KRIBIOLISA® Neutralizing Antibodies to Semaglutide (OZEMPIC™) ELISA

REF : KBN1930

Ver 3.0

RUO

Enzyme Immunoassay for the Qualitative Detection of Neutralizing Antibodies against Semaglutide in human serum

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

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REF KBN1930 Σ 96 tests

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Introduction:

The KRIBIOLISA® ELISA kits are used for assessing the specific biomarker in samples analytes which may be serum and cell culture supernatant as validated with the kit. The kit employs a blocking ELISA technique which engages the blocking pathway to estimate the neutralizing antibodies.

Semaglutide is a glucagon-like peptide 1 (GLP-1) analog used to manage type 2 diabetes along with lifestyle changes, such as dietary restrictions and increased physical activity. GLP-1, a major incretin hormone in humans, acts by numerous mechanisms like augmented insulin secretion (glucose-dependent), inhibition of glucagon release and suppressed hepatic gluconeogenesis. It also causes delayed gastric emptying, reduced appetite and energy intake. Reduction of HbA1c level along with body weight without any risk of hypoglycaemia, provides it a special status for the treatment of obese type 2 diabetic patients.

Semaglutide uses three strategies to improve the residence time of the peptide. First, stearic diacid is used instead of palmitic acid to increase the molecule's affinity for albumin. Second, an extended spacer consisting of two 8-amino3,6- dioxaoctanoic acid (ADO) moieties and a glutamic acid residue further improves albumin binding stability. Third, the peptide's second residue is mutated to aminoisobutyric acid to confer greater DPP-IV resistance. These modifications result in a half-life of 165 hours in humans, making it suitable for once-weekly administration. The fatty di-acid chain attached to the GLP-1R agonist provides semaglutide with a higher affinity for albumin.

Immunogenicity is also a critical concern with this class of therapeutics. Prolonged exposure to the drug could initiate the development of neutralizing antidrug antibodies, which reduces safety and efficacy. The production of ADAs is generally a treatment risk with therapeutic peptides and can lead to an altered pharmacokinetic (PK) profile, which in turn may impact the safety and efficacy of therapeutic peptides (Wang, L., Wang, N., Zhang, W. et al. Therapeutic peptides: current applications and future directions.). ADAs can be binding, leading to minimal or no impact; clearing, leading to impact through an altered PK profile; sustaining, leading to a prolonged exposure that may change the efficacy and/or safety; or neutralizing, leading to reduced pharmacological activity of the therapeutic antibody (Garcês and Demengeot 2018; Bloem et al. 2017).

Regulatory agencies prefer cell-based assays because they rely on cellular responses to NAb-mediated inhibition of therapeutic antibodies (Wu et al. 2016). Because of their mode of detection, these assays have been considered more biologically relevant than non–cell-based assays (Bloem et al. 2017; Liao et al. 2012; Cong et al. 2015; Jolicoeur and Tacey 2012). However, cell-based assays are labor intensive and highly variable and exhibit low serum tolerance and poor drug tolerance (Bloem et al. 2017; Liao et al. 2012; Cong et al. 2015; Jolicoeur and Tacey 2012; Wu et al. 2016). In contrast to cell-based assays, non–cell-based assays often rely on binding of the drug and target for signal detection and quantitation. Competitive ligand-binding assays, used to characterize NAbs, have been shown to provide higher sensitivity, a wider dynamic range, increased precision, and better matrix tolerance than cell-based assays (Wu et al. 2018). Although regulatory agencies generally prefer cell-based assays, the United States Food and Drug Administration and the European Medicines Agency recognize both cell-based and non–cell-based competitive ligand-binding assays as valid measurements of NAbs in some cases (Jolicoeur and Tacey 2012; Wu et al. 2016). The selection of either cell-based or non–cell-based assays is driven by several different variables, including therapeutic mechanism of action (MOA), assay performance characteristics, and risk of immunogenicity, with therapeutic MOA being the primary determinant (Wu et al. 2016).

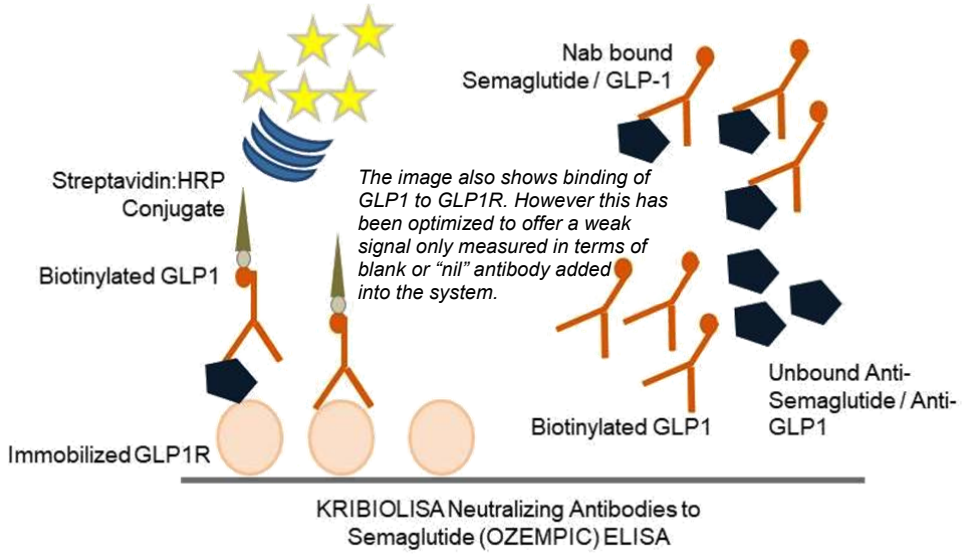
Semaglutide (GLP-1 analog) binds to its ligand GLP-1R which intiaties a cascade that involves activation of membrane bound Adenyl Cyclase (AC) and consequent production of cyclic adenosine monophosphate (cAMP). Downstream of cAMP formation, several signal transduction pathways can be initiated, which generally require activation of either one or both of the cellular cAMP effectors, Protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC).

The KRIBIOLISA® Neutralizing Antibodies to Semaglutide is a receptor-confirmed binding antibody assay that preferentially detects antibodies capable of forming receptor-accessible immune complexes. The kit is validated for assay precision, sensitivity, hook effect, selectivity, robustness, stability, and system suitability.

Intended Use:

The KRIBIOLISA® Neutralizing Antibodies to Semaglutide (OZEMPIC™) ELISA kit is a ligand–receptor complex capture sandwich ELISA for the quantitative detection of neutralizing antibodies (NAb) to GLP-1. Microplate wells are pre-coated with recombinant GLP-1 receptor (GLP-1R). Standards, controls, and samples are first incubated in solution with biotinylated GLP-1, allowing NAb–biotin-GLP-1 immune complexes to form.

The mixture is then transferred to the GLP-1R-coated wells, where complexes containing biotin-GLP-1 are captured via GLP-1 binding to GLP-1R. After washing, bound biotin-GLP-1 is detected using streptavidin-HRP. Addition of TMB substrate produces a color signal measured at 450 nm (with reference 620–630 nm). The absorbance is directly proportional to the concentration of neutralizing antibody present in the sample.



Materials Provided:

Part	Description	Qty
GLP-1R Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with GLP-1R	1 x 96 wells
Uncoated Microtiter Plate	96 well polystyrene uncoated microplate (12 strips of 8 wells)	1 x 96 wells
Neutralizing Anti-Semaglutide Positive Control	Neutralizing Anti-Semaglutide Positive Control Buffered protein base with preservative thiomersal < 0.01% (lyophilized)	2 vials
Biotin:Anti-GLP-1	Biotin:Anti-GLP-1 concentrated Control Buffered protein base with preservative thiomersal < 0.01% (lyophilized)	5 vials
Streptavidin:HRP, concentrated	Streptavidin:HRP Conjugate concentrated	2 vials
Biotin Diluent	Buffered protein base with preservative thiomersal <0.01%	12 ml
Streptavidin:HRP Diluent	Buffered protein base with preservative thiomersal <0.01%	12 ml
(1X) Sample Diluent	Buffered protein base with preservative thiomersal <0.01%	2 x 50 ml
(1X) Control Diluent	Buffered protein base with preservative thiomersal <0.01% with 1:1000 dilution human serum	12 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.

5. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to after reconstitution aliquot and stores the Biotin:Anti-GLP-1 concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C.
2. Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.
3. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.

Sample Preparation and Storage:

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

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.

Samples should be diluted 1:1000 (v/v) for optimal recovery, (for example 1 ul sample + 999 ul sample diluent) prior to assay. In cases where matrix interferences is under or over observed, the samples may be diluted less or more respectively with Sample Diluent accordingly.

The samples may be kept at 2-8°C for up to three days. For long-term storage please store at -20°C.
Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Preparation before Use:

Allow serum or plasma samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

In cases where matrix interferences is under or over observed, the samples may be diluted with Sample Diluent accordingly.

The samples may be kept at 2 - 8°C for up to three days. For long-term storage please store at -20°C.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. **Reconstitute the lyophilized Neutralizing Anti-Semaglutide Positive Controls in 500 ul Control Diluent.**
4. **Control Diluent is the Neutralizing Anti-Semaglutide Negative Control.**
5. **Preparation of Working Streptavidin:HRP - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**
6. **Preparation of Working Biotin:Anti-GLP-1 - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**
7. To make **Wash Buffer (1X)**; dilute **25 ml** of **20X Wash Buffer** in **475 ml of DI water**.

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of bound Nab Semaglutide.
3. It is recommended that the Standards and Samples be assayed in duplicates.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
5. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1) Neutralization Reaction

1. Pipette **100 ul** of prepared **Positive / Negative Control** in duplicate to the respective wells in the uncoated microplate.

2. Pipette **100 ul** of the diluted **Samples solution** into the respective wells in the uncoated microplate.

3. Pipette **100 ul** of the prepared **Biotin:Anti-GLP-1** into all the wells in the uncoated microplate.

4. Seal the plate and **incubate** for **60 minutes** at **37°C.**

2) Binding Reaction

1. Pipette **100 ul** of the **Positive / Negative Control Solution Complex** into the respective wells of the GLP-1R coated microplate from the neutralization reaction plate.

2. Pipette **100 ul** of the diluted **Samples Solution Complex** (from the Neutralization Reaction) into the respective wells of the GLP-1R coated microplate from the neutralization reaction plate.

3. Seal plate and **incubate** for **120 minutes** at **37°C.**

4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.

5. Pipette 100 ul of diluted **Working Streptavidin:HRP Conjugate** to all the wells.

6. Seal plate and incubate for **60 minutes at 37°C.**

7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.

8. Pipette **100 ul** of **TMB Substrate** solution to all the wells.

9. **Incubate** in the dark for **30 minutes** at **37°C.**

10. Stop reaction by adding **100 ul** of **Stop Solution** to each well.

11. **Read Absorbance** at 450 nm within 30 minutes of stopping reaction.

Qualitative Interpretation:

Calculation for Cut Off Values -
Read the sample and positive control wells on microtitre plate reader at 450 nm.

The OD (Optical Density) of NC (Negative Control) in duplicate should be used for calculating the mean and standard deviation. This is the $Negative_{mean}$.

The Cut-Off for Negative Samples is equal to a value greater than ($Negative_{mean}$ + 2*Standard Deviation).

Formula:
Negative Sample Value = OD > (*Negative$_{mean}$ + *2*SD)

Typical example –

Sample Type	*Absorbance #1*	*Absorbance #2*	*Mean*
Negative	*0.108*	*0.018*	*0.063*

Therefore, Cut-Off = Mean + 2*SD
= 0.063 + (2*0.064)
= 0.063 + 0.128
= 0.191

Interpretation of Results:

Positive NAb-Semaglutide Samples *	**> Cut-Off ***
Negative Nab-Semaglutide Samples *	**=< Cut-Off ***

** The cut-off value is based on validation using recombinant antibodies in the assay. Users may set up their own cut-off values based on different patient serum panels from different co-existent diseases, geographic locations or ethnic backgrounds.*

Limitation of the Procedure:

This ELISA test is designed for qualitative and/or quantitative detection of antibodies that bind GLP-1 and still allow receptor interaction, resulting in enhanced retention of ligand on a receptor-coated surface. It does NOT selectively measure antibodies that prevent GLP-1 from binding GLP-1R. Hence it is not a comprehensive neutralizing antibody assay.

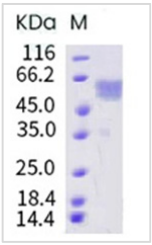
Performance Characteristics:
Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory.

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 and the dilutional linearity assay to assure quality results.

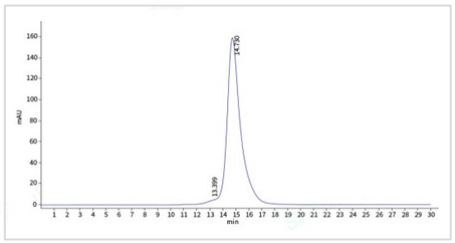
For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Specificity of the Immobilized GLP-1R:

The GLP-1R protein construct used a DNA sequence encoding the human GLP1R (NP_002053.3) (Met1-Tyr145) and was expressed with the Fc region of human IgG1 at the C-terminus.



Gel Image of the Coat/Capture Protein



Chromatogram of the Coat/Capture Protein

Specificity of the Neutralizing Antibody (Positive Control):
The Neutralizing Anti-Semaglutide monoclonal antibody binds to Semaglutide and is offered in a lyophilized form.

Validation of the Incubation between Positive Control / Samples and Biotin:GLP1 When Incubated within the Pre-Coated Plate
Pre-incubation is a solution-phase complex formation step that maximizes complex yield and stability. In-well incubation introduces competition by the coated receptor plus surface/steric/kinetic limitations, leading to a reduction in the signal / absorbance.

Semaglutide NAB (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.136	--	--
PC (1:4)	0.138	144.2	57.7
PC (1:2)	0.148	598.2	119.6
PC (1:1)	0.159	1003.5	100.4
PC	0.190	1985.2	99.3

When you add Ab + biotin-GLP-1 directly into a GLP-1R-coated well, a few things reduce the amount of captured biotin:
1) GLP-1R immediately competes for free GLP-1
As soon as biotin-GLP-1 is added, GLP-1R binds it, pulling GLP-1 out of solution and immobilizing it. That reduces the free biotin-GLP-1 available to form Ab–GLP-1 complexes in solution.

This creates a three-way competition:
- Ab binds GLP-1 (solution)
- GLP-1R binds GLP-1 (surface)
- complex formation now happens under “depleted ligand” conditions
Net effect: fewer Ab–biotin-GLP-1 complexes available for capture → lower signal.

2) Surface binding can create a “dead-end” orientation
If biotin-GLP-1 binds GLP-1R first, the way it sits on the receptor can sterically hinder antibody binding (or slow it), especially if:
- the biotin is on/near a region that changes accessibility when GLP-1 is receptor-bound,
- the Ab epitope is partially buried or conformationally altered upon receptor engagement.
So even if Ab can bind GLP-1, it may bind worse once GLP-1 is already captured to GLP-1R.

3) Kinetics: complex formation is faster in bulk than at a surface
In solution, molecules collide freely. On a surface, diffusion to the surface and orientation constraints make binding slower. If your incubation time is optimized for pre-mix, in-well complexing often needs longer and still won’t reach the same effective equilibrium.

4) Bivalency/bridging is less efficient in-well
In solution, an IgG can “bridge” two GLP-1 molecules (one may be biotinylated), stabilizing the complex. On the surface, we may Hend up with:
- GLP-1R-bound GLP-1 that is not well positioned for the second arm of the Ab,
- or Ab that binds monovalently and dissociates more easily during washes.
Result: lower retained biotin after washing → reduced OD.

Note: Pre-incubation is critical for assay sensitivity. Performing the incubation step directly in coated wells can reduce signal due to competitive binding of GLP-1 to immobilized GLP-1R before immune complex formation.

Note:
For researchers and clinical companies looking to use the kit for demonstration and estimation of their own developed antibody to Semaglutide with their own Semaglutide, it is recommended to first run similarity assay using the kit provided Semagalutide. Subsequently, you may use your internal Semaglutide in the assay once the parameters are established.

In case the demonstrated variance is beyond the defined quality limits when using your own Semaglutide, we would recommend to use the kit provided Semaglutide only.

In case the recoveries obtained are not as per the desired results, please connect with us (email: sales1@krishgen.com) to help you optimize the assay using our own differently formulated diluents to best optimize on the kit.

Safety Precautions:

- **This kit is For Research Use Only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (<0.1 % w/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from **Human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance
1A 2A	Negative Control Negative Control		
1B 2B	Postive Control Positive Control		
1C 2C	Sample Sample		
1D 2D	Sample Sample		
1E 2E	Sample Sample		
1F 2F	Sample Sample		
1G 2G	Sample Sample		
1H 2H	Sample Sample		
3A 4A	Sample Sample		
3B 4B	Sample Sample		
3C 4C	Sample Sample		

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SYMBOLS KEY

COATED MTP	GLP-1R Coated Microtiter Plate (12 x 8 wells)
UNCOATED MTP	Uncoated Microtiter Plate (12 x 8 wells)
PC	Positive Control (lyophilized)
BIO Anti-GLP-1	Biotin:Anti-GLP-1 concentrated (lyophilized)
STRP HRP	Streptavidin:Horseradish Peroxidase concentrated
BIO DIL	Biotin Diluent
STRP HRP DIL	Streptavidin:HRP diluent
1X CNTRL DIL	(1X) Control Diluent
1X SAMP DIL	(1X) Sample Diluent
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature