KRIBIOLISA™ Liraglutide ELISA

REF : KBI5020

Ver 4.0

RUO

Immunoassay for the Quantitative Estimation of Liraglutide in serum, plasma and cell culture supernatant

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 KBI5020 Σ 96 tests

KRISHGEN BioSystems for US / Europe: toll free +1(888)-970-0827 | tel: +1(562)-568-5005
for Asia / India: tel: +91(22)-49198700
email: sales@krishgen.com

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 ELISA is used as for the quantitative determination of Liraglutide in serum, plasma and cell culture supernatant.

Principle:
The method employs a competitive immunoassay for the determination of Liraglutide. The Anti-Liraglutide Antibodies are coated on microtiter plate. A constant concentration of Biotinylated Liraglutide and varying concentration of unlabeled standard or sample compete for binding to anti-Liraglutide antibodies. Captured Biotinylated Liraglutide is subsequently bound by HRP conjugate 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 inversely proportional to the amount of bound Liraglutide present in the standards or samples.

Materials Provided:
1. Anti-Liraglutide Coated Microtitre Plate (12 x 8 wells) – 1 no.
2. Liraglutide Standard (concentrated, 6 mg/ml) – 1 vial
3. Biotinylated Liraglutide – 1 vial
4. Streptavidin-HRP Conjugate – 1 vial
5. (20X) Wash Buffer – 25ml
6. Assay Diluent – 50ml
7. HRP Conjugate Diluent – 12ml
8. TMB Substrate – 12ml
9. Stop Solution – 12ml
10. Instruction Manual

Materials to be provided by the End-User:
1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Handling/Storage:
1. All reagents should be stored at 2ºC to 8ºC for stability.
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
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 Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80ºC. Avoid repeated freeze-thaw cycles.

For Serum / Plasma - Samples have to be diluted 1:1000 (v/v), e.g. 1 ul Sample + 999 ul Assay Diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

Preparation before Use
Allow samples to reach room temperature prior to assay. Take care to agitate samples gently in order to ensure homogeneity.

Reagent Preparation (all reagents should be diluted immediately prior to use):
1. Bring all kit components and samples to room temperature (18-25°C) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.
2. **Standard:** The concentration of the standard in the stock solution is **6 mg/ml**. Prepare the standards as per the table given below using the provided Standard Concentration and Assay Diluent.

Standard Concentration	Standard No	Dilution Particulars
15 ug/ml	--	2.5 ul Original Standard (**6 mg/ml**) + 997.5 ul of Assay Diluent
3.0 ug/ml	Standard No.5	200 ul Standard (15 ug/ml) + 800 ul of Assay Diluent
2.5 ug/ml	Standard No.4	833 ul Standard No.5 + 167 ul Assay Diluent
1.0 ug/ml	Standard No.3	400 ul Standard No.4 + 600 ul Assay Diluent
0.5 ug/ml	Standard No.2	500 ul Standard No.3 + 500 ul Assay Diluent
0.1 ug/ml	Standard No.1	200 ul Standard No.2 + 800 ul Assay Diluent

Mix each tube thoroughly before the next transfer. Set up 5 points of diluted standard such as 3 ug/ml, 2.5 ug/ml, 1.0 ug/ml, 0.5 ug/ml, 0.1 ug/ml and 0 ug/ml. It is recommended to freshly prepare the standards before the assay and discard unused standards.

Procedural Notes:
1. For good assay reproducibility and sensitivity, proper washing of the ELISA plate to remove excess/unbound reagents is essential.
2. If the concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect. To overcome Hook Effect samples to be assayed should be sufficiently diluted with our recommended diluent.
3. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity of the conjugate resulting in under-estimation of the antibodies.
4. All Standards and Samples should be assayed at least in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromising the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1) Bring all reagents to room temperature prior to use. It is strongly recommended that all Controls and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at 37°C.
2) Pipette out **100 ul** of **Sample / Standard (Liraglutide)** into each well.
3) Cover the plate and incubate for 90 minutes at 37°C.
4) Add **50 ul** of **Biotinylated Liraglutide** in each well.
5) Cover the plate and incubate for 90 minutes at 37°C.
6) 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.
7) Pipette without delay in the same order **100 ul** of **Streptavidin-HRP Conjugate** into each well.
8) Cover the plate and incubate for 1 hour at 37°C.
9) Aspirate and wash plate 4 times with **Wash Buffer (1X)** as in step 6 above.
10) Add **100 ul** of **TMB Substrate** in each well.
11) Incubate the plate at 37°C for 15-30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
12) Pipette **100 ul** of **Stop Solution** in all wells. Wells should turn from blue to yellow in color.
13) Read the absorbance at 450 nm with a microplate reader.

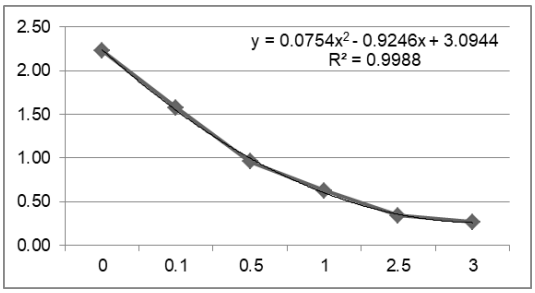
Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) 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 Liraglutide Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate 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 3 ug/ml standard.

Typical Graph



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. 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.

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 0.05 ug/ml

Specificity:
The antibodies used in the kit are monoclonal for high specificity. The standards have been calibrated against commercially sourced Victoza® Injection manufactured by Novo Nordisk.

Linearity:
Standards provided in the kit were used for measuring the linearity range of Liraglutide present in matrix. The Detection range provided is 0 - 3 ug/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 (0.1 ug/ml), medium (1 ug/ml) and high (3 ug/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	<12%	<15%
Medium	<10%	<12%
High	<10%	<12%

Recovery:
Known amount of Liraglutide was "spiked" into sample diluent (1X), diluted normal human serum at varying concentrations and diluted normal human plasma at varying concentrations. The samples were then run in the ELISA.

The resulting concentration, or "recovery" of the spiked material, demonstrates if the expected value can be measured accurately. If the recovered value differs significantly from the amount expected, this may be a sign that some factor in the sample matrix may be causing a falsely elevated or falsely depressed value.

Liraglutide Standard (1:10 serum)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	0.454	0.403	0.429	0.00	---
0.1	0.172	0.224	0.198	14.18	14.18
0.5	0.164	0.236	0.200	14.04	2.81
1.0	0.170	0.156	0.163	*	*
2.5	0.156	0.134	0.145	*	*
3.0	0.143	0.127	0.135	*	*

Liraglutide Standard (1:100 serum)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	0.849	0.772	0.810	0.00	---
0.1	0.443	0.368	0.405	175.86	175.86
0.5	0.249	0.291	0.270	699.13	139.83
1.0	0.334	0.241	0.287	588.45	58.84
2.5	0.154	0.146	0.150	2468.9	98.76
3.0	0.101	0.120	0.111	3853.54	128.45

Liraglutide Standard (1:1000 serum)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	1.009	0.881	0.945	0.00	---
0.1	0.488	0.466	0.477	183.05	183.05
0.5	0.357	0.360	0.358	484.58	96.92
1.0	0.256	0.237	0.247	1099.72	109.97
2.5	0.136	0.140	0.138	2353.03	94.12
3.0	0.108	0.098	0.103	2989.96	99.67

Liraglutide Standard (1:2000 serum)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	0.482	0.564	0.523	0.00	---
0.1	0.481	0.557	0.519	7.52	7.52
0.5	0.297	0.326	0.311	455.07	91.01
1.0	0.165	0.171	0.168	1108.14	110.81
2.5	0.132	0.136	0.134	1700.71	68.03
3.0	0.104	0.097	0.101	*	*

Concentration in ug/ml Abs = Absorbance at 450 nm Interpolated Conc = Recovery Concentration post spiking with known standard
** indeterminate value*

Liraglutide Standard (1:10 plasma)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	0.434	0.405	0.420	0.00	---
0.1	0.539	0.584	0.562	*	*
0.5	0.426	0.342	0.384	587.02	117.40
1.0	0.333	0.254	0.293	995.84	99.58
2.5	0.191	0.147	0.169	2089.94	83.60
3.0	0.127	0.147	0.137	*	*

Liraglutide Standard (1:100 plasma)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	0.334	0.405	0.370	0.00	---
0.1	0.539	0.584	0.562	*	*
0.5	0.461	0.341	0.401	536.87	107.37
1.0	0.323	0.244	0.283	999.41	99.94
2.5	0.171	0.157	0.164	2200.54	88.02
3.0	0.166	0.147	0.157	*	*

Liraglutide Standard (1:1000 plasma)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	1.438	1.246	1.342	0.00	---
0.1	0.788	0.747	0.767	83.03	83.03
0.5	0.404	0.328	0.366	474.22	94.84
1.0	0.252	0.241	0.246	1019.14	101.91
2.5	0.142	0.140	0.141	3046.74	121.87
3.0	0.140	0.162	0.151	2642.42	88.08

Liraglutide Standard (1:2000 plasma)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	1.239	1.022	1.130	0.00	---
0.1	0.749	0.750	0.749	117.19	117.19
0.5	0.341	0.327	0.334	468.47	93.69
1.0	0.235	0.204	0.220	994.32	99.43
2.5	0.162	0.158	0.160	3127.05	125.08
3.0	0.156	0.163	0.159	3272.76	109.09

Concentration in ug/ml Abs = Absorbance at 450 nm Interpolated Conc = Recovery Concentration post spiking with known standard
** indeterminate value*

The above Absorbances were read on a Tecan Safire2® and the data extrapolated using GraphPad Prism® ver8.0. Normal human serum and plasma was sourced from commercial laboratories. The recovery for Liraglutide spiked in the Assay Diluent is presented herein below :

Liraglutide Standard (1:2000 plasma)	Abs1	Abs2	Mean	Interpolated Concentration	% Concentration against known Concentration
0	2.30	2.16	2.23	---	---
0.1	1.60	1.56	1.58	0.08	84.73
0.5	0.92	1.00	0.96	0.42	83.50
1	0.54	0.71	0.63	1.01	101.30
2.5	0.34	0.34	0.34	2.66	106.21
3	0.29	0.24	0.27	3.68	122.66

Liraglutide used for the above spike and recovery studies was commercially sourced Victoza® Injection manufactured by Novo Nordisk.

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.

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/w) 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.

Example of a Work List

Well #	Contents	Abs at 450nm	Mean Absorbance	ug/ml Liraglutide equiv.
1A	Zero Std			
2A	Zero Std			
1B	0.1 ug/ml			
2B	0.1 ug/ml			
1C	0.5 ug/ml			
2C	0.5 ug/ml			
1D	1.0 ug/ml			
2D	1.0 ug/ml			
1E	2.5 ug/ml			
2E	2.5 ug/ml			
1F	3.0 ug/ml			
2F	3.0 ug/ml			
1G	Sample			
2G	Sample			
1H	Sample			
2H	Sample			
3A	Sample			
4A	Sample			

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