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KRIBIOLISA® Liraglutide (Victoza™) ELISA

REF : KBI5020

Ver 5.5

RUO

This Kit has been calibrated against commercially sourced referenced therapeutic product, Victoza™ Injection.

Enzyme Immunoassay for the Quantitative Estimation of Liraglutide in human serum and plasma

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

For Research Use Only. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Krishgen Biosystems Private Limited is strictly prohibited.

REF **KBI5020** **Σ 96 tests**

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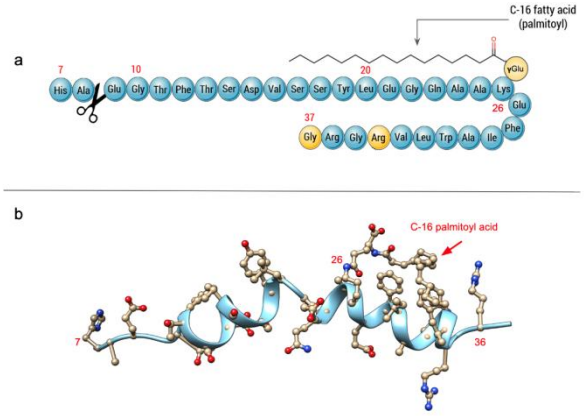
Introduction:

Liraglutide (NN2211) is a derivative of a human incretin (metabolic hormone), glucagon-like peptide-1 (GLP-1) that is used as a long-acting glucagon-like peptide-1 receptor agonist, binding to the same receptors as does the endogenous metabolic hormone GLP-1 that stimulates insulin secretion. Marketed under the brand name Victoza, it is an injectable drug developed by Novo Nordisk for the treatment of type 2 diabetes.



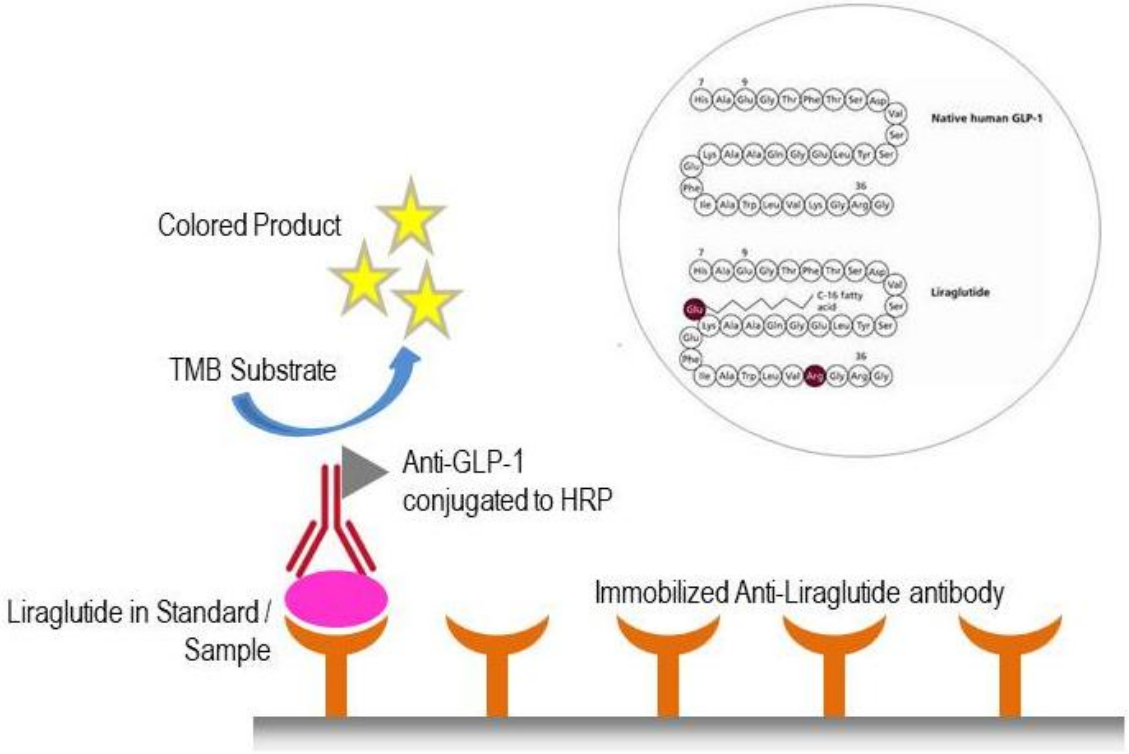
In 2015, Novo Nordisk began marketing a separate strength in the U.S. and E.U. under the brand name Saxenda as a treatment for adults who are obese or overweight with at least one weight-related comorbid condition.

Intended Use:

The KRIBIOLISA® Liraglutide (Victoza™) ELISA is used as for the quantitative determination of Liraglutide in human serum and plasma.

Principle:

The method employs a sandwich immunoassay for the determination of Liraglutide. The anti-Liraglutide Antibodies are coated on microtiter plate. Liraglutide standard and Liraglutide present in the samples will bind to coating antibody. Anti-GLP-1 antibody conjugated to HRP is then added which produces a soluble colored product after addition of 3,3',5,5' Tetra Methyl Benzidine (TMB) substrate. 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 bound Liraglutide present in the standards or samples.



PRINCIPLE OF THE KRIBIOLISA™ LIRAGLUTIDE ELISA

Materials Provided:

Part	Description	Qty
Anti-Liraglutide Coated Microtitre Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Liraglutide antibody	1 x 96 wells
Liraglutide Standard	Lyophilized Liraglutide Standard with Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane (concentrated – 3000 ng/ml)	2 vials
Anti-GLP-1:HRP Conjugate concentrated	Anti-GLP-1:HRP Conjugate to Horseradish Peroxidase concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	12 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane with 1:1000 dilution normal human serum	10 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 aliquot and store the Anti-GLP-1:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Anti-GLP-1:HRP Conjugate 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.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

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. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

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 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. To make **Wash Buffer (1X)**; dilute **25 ml** of **20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 3000 ng/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 853.3 ul of reconstituted original **Standard (3000 ng/ml)** with 146.7 ul of Standard Diluent (1X) to generate a **2560 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
3000 ng/ml	Reconstituted Standard	Lyophilized Standard provided in the Kit + 1 ml Standard Diluent (1X)
2560 ng/ml	Standard No.7	853.3 ul Reconstituted Standard + 146.7 ul Standard Diluent (1X)
1280 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
640 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
320 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
160 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
80 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
40 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No. 0	Only Standard Diluent (1X)

Use the Standards as soon as possible upon reconstitution. Discard balance standard after use.

5. **Working Anti-GLP-1:HRP Conjugate** – **Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

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 Liraglutide.
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. Pipette **100 ul** of **Standards** and **Samples** to the respective wells.

2. Seal plate and incubate for 1 hour at 37ºC.

3. Wash plate 5 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.

4. Add **100 ul** of **Working Anti-GLP-1:HRP Conjugate** to each well.
5. Seal plate and incubate for 1 hour at 37ºC.
6. Wash plate 5 times with **Wash Buffer (1X)** same as mentioned in step 3.
7. Pipette **100 ul** of **TMB Substrate solution** in all wells.
8. Incubate in the dark for 30 minutes at 37ºC.
9. Stop reaction by adding **100 ul** of **Stop Solution** to each well.
10. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate Standards and Samples. Using standard graph paper, plot the average value (absorbance 450 nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Liraglutide concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

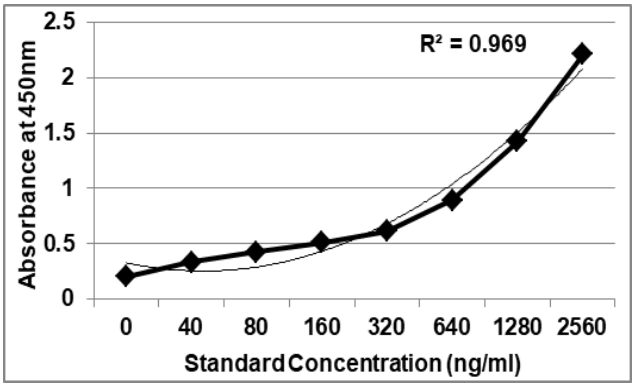
Software which is able to generate a polynomial regression (2nd order) or a cubic spline curve-fit is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 2560 ng/ml standard.

Typical Data (representative only)

Standard Concentration (ng/ml)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.202	--	--
40	0.335	46.9	117.3
80	0.427	114.7	143.4
160	0.513	189.0	118.1
320	0.613	285.2	89.1
640	0.895	599.1	93.6
1280	1.422	1304.3	101.9
2560	2.213	2562.1	100.1

Typical Graph (representative only)



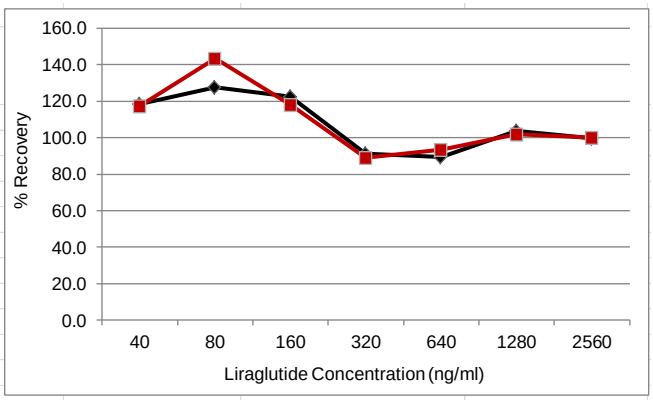
Abs = absorbance at 450nm

Quality Control:
It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics of the Kit:
This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2*SD. 10 replicates of '0' standards were evaluated and the LOD was found to be less than 40 ng/ml.

Specificity:
The capture antibody used in the kit is a rat polyclonal with ~95% purity by SDS page. The standard has been calibrated against commercially sourced Victoza™ Injection manufactured by Novo Nordisk.



Standard Concentration (ng/ml)	Victoza % Recovery	Kit Standard % Recovery
0	--	--
40	117.3	118.2
80	143.4	127.6
160	118.1	122.4
320	89.1	91.3
640	93.6	89.3
1280	101.9	103.7
2560	100.1	99.8

Note: Liraglutide is chemically similar to human glucagon-like peptide-1 (GLP-1), but there are some differences in their peptide chains namely Liraglutide includes a C16 fatty acid conjugate and a substitution of Lys 34 to Arg.

The capture antibody used in the kit is not only specific to Liragluitde but has demonstrated cross reactivity of 100-120% against other forms of GLP-1 including exogenous GLP-1, Tirzapatide and Semaglutide salts like Semaglutide Acetate. Hence the kit has limitations when detecting Liraglutide in the presence of other GLP-1 agonists.

Linearity:
Standards provided in the kit were used for measuring the linearity range of Liraglutide present in matrix. The Detection range provided is 0 - 2560 ng/ml.

Precision:
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (40 ng/ml), medium (320 ng/ml) and high (2560 ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	<15%	<12%
Medium	<12%	<10%
High	<12%	<10%

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.

SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.

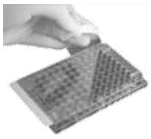
Components (2-8ºC) → 30 mins RT Before Use → 

→ Thaw at Room Temperature (18-24ºC) → USE NOW

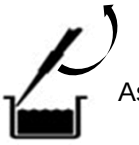
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

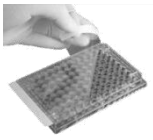
3.  Pipette **100 ul Standards** and **diluted Samples** into the respective wells.

4. Cover plate  and **incubate** for 

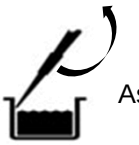
at 37°C.

5.  Aspirate and wash wells 5 times with **Wash Buffer (1X).**

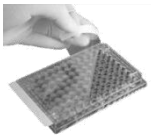
6.  Pipette **100 ul Working Anti-GLP-1:HRP Conjugate** into each well.

7. Cover plate  and **incubate** for 

at 37°C.

8.  Aspirate and wash wells 5 times with **(1X) Wash Buffer**.

9.  Pipette **100 ul TMB Substrate** into each well.

10. Cover plate  and **incubate** for 

at 37°C.

11.  Pipette **100 ul Stop Solution** into each well.

12. Read absorbance at 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450 nm	Mean Absorbance	ng/ml Liraglutide equivalent
1A 2A	0 Standard 0 Standard			
1B 2B	40 ng/ml 40 ng/ml			
1C 2C	80 ng/ml 80 ng/ml			
1D 2D	160 ng/ml 160 ng/ml			
1E 2E	320 ng/ml 320 ng/ml			
1F 2F	640 ng/ml 640 ng/ml			
1G 2G	1280 ng/ml 1280 ng/ml			
1H 2H	2560 ng/ml 2560 ng/ml			
3A 4A	Sample Sample			
3B 4B	Sample Sample			

LIMITED WARRANTY

Krishgen Biosystems Private Limited does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the Products; against defects in products or components not manufactured by Krishgen Biosystems Private Limited, or against damages resulting from such non-Krishgen Biosystems Private Limited made products or components. Krishgen Biosystems Private Limited passes on to customer the warranty it received (if any) from the maker thereof of such non Krishgen made products or components.

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THANK YOU FOR USING KRISHGEN PRODUCT!

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Victoza™ is the registered trademark of Novo Nordisk.

SYMBOLS KEY

MTP	**Anti-Liraglutide Coated Microtitre Plate (12x8 wells)**
STD	**Liraglutide Standard, Lyophilized**
HRP CONJ	**Anti-GLP-1:HRP Conjugate concentrated**
DETN DIL	**Detection Diluent**
1X SAMP DIL	**(1X) Sample Diluent**
1X STD DIL	**(1X) Standard 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**