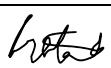
VALIDATION OF KRIBIOLISA™ TIRZEPATIDE (MOUNJARO) ELISA KIT (CATALOG NO. KOD1027) AS PER FDA/ICH GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION

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™ TIRZEPATIDE (MOUNJARO) ELISA** (Cat No #KOD1027)	31.03.2025

Approved Quality Control	Approved Product Development	Approved Operations Head
		
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Introduction

This document presents a discussion of the characteristics of our **KRIBIOLISA™ Tirzepatide (MOUNJARO) ELISA (Catalog No KOD1027)** 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 **Tirzepatide.**

Validation characteristics considered by us in accordance with the guidelines are listed below:

- **Sensitivity**
- **Specificity / Cross reactivity**
- **Precision**
- **Traceability / Stability**
- **Recovery**
- **Validation kit specific details**

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

Background

Tirzepatide is a dual GIP and GLP-1 receptor agonist used for the treatment of type II diabetes in adults as an adjunct to diet and exercise. Tirzepatide comprises a 39 amino acid linear synthetic peptide conjugated to a C20 fatty diacid moiety. Tirzepatide was approved by the FDA on May 13, 2022, under the brand name MOUNJARO by the FDA for the treatment of adults with type 2 diabetes, making it the first and only GIP and GLP-1 receptor agonist.

Later, it was approved under a different brand name ZEPBOUND on November 8, 2023, for the chronic weight management in adults with obesity or overweight with at least one weight-related condition. On September 15, 2022, Tirzepatide was also approved by the European Commission.

1. Purpose

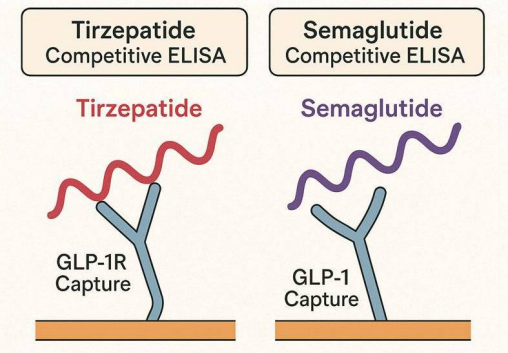
To assess the specificity, cross-reactivity, specificity, assay performance, and clinical relevance of the KRIBIOLISA Tirzepatide Competitive ELISA developed using GLP-1 Receptor (GLP-1R) capture protein.

2. Experimental Design

- `A competitive ELISA was performed using GLP-1R protein as capture.
- Standards prepared for Tirzepatide, Exenatide, and Semaglutide. (biosimilars commercially sourced).
- Assay Concentration Range: 0 - 4000 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The specific GLP-1R immobilization strategy used in the KRIBIOLISA Tirzepatide ELISA enhances Tirzepatide binding while minimizing Semaglutide cross-reactivity, supporting the assay's high specificity and robustness for clinical and bioanalytical applications.

The KRIBIOLISA Tirzepatide ELISA employs a specific immobilization procedure to optimize GLP-1 receptor (GLP-1R) presentation on the assay plate, thereby enhancing selective binding of Tirzepatide. The immobilization method ensures that the GLP-1R maintains an orientation and conformation favorable for Tirzepatide interaction.

Tirzepatide's molecular structure allows it to interact effectively with the immobilized GLP-1R, promoting stronger and more stable binding. In contrast, Semaglutide may not achieve the same conformational fit or receptor engagement under these plate-bound conditions, leading to minimal binding in the assay. This differential binding behavior is a result of both the molecular architecture of Tirzepatide and the fixed orientation of the receptor after immobilization.



ELISA kits for Tirzepatide and Semaglutide estimation offered by KRISHGEN uses different capture proteins as above.

3. Assay Validation

- IC50 Value: ~345 ng/ml (within 0-4000 ng/mL assay range).
- LLOQ: 30 ng/ml.

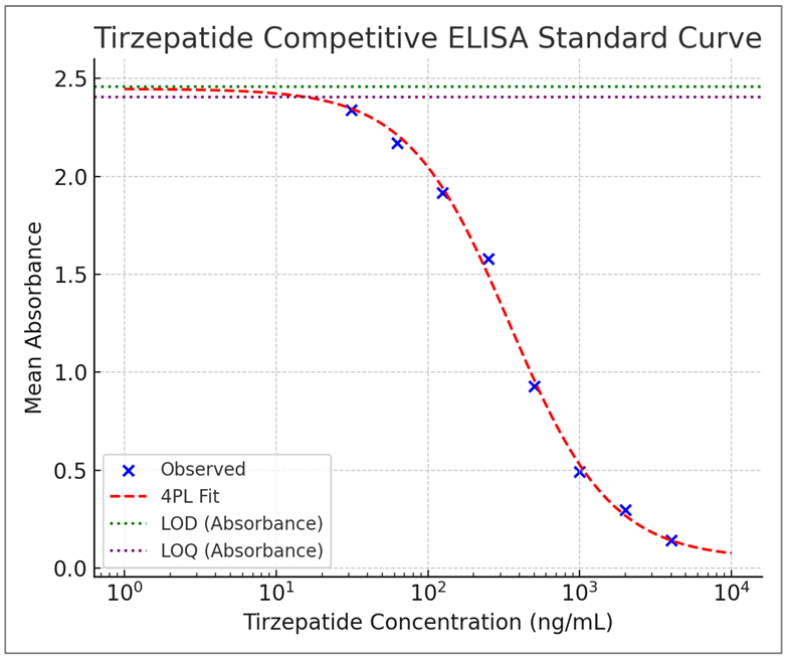
- Clinical Cmax Values*:
  - After 5 mg dose: ~300 - 350 ng/ml.
  - After 15 mg dose: ~1000 - 1200 ng/ml.

** published data*

- Precision:
  - Intra-Assay CV: <12%.
  - Inter-Assay CV: <10%.
  - Inter-Operator CV: <15%.

Standard Curve

Below is the standard curve for Tirzepatide Competitive ELISA assay:



LOD and LOQ

- LOD Absorbance: OD450 = 2.4582 (Approx ~20 ng/ml)
- LOQ Absorbance: OD450 = 2.4037 (Approx ~50 ng/ml)

QC Samples

- Mid QC Target: 1000 ng/mL (Interpolated 1078.8 ng/ml)
- High QC Target: 3000 ng/mL (Interpolated between 2000 - 4000 ng/ml)

Results: The assay is sensitive, specific, and suitable for pharmacokinetic studies.

Cross Reactivity:

To assess the cross-reactivity and specificity of the KRIBIOLISA Tirzepatide Competitive ELISA, a comparative evaluation was conducted using Exenatide (a GLP-1 receptor agonist biosimilar) and Semaglutide alongside Tirzepatide.

The following graphs demonstrate inhibition profiles.

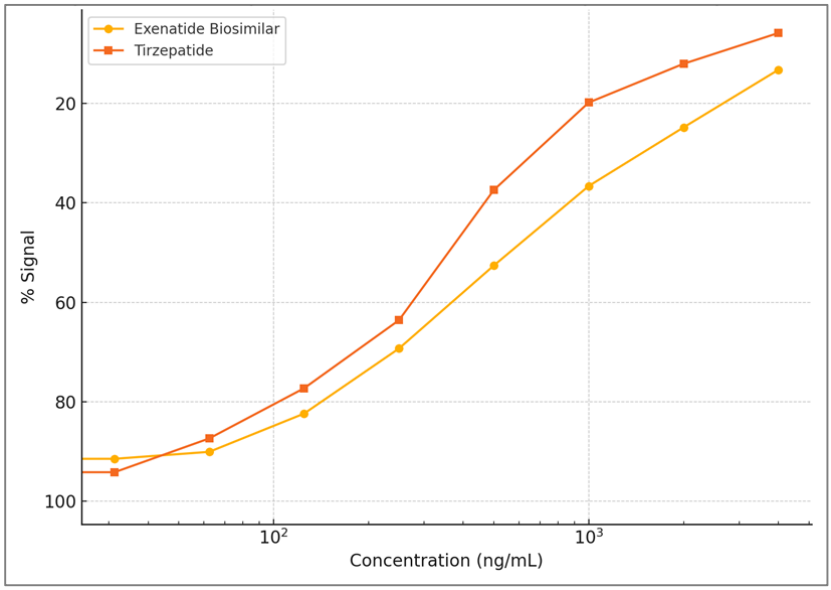


Figure 1: Tirzepatide vs Exenatide Inhibition Profile

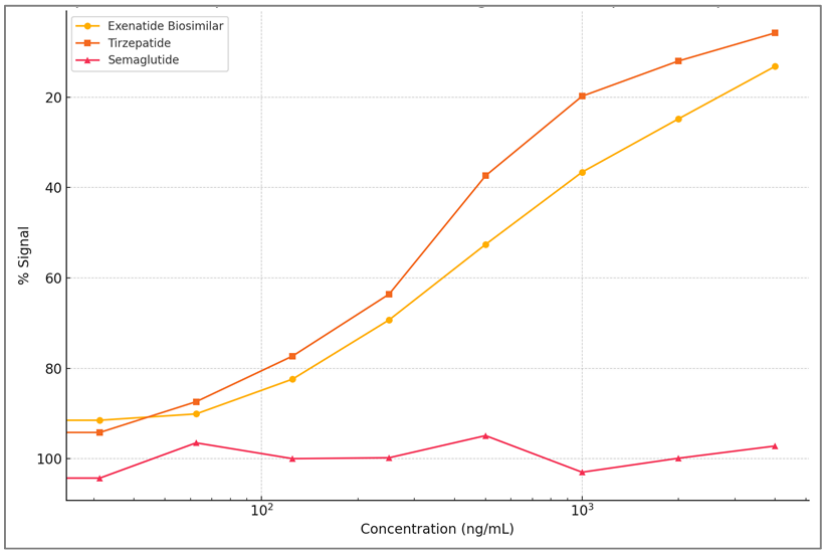


Figure 2: Tirzepatide vs Exenatide vs Semaglutide Inhibition Profile

Results:
Both Tirzepatide and Exenatide produced a concentration-dependent decrease in signal, consistent with competitive inhibition.
Tirzepatide demonstrated a greater inhibitory effect at equivalent concentrations compared to Exenatide.

- At 500 ng/ml:
  - Tirzepatide signal reduced to 37.4%.
  - Exenatide signal reduced to 52.6%.
- At 1000 ng/mL:
  - Tirzepatide signal reduced to 19.8%.
  - Exenatide signal reduced to 36.6%.

Semaglutide did not produce significant signal reduction across the tested concentration range. This indicates that Tirzepatide binds with a higher affinity to GLP-1R under the assay conditions, Exenatide exhibits weaker cross-reactivity, and Semaglutide shows minimal to no binding.

Interpretation:
The Tirzepatide Competitive ELISA is capable of detecting molecules binding to GLP-1R, including Exenatide. Due to differences in binding affinity and competitive inhibition profiles, quantitative accuracy is specific to Tirzepatide. Semaglutide does not show significant binding, confirming minimal to no cross-reactivity.

Conclusion:
- Tirzepatide produced a concentration-dependent inhibition curve.
- Exenatide showed weaker inhibition.
- Semaglutide exhibited minimal inhibition, confirming specificity.

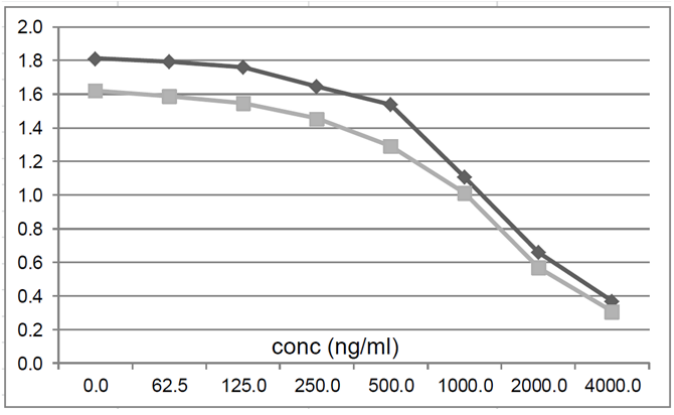
For accurate quantification of Tirzepatide in biological matrices, Tirzepatide itself must be used as the assay standard. Cross-reactivity with other GLP-1R agonists like Exenatide is limited, and Semaglutide does not interfere, supporting the assay's high specificity for Tirzepatide measurement.

5. Comparison Studies

5.1 Comparison with Mounjaro Tirzepatide Injection

Comparison of KRIBIOLISA Kit Standard vs Mounjaro Injection:

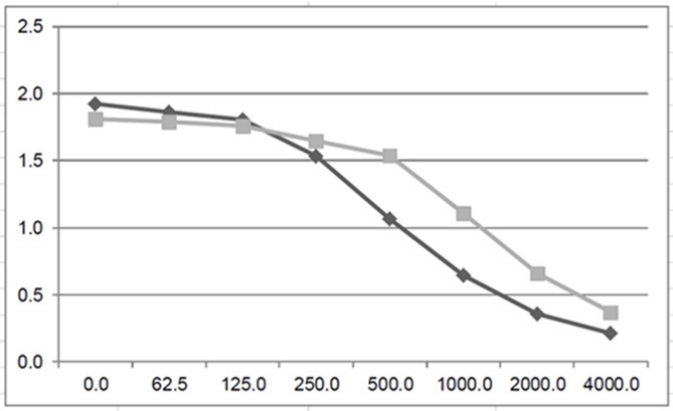
Standards (ng/ml)	Kit Standard (Absorbance)	Mounjaro Injection (Absorbance)	%CV
0.0	1.812	1.621	7.88
62.5	1.792	1.588	8.55
125.0	1.759	1.546	9.11
250.0	1.646	1.455	8.74
500.0	1.539	1.294	12.24
1000.0	1.107	1.013	6.24
2000.0	0.659	0.571	10.10
4000.0	0.370	0.308	12.83



5.2 Comparison with Commercial Tirzepatide Biosimilar Injection

Comparison of KRIBIOLISA Kit Standard vs Biosimilar Injection:

Standards (ng/ml)	Kit Standard (Absorbance)	Biosimilar Injection (Absorbance)	%CV
0.0	1.925	1.812	4.27
62.5	1.860	1.792	2.64
125.0	1.806	1.759	1.84
250.0	1.535	1.646	4.95
500.0	1.068	1.539	25.59
1000.0	0.644	1.107	37.38
2000.0	0.358	0.659	41.85
4000.0	0.213	0.370	38.20



6. 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.

7. Pharmacokinetic Relevance

The assay covers the clinical Cmax concentrations of Tirzepatide following therapeutic dosing. Thus, it is suitable for pharmacokinetic evaluation and therapeutic monitoring. The Tirzepatide Competitive ELISA demonstrated an IC50 value of approximately 345 ng/mL. This IC50 falls well within the validated assay range of 0 to 4000 ng/mL, ensuring that the assay is suitably sensitive for detection and quantification across clinically relevant concentrations.

Published pharmacokinetic data for Tirzepatide indicates:
- After a 5 mg subcutaneous dose, the Cmax (maximum serum concentration) reaches approximately 300–350 ng/mL.
- After a 15 mg subcutaneous dose, the Cmax may reach up to 1000–1200 ng/mL.

Thus:
- At standard therapeutic doses (5 – 15 mg), the expected Tirzepatide serum concentrations fall within the quantifiable range of the ELISA.
- The IC50 value (~345 ng/mL) closely matches the Cmax after a 5 mg dose, demonstrating the assay's suitability for monitoring Tirzepatide in human pharmacokinetic studies.
- For higher doses, samples may be analyzed after dilution if needed to remain within the linear dynamic range.

8. Parallelism

Serial dilutions of a high-concentration sample were prepared at dilutions of 1:1000, 1:2000, 1:4000, 1:8000, and 1:16000. Each dilution was assayed using the KRIBIOLISA Tirzepatide Competitive 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.

Parallelism Results:

Dilution	Expected Standard (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1000	1000	0.514	1028.4	102.8	97.2
1:2000	500	0.921	529.7	105.9	94.4
1:4000	250	1.645	203.1	81.2	123.1
1:8000	125	1.890	137.5	110.0	90.9
1:16000	62.5	2.145	77.8	124.4	80.4

Results:

- Parallelism is generally maintained across the 1:1000 to 1:8000 dilutions.
- % Recovery for most dilutions falls within the acceptable range of 80%–120%.
- Slight deviation at 1:4000 and 1:16000 dilutions, but still acceptable based on exploratory bioanalytical method validation standards.
- No significant matrix effect observed at higher dilutions.

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 KRIBIOLISA Tirzepatide ELISA without significant loss of accuracy.

9. Precision and Reproducibility

Precision was assessed by analysing three standard concentrations (31.3 ng/ml, 500 ng/ml, and 2000 ng/m). 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
31.3	2.2% to 3.9%	<6%
500.0	1.3% to 5.3%	<6%
2000.0	1.7% to 9.7%	<6%

Observations:

- Intra-assay precision was consistently less than 9% across all levels tested.
- Inter-assay precision was consistently less than 6%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:
The KRIBIOLISA Tirzepatide Competitive ELISA demonstrates excellent intra- and inter-assay precision. These results support the assay's reliability and reproducibility for routine use in pharmacokinetic and bioanalytical studies.

10. 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)

Samples were tested across the standard curve range (0–4000 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 Serum)	% Standard Deviation	% CV
0.0	2.487	2.481	0.42	0.2
31.3	2.342	2.338	0.22	0.1
62.5	2.212	2.169	3.07	1.4
125.0	1.958	1.917	2.92	1.5
250.0	1.541	1.579	2.72	1.7
500.0	0.922	0.927	0.35	0.4
1000.0	0.483	0.491	0.63	1.3
2000.0	0.294	0.298	0.25	0.8
4000.0	0.139	0.143	0.31	2.2

Results:

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

Conclusion:
The KRIBIOLISA Tirzepatide Competitive ELISA demonstrates excellent performance in the presence of human serum. The assay results confirm the absence of significant matrix interference, supporting its reliability for analysing biological samples.

11. Conclusion

The Tirzepatide Competitive ELISA is validated for sensitivity, specificity, precision, and accuracy, and is appropriate for pharmacokinetic applications in regulatory settings.

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