ELISA VALIDATION GUIDE

KRIBIOLISA® Semaglutide (Ozempic™) ELISA

KRISHGEN BioSystems

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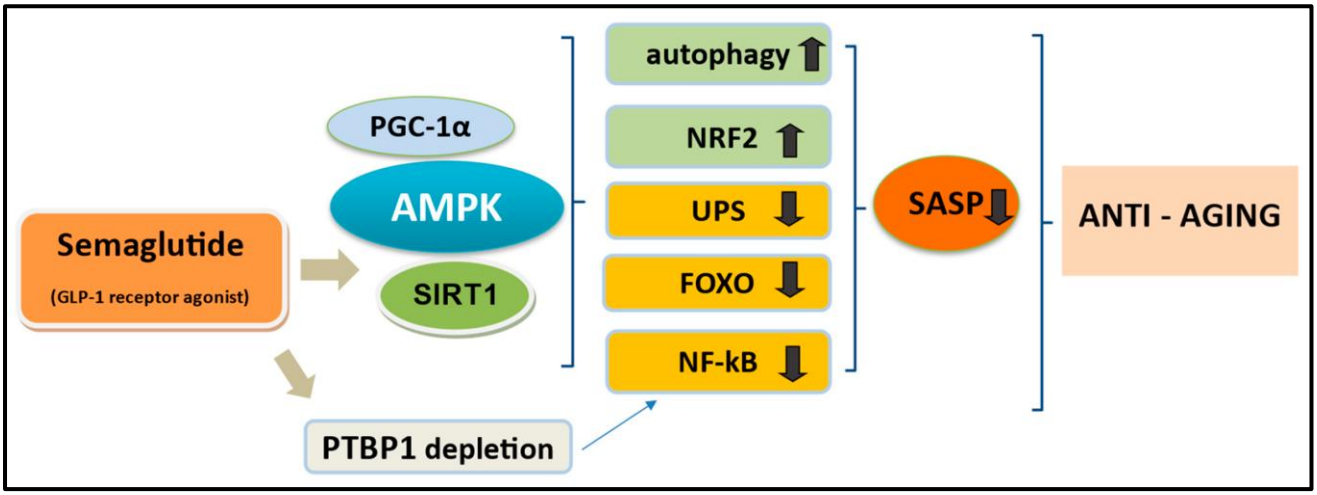
Background

1. Introduction to Semaglutide (Ozempic)

Semaglutide is a category of glucagon-like peptide-1 (GLP-1) receptor agonist primarily utilized for the treatment of type 2 diabetes mellitus and severe weight management. It has been considered as an acting synthetic analog of human GLP-1 which is engineered for resisting enzymatic degradation by DPP-4 which activates its half-life and therapeutic efficacy. Semaglutide comprises of 31 different types of amino acids and it is conjugated with a fatty acid side chain so that it can increase the binding specificity of albumin which will help in increasing its retention time in blood.

FDA first approved Semaglutide under the brand name of OZEMPIC, whose primary aim was treating adults who are suffering from type 2 diabetes. This was designed to be used as an adjunct to diet along with exercises in order to improve the range of glycemic control. Later Semaglutide was again approved under the brand name RYBELSUS and was marketed as the first oral GLP-1 receptor agonist for treatment of diabetes in adults. Subsequently, on June 4, 2021 another FDA approval was received by Semaglutide under the brand name of WEGOVY for its role in chronic weight management. It was developed in order to treat overweight or obesity in adults in association with decreased level of calorie intake and enhances physical activity. Semaglutide has also been approved by the European Medicines Agency (EMA) under the same brand names (Ozempic, Rybelsus, and Wegovy) for corresponding indications, further supporting its widespread clinical use in the management of diabetes and obesity.



2. Clinical Relevance of Semaglutide Monitoring

TDM or Therapeutic drug monitoring of Semaglutide is crucial for optimization of dosage regiments, especially in patients who have diverse absorption profiles or comorbidities like hepatic or renal impairment. In addition to this, critically monitoring the Semaglutide level helps in pharmacokinetics (PK) assessment, validation of therapeutic efficacy along with declining the risk of adverse side effects like hypoglycemia or gastrointestinal intolerance.

TDM also aids in correlating drug exposure with glycemic control and weight loss outcomes, thereby supporting personalized management strategies in type 2 diabetes and obesity treatment.

Scope of Validation

This document presents a discussion of the characteristics of our KRIBIOLISA™ Semaglutide (Ozempic™) ELISA KIT (CATALOG NO. KBI5030) 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 Semaglutide.

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).
- Sample Handling and Storage Conditions.
- References (Semaglutide C_{max}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 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

To assess the specificity, cross-reactivity, specificity, assay performance, and clinical relevance of the KRIBIOLISA Semaglutide Competitive ELISA developed using GLP-1 monoclonal antibody (GLP-1).

Principle of the Assay

This ELISA is based on the principle of competitive binding. A known amount of antigen is pre-coated on a 96-well microplate. When samples or standards containing the target antigen are added to the wells along with a specific antibody, a competition occurs between the plate-bound antigen and the free antigen in the sample for the limited binding sites of the antibody. The more antigens present in the sample, the less antibody is available to bind to the plate-coated antigen.

After incubation, unbound antibodies are washed away, and an HRP-conjugated secondary antibody specific to the primary antibody is added. Following another wash step to remove unbound conjugate, TMB substrate is added, producing a colorimetric reaction. The reaction is stopped by adding stop solution, and the optical density (OD) is measured at 450 nm. The intensity of color is inversely proportional to the concentration of antigen in the sample—the higher the antigen levels in the sample, the lower the OD.

Experimental Design

- A competitive ELISA was performed using GLP-1 monoclonal antibody as capture antibody.
- Standards prepared for Semaglutide.
- Assay Concentration Range: 0 - 4000 ng/ml.
- Signal (% absorbance) plotted versus concentration.
- The GLP-1 immobilization strategy used in the KRIBIOLISA Semaglutide ELISA is optimized to enhance the binding affinity of Semaglutide, while decreasing non-specific or competitive binding from structurally related peptides like Tirzepatide and Exenatide. This concept aligns with the assay's high specificity, sensitivity, and reliability for use in clinical monitoring and bio analytical quantification of Semaglutide in biological matrices.

The KRIBIOLISA Semaglutide ELISA follows a specific immobilization protocol for optimization of GLP-1antibody presentation on the assay plate, enhancing and improving the selective and high-affinity binding of Semaglutide. The immobilization technique ensures and looks after the fact that the GLP-1 regains a conformation and spatial orientation that is highly favorable for interaction with the molecular structure of Semaglutide, which closely mimics endogenous GLP-1. The structural modification of Semaglutide includes its fatty acid side chain and stabilized amino acid sequence, which facilitates robust and stable engagement with the immobilized receptor. However, peptides such as Tirzepatide, because of their dual agonist design and differences in structural orientation, may portray altered binding behavior under these conditions, resulting in reduced or minimal interaction with the plate-bound GLP-1. This differential binding emphasizes on the molecular specificity of Semaglutide and the critical role of receptor orientation in activating selective detection, thereby validating the assay's precision, specificity, and utility in Semaglutide quantification.

Validation Parameters and Acceptance Criteria

1. Semaglutide C_{max} Values and Recommended ELISA Range

This table summarizes Semaglutide C_{max} levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected Semaglutide Range (ng/ml)	Recommended ELISA Range (ng/ml)
Post low-dose (e.g., 0.25–0.5 mg/week) administration	1-4	0–10
Standard maintenance dose (1.0–2.0 mg/week)	10-30	0–50
High dose regimens (e.g., 2.4 mg/week of obesity)	25-50	0-100
Pharmacokinetic studies/long-acting analog evaluations	40-100	0–150

Note: Assay sensitivity <1 ng/ml recommended for baseline detection; upper limit ≥100 ng/ml advised for CRS monitoring.

The KRIBIOLISA Semaglutide ELISA kit is developed using an assay range of 0 - 4000 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 6400-fold dilution and the values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity
The capture and detection antibodies used in the Semaglutide ELISA are highly specific monoclonal antibodies that recognize unique epitopes within the Semaglutide molecule, particularly within its GLP-1 analog structure. These antibodies demonstrate strong affinity toward both the native and modified (acylated) forms of Semaglutide, ensuring precise quantification without cross-detection of endogenous GLP-1 or other incretin mimetics.

2.2 Selectivity
The ELISA exhibits negligible cross-reactivity with endogenous GLP-1, GLP-1 receptor agonists such as Liraglutide or Exenatide, or unrelated peptide hormones like GIP and insulin. It also shows no interference from serum components or plasma proteins, affirming high assay selectivity and reliability in complex biological matrices.

2.3Clinical C_{max} Values*:
- After 5 mg dose: ~432 ng/ml.
- After 20 mg dose: ~1673 ng/ml.

2.4 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 Semaglutide ELISA = 23.8 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).

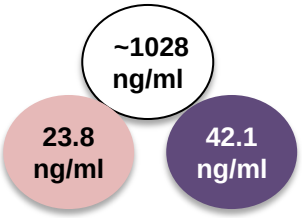
LOQ for KRIBIOLISA Semaglutide ELISA – 42.1 ng/ml

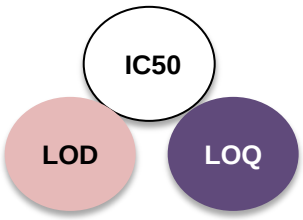
IC_{50} in ELISA (Half Maximal Inhibitory Concentration)
IC_{50} = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

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 Semaglutide ELISA = ~1028 ng/ml
Summary:

Parameter	Value (ng/mL)
LOD	23.8 ng/ml
LOQ	42.1 ng/ml
IC_{50}	1028 ng/ml

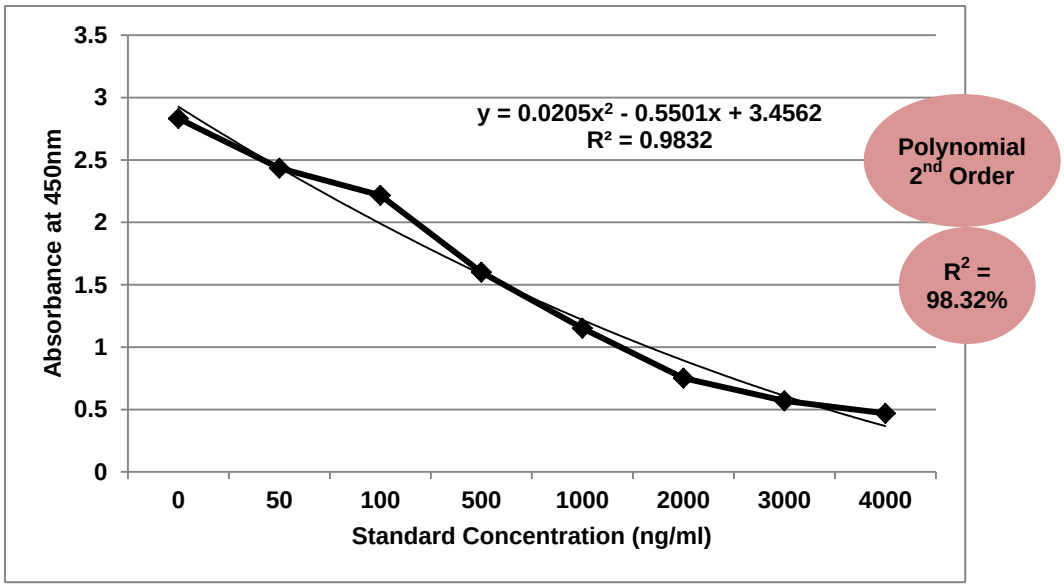


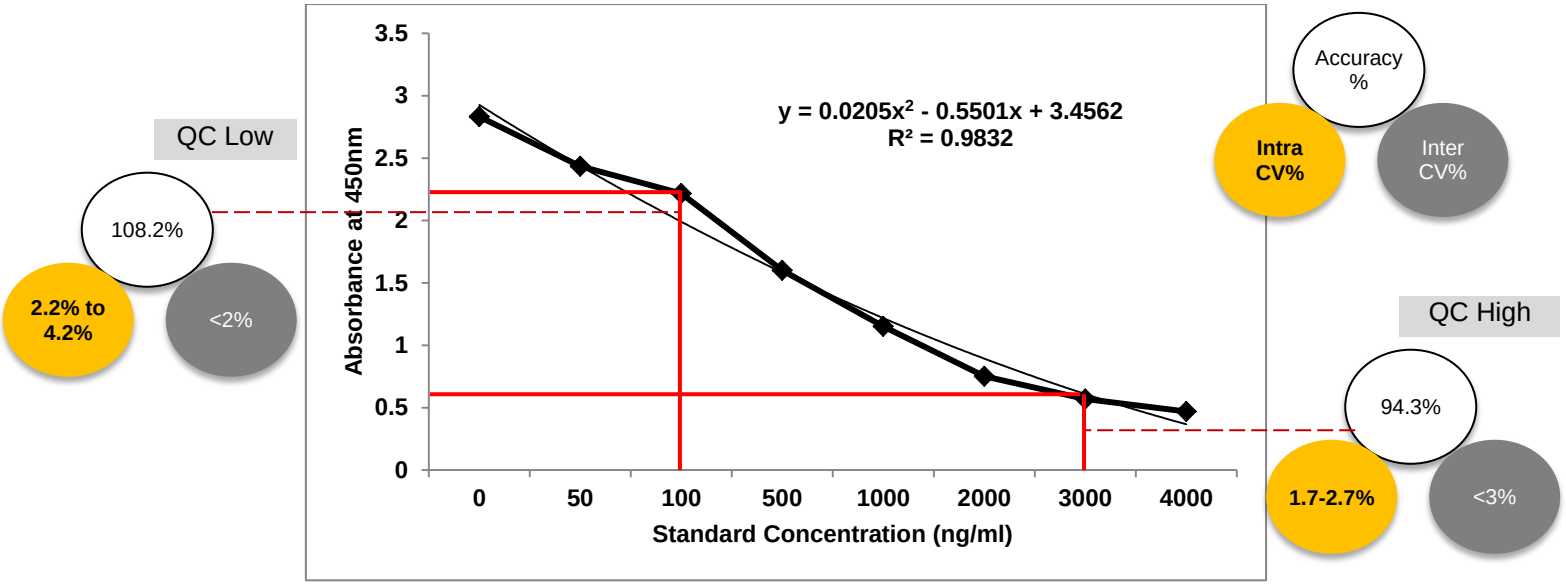


Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% **S/N = Signal / Noise Ratio*

3. Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery
0	2.832	--	---
50	2.435	49.9	99.8
100	2.217	108.5	108.5
500	1.601	452.0	90.4
1000	1.152	1029.1	102.9
2000	0.751	2138.2	106.9
3000	0.569	3055.1	101.8
4000	0.469	3764.6	94.1
Positive Control (1000 ng/ml)	1.112	1080.5	108.1
Low QC Control (100 ng/ml)	2.218	108.2	108.2
High QC Control (3000 ng/ml)	0.620	2830.2	94.3





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

Precision was assessed by analyzing three standard concentrations (50 ng/ml, 1000 ng/ml, and 4000 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
50	2.2% to 4.2%	<2%
1000	1.3% to 2.1%	<4%
4000	1.7% to 2.7%	<4%

Conclusion:

The KRIBIOLISA Semaglutide 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. Pharmacokinetic Relevance

The assay covers the clinical C_{max} concentrations of Semaglutide following therapeutic dosing. Thus, it is suitable for pharmacokinetic evaluation and therapeutic monitoring. The Semaglutide Competitive ELISA demonstrated an IC_{50} value of approximately 1028 ng/ml. This IC_{50} 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 Semaglutide indicates:

- After a 5 mg dose, the C_{max} (maximum serum concentration) reaches approximately 423 ng/ml.
- After a 20 mg dose, the C_{max} may reach up to 1673 ng/ml.

Thus:

- At standard therapeutic doses (5 – 15 mg), the expected Semaglutide serum concentrations fall within the quantifiable range of the ELISA.
- The IC_{50} value (~1028 ng/ml) is between the range of the C_{max} values, demonstrating the assay's suitability for monitoring Semaglutide in human pharmacokinetic studies.
- For higher doses, samples may be analyzed after dilution if needed to remain within the linear dynamic range.

5. Parallelism

Serial dilutions of a high-concentration sample were prepared at dilutions of 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400 for both human serum and human plasma. Each dilution was assayed using the KRIBIOLISA Semaglutide 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
1:200	2000	0.750	2142.3	107.1	93.4
1:400	1000	1.107	1115.4	111.5	89.7
1:800	500	1.584	467.0	93.4	107.1
1:1600	250	2.000	209.7	83.9	119.2
1:3200	125	2.172	123.8	99.0	101.0
1:6400	62.5	2.396	58.7	93.9	106.5

B) Human Plasma:

Dilution	Expected Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:200	2000	0.669	1882.2	94.1	106.3
1:400	1000	1.028	968.0	96.8	103.3
1:800	500	1.407	472.4	94.5	105.8
1:1600	250	1.774	215.0	86.0	116.3
1:3200	125	2.100	87.4	70.0	118.6
1:6400	62.5	2.207	59.7	95.5	104.7

Results:

i. Parallelism is generally maintained across the 1:200 to 1:6400 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 Semaglutide ELISA kit was tested for matrix effect on human serum, plasma and physiological buffer 7.4 to mimic tear fluid samples.

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 Semaglutide ELISA without significant loss of accuracy.

6. 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 + 1:100 human serum)
- Assay buffer spiked with human serum (buffer + 1:100 human plasma)

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 + 1:100 Human Serum)	% Standard Deviation	% CV
0	2.728	2.832	7.4	2.6
50	2.311	2.435	8.8	3.7
100	2.110	2.217	7.5	3.5
500	1.458	1.601	10.1	6.6
1000	1.002	1.152	10.6	9.8
2000	0.601	0.751	10.6	15.6
3000	0.502	0.569	4.7	8.8
4000	0.398	0.469	5.0	11.5

Standard (ng/ml)	Mean Absorbance (Buffer)	Mean Absorbance (Buffer + 1:100 Human Plasma)	% Standard Deviation	% CV
0	2.728	2.689	2.8	1.0
50	2.311	2.214	6.9	3.0
100	2.110	2.089	1.5	0.7
500	1.458	1.389	4.9	3.4
1000	1.002	1.005	0.2	0.2
2000	0.601	0.628	1.9	3.1
3000	0.502	0.412	6.4	13.9
4000	0.398	0.312	6.1	17.1

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 Semaglutide Competitive 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 analysing biological samples.

7. Cross Reactivity:

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

The following graphs demonstrate inhibition profiles

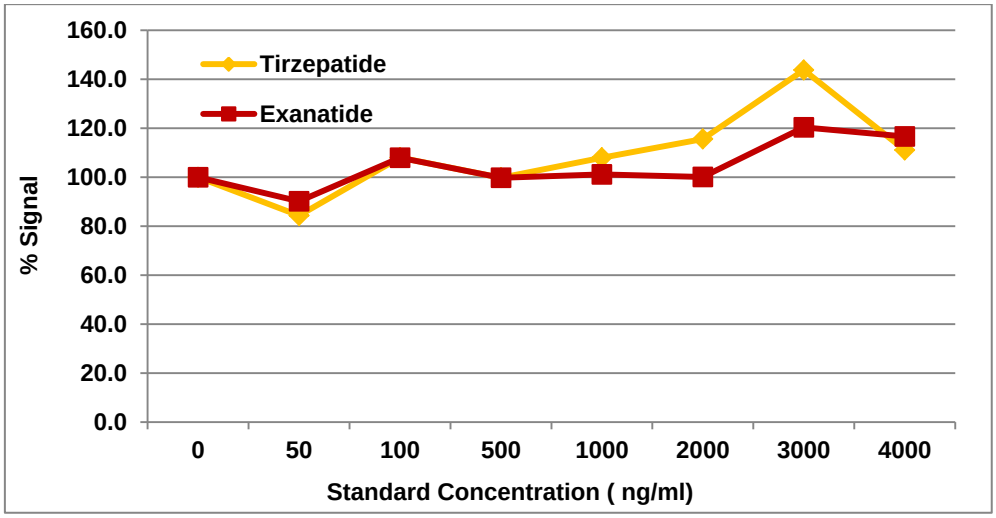


Figure 1: Tirzepatide vs Exenatide Inhibition Profile

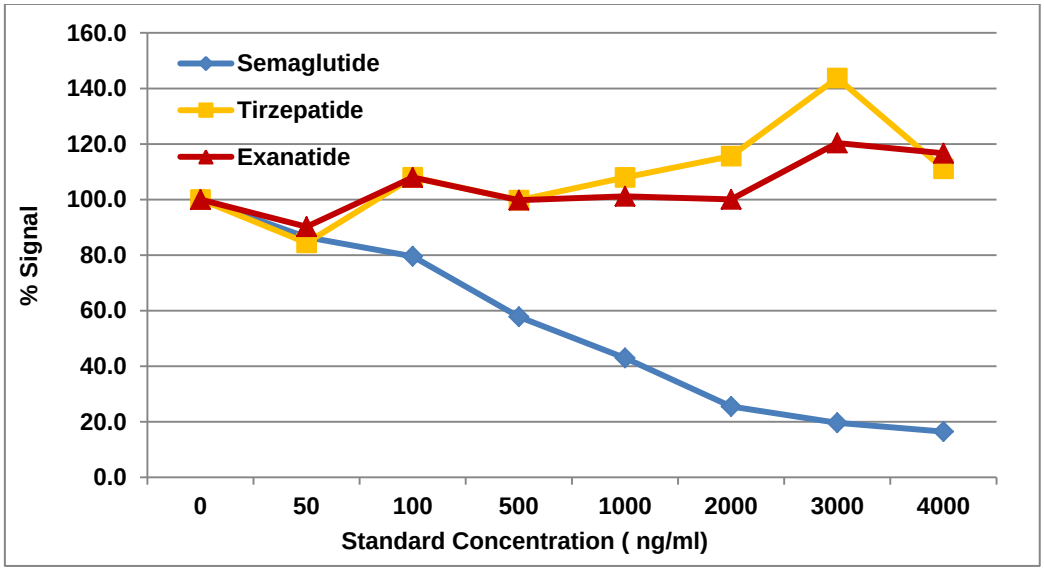


Figure 2: Tirzepatide vs Exenatide vs Semaglutide Inhibition Profile

Results:

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

Interpretation:

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

Conclusion:

- Semaglutide produced a concentration-dependent inhibition curve.
- Exenatide and Tirzepatide exhibited minimal inhibition, confirming specificity.

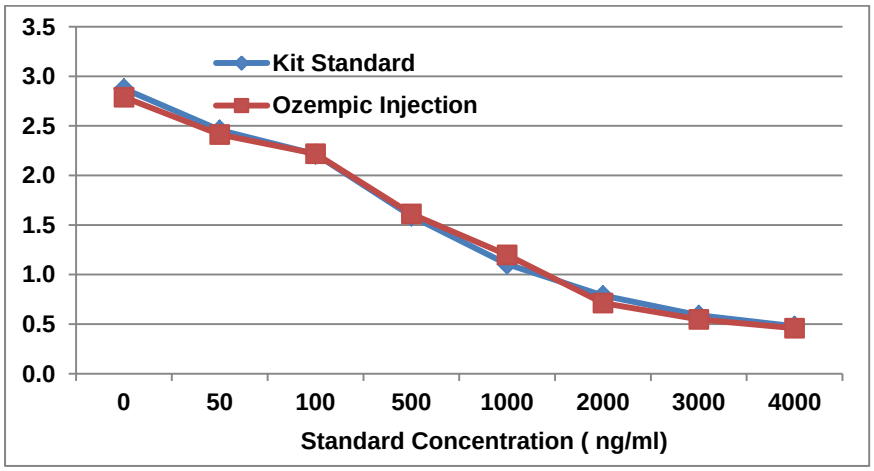
For accurate quantification of Semaglutide in biological matrices, Semaglutide itself must be used as the assay standard. Exenatide and Tirzepatide does not interfere, supporting the assay's high specificity for Semaglutide measurement.

8. Comparison Studies

Comparison with OZEMPIC Semaglutide Injection

Comparison of KRIBIOLISA Kit Standard vs Ozempic Injection:

Standard Concentration (ng/ml)	Absorbance (Standard)	Absorbance (Ozempic Injection)	% CV
0	2.875	2.789	2.1
50	2.458	2.412	1.3
100	2.214	2.219	0.2
500	1.589	1.612	1.0
1000	1.105	1.198	5.7
2000	0.789	0.712	7.3
3000	0.589	0.548	5.1
4000	0.478	0.459	2.9



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.

For Serum & Plasma - Samples have to be **diluted 1:100 (v/v)**, e.g. **1 ul sample + 99 ul (1X) Sample Diluent** prior to assay. The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

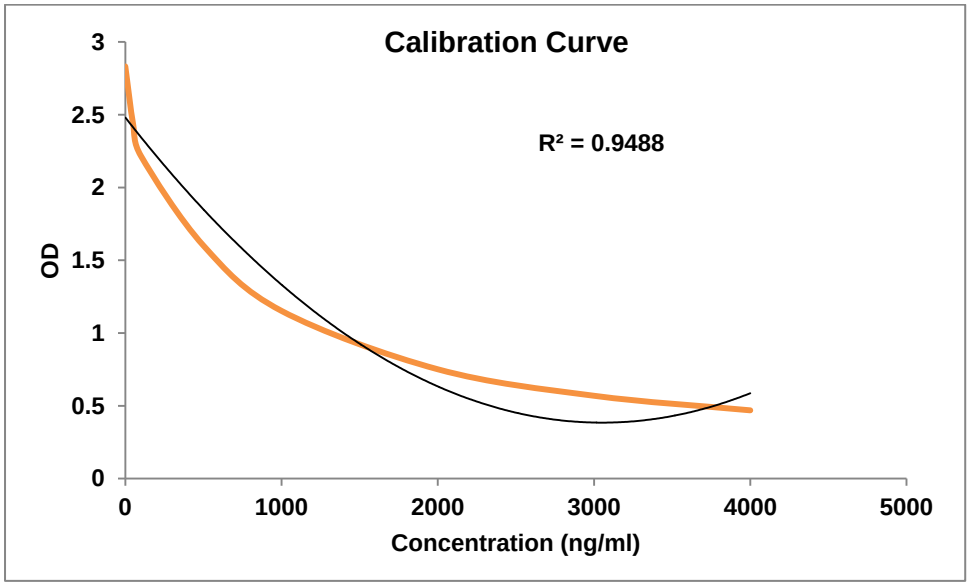
B) Storage Information:

1. It is advisable to aliquot and store the GLP-1 Biotin concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working GLP1-Biotin solution after running your assay.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. 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.
4. 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. Refer to the MSDS online for details.

Graphs, Maps and Appendices:



Matrix Effect Heat Map

	1:200	1:400	1:800	1:1600	1:3200	1:6400
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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