






KRIBIOLISA™ Lixisenatide ELISA

REF : KBI5021

Ver 2.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Lixisenatide 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 |

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 96 tests



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Introduction:

Lixisenatide is a glucagon-like peptide-1 (GLP-1) receptor agonist used in the treatment of type 2 diabetes mellitus (T2DM). It is sold under the brand name Adlyxin by Sanofi-Aventis. Adlyxin received FDA approval July 28, 2016

Intended Use:

The KRIBIOLISA™ Lixisenatide ELISA is used as for the quantitative determination of Lixisenatide in human serum and plasma.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Human GLP-1 antibody is pre-coated onto microwells. Samples and standards are pipetted into microwells and Lixisenatide present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated Human GLP1 antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Exenetide in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:

| Part | Description | Qty |
|--|---|--------------|
| Human GLP-1 antibody Coated Microtiter Plate | 96 well polystyrene microplate (12 strips of 8 wells) coated with Human GLP-1 antibody. | 1 x 96 wells |
| Lixisenatide Standard | Lixisenatide in a buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane – lyophilized (5 ug/ml) | 2 vials |
| Human GLP-1 antibody:HRP Conjugate | Human GLP-1 antibody conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane. | 12 ml |
| (1X) Standard Diluent | Buffered protein base with 1:1000 dilution human serum and protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane | 10 ml |
| (1X) Sample Diluent | Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane | 2 x 50 ml |
| (20X) Wash Buffer | 20-fold concentrated solution of buffered surfactant with preservative thiomersol < 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. All reagents should be stored at 2°C to 8°C for stability.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.

- Before using, bring all components to room temperature (18-25°C). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
- The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

- Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
- 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 -80°C. Avoid repeated freeze-thaw cycles.

Preparation Before Use:

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

Serum Test Sample preparation - Samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul 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.

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

- Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
- Bring all reagents to Room temperature before use.
- To make **Wash Buffer (1X)**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
- Standards Preparation:** Reconstitute the concentrated Standard lyophilized vial with 250 ul of Standard Diluent (1X) to obtain a concentration of 20,000 ng/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 240 ul of original **Standard (20,000 ng/ml)** with 160 ul of Standard Diluent (1X) to generate a **12000 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 |
|------------------------|-------------------|--|
| 20,000 ng/ml | Original Standard | Original Standard provided in the Kit +250 ul Standard Diluent (1X) |
| 12000 ng/ml | Standard No.7 | 240 ul Original Standard (20,000 ng/ml) + 160 ul Standard Diluent (1X) |
| 6000 ng/ml | Standard No.6 | 200 ul Standard No.7 + 200 ul Standard Diluent (1X) |
| 3000 ng/ml | Standard No.5 | 200 ul Standard No.6 + 200 ul Standard Diluent (1X) |
| 1500 ng/ml | Standard No.4 | 200 ul Standard No.5 + 200 ul Standard Diluent (1X) |
| 750 ng/ml | Standard No.3 | 200 ul Standard No.4 + 200 ul Standard Diluent (1X) |
| 375 ng/ml | Standard No.2 | 200 ul Standard No.3 + 200 ul Standard Diluent (1X) |
| 187.5 ng/ml | Standard No.1 | 200 ul Standard No.2 + 200 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.

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. High Dose Hook Effect may be observed in samples with very high concentrations of Lixisenatide. High Dose Hook Effect is due to excess of antibody for very high concentrations of Lixisenatide present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Lixisenatide concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Lixisenatide.
4. It is recommended that all Standards and Samples be assayed 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 compromise of 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. It is strongly recommended that all Standards 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. Add **100 ul** of prepared **Standards** or diluted **Samples** into the respective wells.
3. Cover the 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.
5. Pipette **100 ul** of **Human GLP-1 antibody:HRP Conjugate** into each well.
6. Cover the plate and incubate for 120 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.
8. Add **100 ul** of **TMB Substrate** in each well.
9. Incubate the plate at **37°C** for 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.
10. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.
11. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate Standards and Samples. Using 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 Lixisenatide 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 Lixisenatide Concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit or a 4PL polynomial curve (2nd order) is best recommended for automated results.

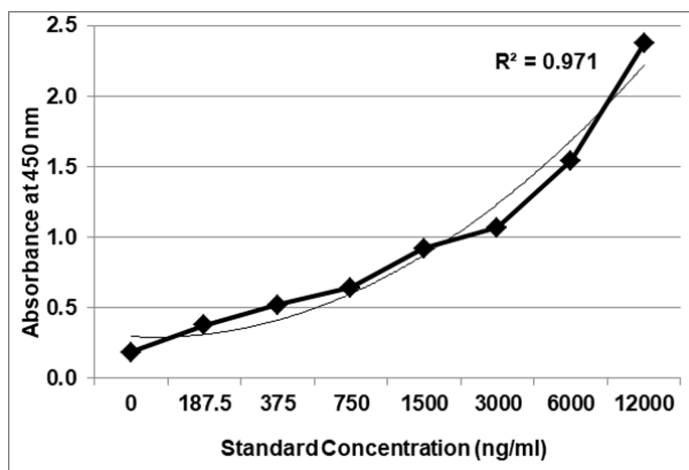
Note:

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 12,000 ng/ml standard.

Typical Data

| Standard Concentration (ng/ml) | Mean Absorbance | Interpolated Concentration | % Interpolated Concentration against Actual Concentration |
|--------------------------------|-----------------|----------------------------|---|
| 0 | 0.186 | | -- |
| 187.5 | 0.378 | 158.3 | 84.4 |
| 375 | 0.518 | 448.4 | 119.6 |
| 750 | 0.643 | 820.8 | 109.4 |
| 1500 | 0.920 | 1795.2 | 119.7 |
| 3000 | 1.068 | 2617.2 | 87.2 |
| 6000 | 1.542 | 5497.0 | 91.6 |
| 12000 | 2.376 | 12426.8 | 103.6 |

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.

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 ~180 ng/ml.

Specificity:

The antibodies used in the kit are monoclonal antibodies, anti-idiotypic and specific for Lixisenatide peptide. There is cross-reactivity observed between GLP-1 analogs like Exenatide, Semaglutide and Liraglutide which has not been validated with this assay.

Linearity:

Standards provided in the kit will be used for measuring the linearity range of Lixisenatide present in matrix.

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 (187.5 ng/ml), medium (3000 ng/ml) and high (12000 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 | <10% | <10% |
| Medium | <5% | <5% |
| High | <5% | <5% |

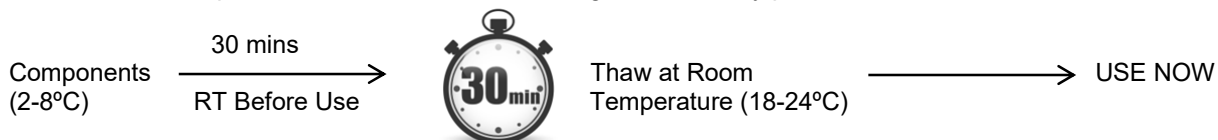
Safety Precautions:

- **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.




SCHEMATIC ASSAY PROCEDURE



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





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

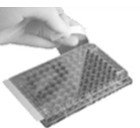




3.  Pipette **100 ul Standards / Samples** into the respective wells.

4.  Cover plate and incubate for  at 37°C.

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X)**.

6.  Pipette **100 ul Human GLP-1 antibody:HRP Conjugate** into each well.

7.  Cover plate and incubate for  at 37°C.

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X)**.

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 450nm with a  microplate reader within  of stopping reaction.

Typical Example of a Work List

| Well # | Contents | Absorbance at 450nm | Mean Absorbance | ng/ml Lixisenatide equivalent |
|--------|-------------|---------------------|-----------------|-------------------------------|
| 1A | zero std | | | |
| 2A | zero std | | | |
| 1B | 187.5 ng/ml | | | |
| 2B | 187.5 ng/ml | | | |
| 1C | 375 ng/ml | | | |
| 2C | 375 ng/ml | | | |
| 1D | 750 ng/ml | | | |
| 2D | 750 ng/ml | | | |
| 1E | 1500 ng/ml | | | |
| 2E | 1500 ng/ml | | | |
| 1F | 3000 ng/ml | | | |
| 2F | 3000 ng/ml | | | |
| 1G | 6000 ng/ml | | | |
| 2G | 6000 ng/ml | | | |
| 1H | 12000 ng/ml | | | |
| 2H | 12000 ng/ml | | | |
| 3A | Sample | | | |
| 4A | Sample | | | |
| 3B | Sample | | | |
| 4B | Sample | | | |

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Krishgen Biosystems 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, or against damages resulting from such non-Krishgen Biosystems made products or components. Krishgen Biosystems passes on to customer the warranty it received (if any) from the maker thereof of such non Krishgen made products or components. This warranty also does not apply to Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.



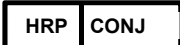









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This Limited Warranty states the entire obligation of Krishgen Biosystems with respect to the Products. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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THANK YOU FOR USING KRISHGEN PRODUCT!

SYMBOLS KEY

| | |
|---|---|
|  | Human GLP-1 antibody Coated Microtiter Plate (12x8 wells) |
|  | Lixisenatide Standard |
|  | Conjugate Horseradish Peroxidase |
|  | Sample Diluent |
|  | Standard Diluent |
|  | (20X) Wash Buffer |
|  | TMB Substrate |
|  | Stop Solution |
|  | Consult Instructions for Use |
|  | Catalog Number |
|  | Expiration Date |
|  | Storage Temperature |