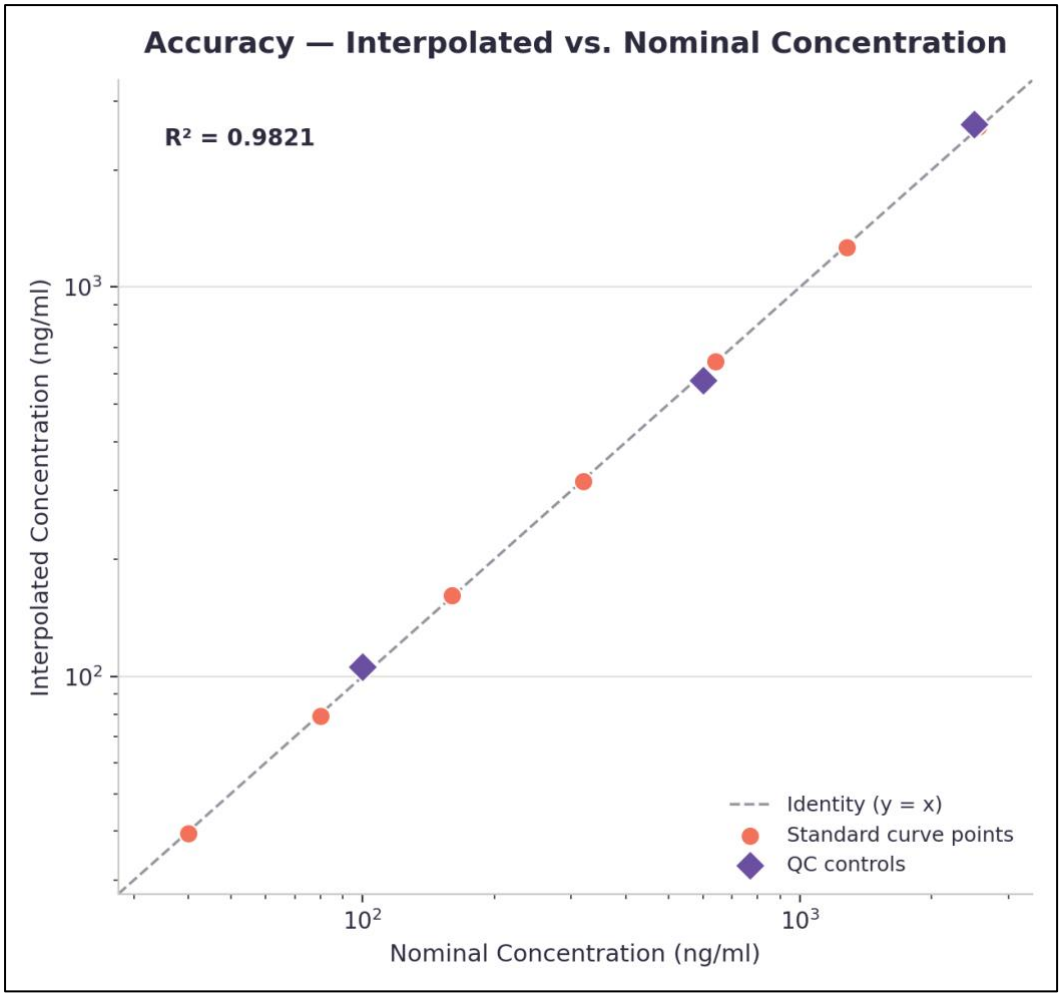


Figure 2. Accuracy — Interpolated vs. Nominal Concentration (Log-Log)



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

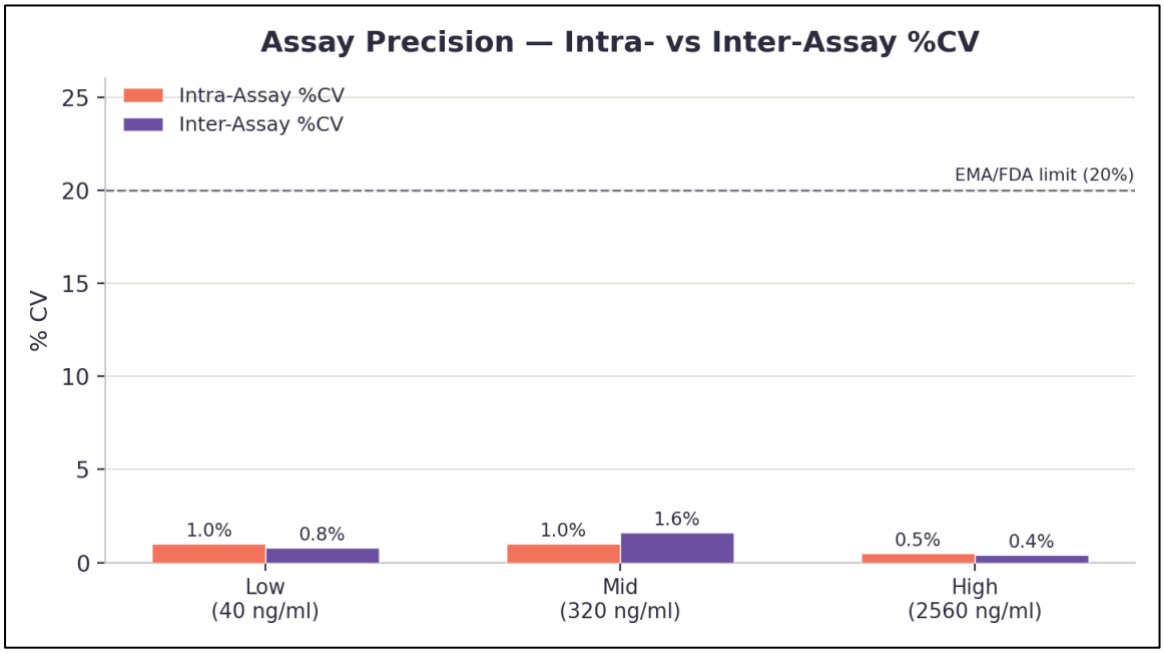
A) Intra-Assay:

Standard (ng/ml)	Mean OD450	SD	%CV
40	0.187	0.15	1.0
320	1.011	0.82	1.0
2560	2.337	0.89	0.5

B) Inter assay:

Standard (ng/ml)	Mean OD450	SD	%CV
40	0.199	0.13	0.8
320	0.957	1.29	1.6
2560	2.421	0.82	0.4

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



5. Parallelism and Matrix Effect

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

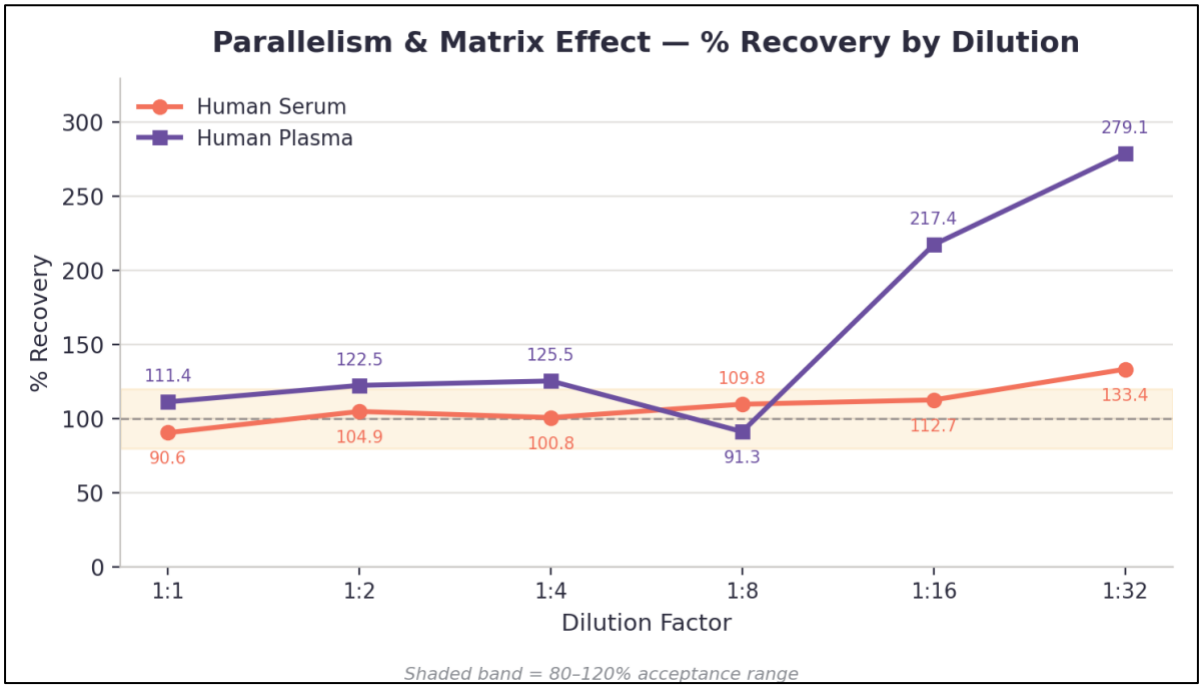
A) Serum:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	1280	2.006	1159.4	90.6	110.4
1:2	640	1.571	671.1	104.9	95.4
1:4	320	0.883	322.5	100.8	99.2
1:8	160	0.471	175.7	109.8	91.1
1:16	80	0.244	90.2	112.7	88.7
1:32	40	0.167	53.3	133.4	75.0

B) Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	1280	1.684	1425.7	111.4	89.8
1:2	640	1.116	783.9	122.5	81.6
1:4	320	0.582	401.6	125.5	79.7
1:8	160	0.217	146.0	91.3	109.6
1:16	80	0.251	173.9	217.4	46.0
1:32	40	0.179	111.6	279.1	35.8

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



Results:

i) Parallelism is maintained across the 1:1 to 1:16 dilutions in serum samples whereas most plasma dilutions remained within the acceptable range.

ii) % Recovery for most dilutions falls within the acceptable range of 80%–120% except 1:16 and 1:32 dilution in plasma samples showed below 50% deviation.

iii) Matrix-related interference or reduced analyte recovery at higher dilutions in plasma samples can be seen.

iv) The KRIBIOLISA® Pertuzumab ELISA kit was tested for matrix effect on human serum and human plasma.

5. Disease State Sample Recovery

Recovery was assessed by analysing diseased plasma (n=1) and diseased serum samples (n=1) 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 Pertuzumab in clinically relevant diseased plasma and diseased serum matrices.

Sample Details:

Diseased-state sample validation was performed using two clinically relevant human biological matrix samples (plasma and off-the-clot serum) from female patients with Stage 4 HER2-positive breast cancer. The samples were from 58-year-old subjects of Caucasian demographic, with reported pathogen-negative status and documented non-diabetic or unknown diabetic profiles. 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	Antico-agulant
PLPL060717C	Human Plasma, Breast Cancer HER2+, stage 4	Caucasian 58/Female	No/No	Negative	Nil	Nil	K2 EDTA
SER060717C	Human Serum, Breast Cancer, HER2+, Stage 4	Caucasian 58/Female	No/No	Negative	Nil	Nil	Nil

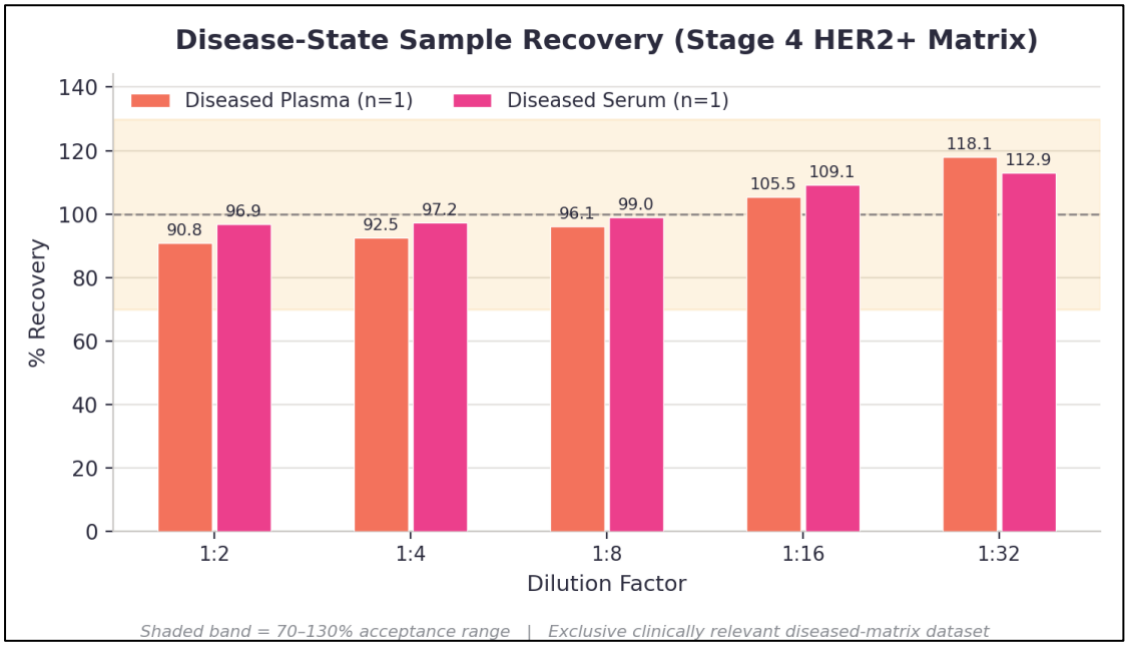
Acceptance Criteria:
• Recovery within 70–130% is generally considered acceptable for ligand-binding assays.
• Consistency across dilutions indicates minimal matrix interference.

Diseased State Sample Impact on Pertuzumab Recovery

Sample Matrix	1:2 Dilution (%)	1:4 Dilution (%)	1:8 Dilution (%)	1:16 Dilution (%)	1:32 Dilution (%)
Diseased Plasma (n=1)	90.8	92.5	96.1	105.5	118.1
Diseased Serum (n=1)	96.9	97.2	99.0	109.1	112.9

Observations:
- Recovery across all dilution levels falls within the acceptable range (70–130%), indicating minimal matrix interference in diseased plasma and diseased serum samples.
- Consistent recovery is observed from 1:2 to 1:32 dilutions, demonstrating robustness of the assay across a broad dilution range.
- The data indicate reliable and reproducible analyte recovery in diseased plasma and diseased serum matrices without the need for extensive sample pre-treatment.
- The assay is therefore suitable for accurate quantification of Pertuzumab in diseased plasma and diseased serum samples across clinically relevant conditions.

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°C. Avoid repeated freeze/thaw cycles.

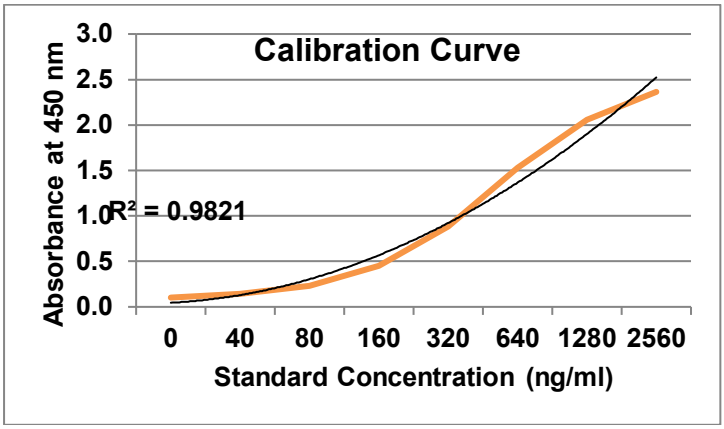
Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x *g*. Remove serum layer and assay immediately or store serum samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

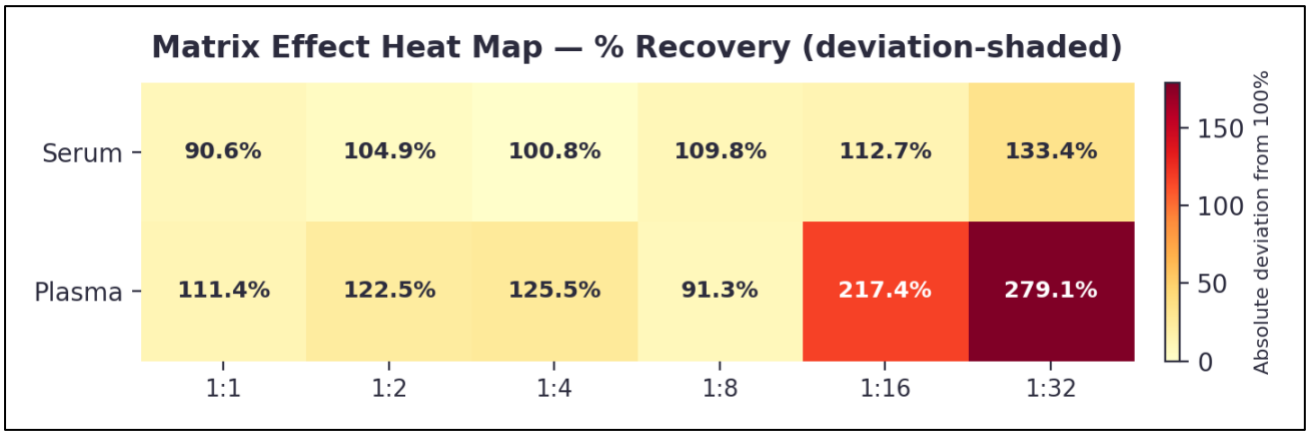
B) Storage conditions:

- Store main kit components at 2-8°C.
- Store recombinant lyophilized standard at 2-8°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°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%	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30%	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%

References

Baselga J, Cortés J, Kim S-B, et al. Pertuzumab plus Trastuzumab plus Docetaxel for Metastatic Breast Cancer. *New England Journal of Medicine*. 2012;366(2):109–119. DOI: 10.1056/NEJMoa1113216

Swain SM, Baselga J, Kim S-B, et al. Pertuzumab, Trastuzumab, and Docetaxel in HER2-Positive Metastatic Breast Cancer. *New England Journal of Medicine*. 2015;372:724–734. DOI: 10.1056/NEJMoa1413513

Pharmacokinetic and exposure–response analysis of pertuzumab in patients with HER2-positive metastatic gastric or gastroesophageal junction cancer.