Liraglutide ELISA

For the quantitative determination of Liraglutide (Victoza/Saxenda) in human serum and plasma.

Store the Anti-GLP-1 HRP Conjugate concentrate at -20°C upon receipt.

For Research Use Only. Not for Use in Diagnostic Procedures.

Catalog Number: 07-LIRHU-E01

Size: 96 determinations

Version: 5.4 **ALPCO**: 1.0

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INTENDED USE

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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 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. This ELISA Kit has been calibrated against commercially-sourced referenced therapeutic product Victoza® Injection.

PRINCIPLE OF THE ASSAY

The method employs a sandwich immunoassay for the determination of Liraglutide. The anti-Liraglutide Antibodies are coated on a microtiter plate. Liraglutide standard and Liraglutide present in the samples will bind to the coating antibody. Anti-GLP-1 antibody conjugated to HRP is then added which produces a soluble colored product after addition of 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 and samples.

MATERIALS SUPPLIED

07-LIRHU-E01			
Component	**Description**	**Quantity**	**Preparation**
Anti-Liraglutide Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Liraglutide antibody	1 x 96 wells	Ready-to-Use
Liraglutide Standard	Lyophilized Liraglutide Standard (concentrated – 3000 ng/ml)	2 vials	Lyophilized, see Reagent Preparation
*Anti-GLP-1: HRP Conjugate concentrated	Anti-GLP-1:HRP Conjugate to Horseradish Peroxidase concentrated (1 mg/ml). *Store frozen at -20°C. Dilution factor is lot-specific.* .	1 vial	See Lot-Specific Reagent Preparation Sheet
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml	Ready-to-Use
Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	2 x 50 ml	Ready-to-Use
Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02%	10 ml	Ready-to-Use

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	bromonitrodioxane with 1:1000 dilution normal human serum		
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 mL	See Reagent Preparation
TMB Substrate	Stabilized Chromogen	12 mL	Ready-to-Use
Stop Solution	0.73M Phosphoric Acid	12 mL	Ready-to-Use
Instruction Manual		1	
Reagent Preparation Sheet		1	

**See Lot-Specific Reagent Preparation Instructions included in Kit.*

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

- Microtiter Plate Reader able to measure absorbance at 450 nm.
- Adjustable pipettes to measure volumes ranging from 25 ul to 1000 ul
- Deionized (DI) water
- Wash bottle or automated microplate washer
- Graph paper or software for data analysis
- Absorbent paper
- 37°C Incubator
- Timer

PRECAUTIONS

- This kit is for Research Use Only. Follow the instructions carefully.
- Observe the expiration dates and stability information stated on the kit.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shifts during pipetting of reagents.
- Keep reagents in the original shipping containers.
- Some reagents contain a small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to touch skin or mucosa.

- 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.

- Use Good Laboratory Practice.

REAGENT HANDLING and STORAGE CONDITIONS

1. Aliquot and store the Anti-GLP-1 HRP Conjugate concentrate at **-20°C** upon receipt. Immediately discard any excess 1x Working Anti-GLP-1 HRP Conjugate after running the assay.
2. Store the rest of the kit components at 2-8°C.
3. Use all reagents and wash solutions within 12 months of manufacturing date.
4. Before use, bring all components to room temperature (18-25ºC).
5. Upon assay completion, ensure all kit components are returned to appropriate storage conditions.

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6. The substrate is light-sensitive and should be protected from direct sunlight or UV sources.

SAMPLE COLLECTION, PREPARATION, AND STORAGE

Samples should be clear and non-hemolyzed. Samples should be run at several dilutions to ensure accurate quantitation. *Grossly hemolyzed samples are not suitable to use in this assay.*

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 store 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 are 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.

REAGENT PREPARATION

All reagents should be diluted immediately before use. See Reagent Preparation Sheet included in kit for lot-Specific 1x Working Anti-GLP-1 HRP Conjugate Preparation.

1. Label any aliquots made with the kit Lot Number and Expiration Date and store as directed.

2. Bring all reagents to room temperature before use.

3. To prepare **1x working Wash Buffer**; 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. Let sit and agitate gently for 15 mins before preparing more 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 per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Preparation Instructions
3000 ng/ml	Original Standard	Original Standard provided in the Kit + 1 ml Standard Diluent (1X)
2560 ng/ml	Standard No.7	853.3 ul Original Standard (3000 ng/ml) + 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)

Note: Use standards as soon as possible upon reconstitution. Discard leftovers.

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5. **Lot-Specific 1x Working Anti-GLP-1 HRP Conjugate Preparation:**
   Important: It is recommended to perform a two-step (2-step) dilution immediately before running the assay to avoid dilution error. Discard any unused 1x working Anti-GLP-1 HRP Conjugate concentrate.

   IMPORTANT: THE DILUTION FACTOR PROVIDED IS LOT-SPECIFIC AND VARIES FROM LOT TO LOT. REFER TO THE "REAGENT PREPARATION SHEET" INCLUDED IN THE KIT FOR LOT-SPECIFIC INSTRUCTIONS.

PROCEDURAL NOTES

1. To achieve good reproducibility and sensitivity, proper washing is essential.
2. Avoid using samples containing sodium azide (NaN3), as it could destroy the HRP activity resulting in underestimation of the amount of Liraglutide.
3. It is recommended that all Standards and Samples be assayed in duplicate or triplicate.
4. Maintain a repetitive timing sequence from well to well for all steps to ensure consistent incubation times for each well.
5. If the Substrate has a distinct blue color prior to use, it may be contaminated, and use of such substrate can compromise assay sensitivity.
6. The plates should be read within 30 minutes after addition of Stop Solution.
7. It is recommended to make a plate map to identify the location of standards and samples.

ASSAY PROCEDURE

It is strongly recommended that all Standards and Samples be run in duplicate or triplicate. A standard curve is required for each assay.

1. Pipette **100 µl of prepared Standards** and **Samples** into the respective wells.
2. Cover the plate and incubate for 60 minutes at 37°C.
3. Wash the plate 5 times with 300 ul **1x working Wash Buffer** and blot residual buffer by firmly tapping the plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells as residue can interfere in the reading step. All washes should be performed this way.
4. Add **100 ul** of **1x working Anti-GLP-1 HRP Conjugate** to each well, prepared according to the Reagent Preparation Sheet included in the kit.
5. Seal the plate and incubate for 60 minutes at 37ºC.
6. Wash the plate 5 times with 300 ul **1x working Wash Buffer** and blot residual buffer by firmly tapping the plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells.
7. Pipette **100 ul** of **TMB Substrate solution** into all wells.
8. Incubate in the dark for 30 minutes at 37ºC.
9. Stop the reaction by adding **100 ul** of **Stop Solution** to each well.
10. Read the absorbance at 450 nm within 30 minutes of stopping the reaction.

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 capable of generating

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a polynomial regression (2nd order) or a cubic spline curve-fit is recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following case:
- If the sample absorbance value is below the lowest standard.

QUALITY CONTROL

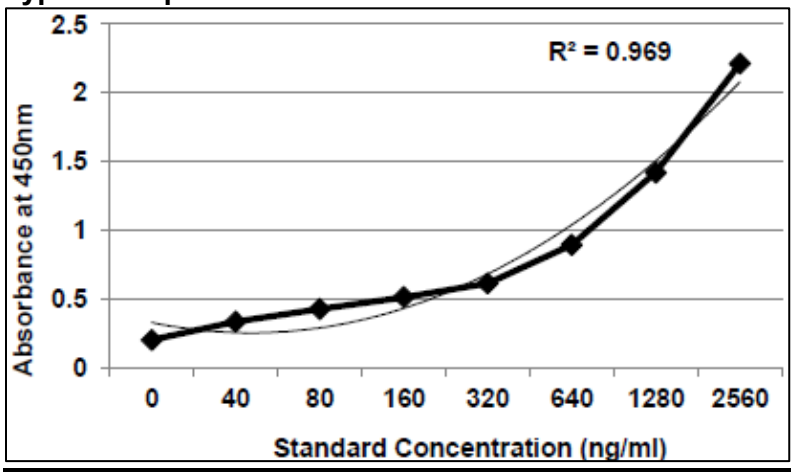
It is recommended that each laboratory assay appropriate quality control samples with each run to ensure that all reagents and procedures are correct.

Typical Data

For demonstration purposes only. A standard curve must be run for each plate.

Standards (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



PERFORMANCE CHARACTERISTICS

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

Sensitivity:

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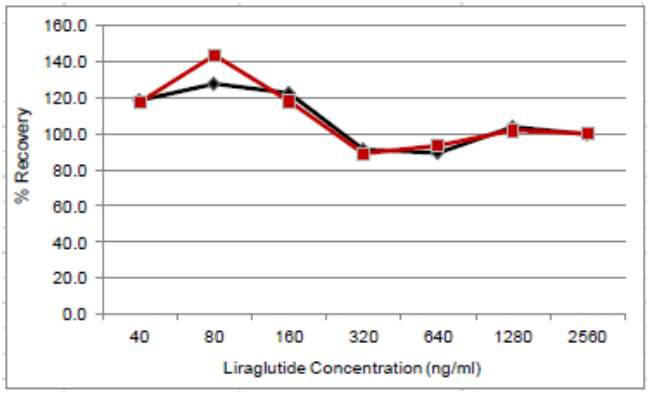
Limit Of Detection: LoD is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2*SD. 10 replicates of '0' standards were evaluated and the LOD was found to be less than 40 ng/ml.

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 from 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%

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