KRIBIOLISA™ Leuprolide / Leuprorelin (Lupron) ELISA

REF : KBI5019

Ver2.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Leuprolide 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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REF KBI5019 Σ 96 tests

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Introduction:

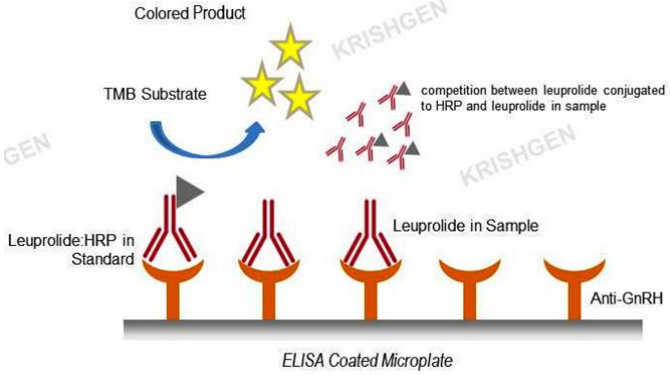
Leuprorelin, also known as leuprolide, is a manufactured version of a hormone used to treat prostate cancer, breast cancer, endometriosis, uterine fibroids. Leuprorelin is in the gonadotropin-releasing hormone (GnRH) analogue family of medications. It works by decreasing gonadotropin and therefore decreasing testosterone and estradiol.

Intended Use:

The KRIBIOLISA™ Leuprolide / Leuprorelin (Lupron™) is used as an analytical tool for quantitative determination of Leuprolide in human serum and plasma.

Principle:

The Leuprolide ELISA is a competitive immunoassay for the determination of Leuprolide in human serum and plasma. Plates are coated with Anti-GnRH antibody. A varying concentration of unlabeled standard or sample and constant concentration of Leuprolide:HRP conjugate will bind in sequence to the antibodies coated on the microplate. Upon washing, unbound Leuprolide:HRP Conjugate will be removed. Bound Leuprolide:HRP Conjugate complex will produce a soluble blue colored product after the 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 Leuprolide present in the standards or samples.



Materials Provided:

Part	Description	Qty
Anti- GnRH antibody Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti- GnRH antibody Coated Microtiter Plate	1 x 96 wells
Leuprolide standard	Leuprolide standard (lyophilized, concentration - 10,000 ng/ml)	2 vials
Leuprolide:HRP Conjugate	Leuprolide:HRP Conjugate prepared in buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative thiomersol < 0.01%	50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane with 1:10 dilution human serum	10 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	2N Sulfuric Acid	12 ml
Instruction Manual		1 no

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 - Samples have to be diluted 1:10 (v/v), e.g. 1 ul sample + 9 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.

For Plasma - Samples have to be diluted 1:10 (v/v), e.g. 1 ul sample + 9 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.

Preparation Before Use:

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

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 **(1X) 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 to obtain 10,000 ng/ml. Keep the vial for 15 mins with gentle agitation and then run the assay procedure. Use the Standard Diluent as the zero standard. Below table shows the calculation for the standard range.

Standard Concentration (ng/ml)	Standard No.	Dilution Particulars
10,000 ng/ml	Lyophilized standard	Lyophilized Standard + 1ml of Standard Diluent
3000 ng/ml	Standard No.10	300 ul Reconstituted Standard + 700 ul Standard Diluent (1X)
2000 ng/ml	Standard No.9	200 ul Reconstituted Standard + 800 ul Standard Diluent (1X)
1000 ng/ml	Standard No.8	500 ul Standard No.9 + 500 ul Standard Diluent (1X)
500 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent (1X)
250 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
31.25 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
15.6 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.1	500 ul Standard Diluent

Mix each tube thoroughly before the next transfer. Use the standards for experiment within one hour of preparation of standard. Discard 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. 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.
3. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
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.
8. Making serial dilution in the wells directly is not permitted.
9. Prepare the Standard within 15 minutes prior to running the assay.
10. Please carefully dilute Standards according to the instruction, and avoid foaming. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated.
11. If crystals have formed in the Wash Solution (20X) concentrate, warm to room temperature and mix gently until the crystals are completely dissolved.
12. Contaminated water or container for reagent preparation will influence the detection results.

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 RT.

2. Pipette out **100 ul of Standards and samples** to the respective wells.

3. Add **100 ul Leuprolide:HRP conjugate** to each well.

4. Cover the plate and incubate for 120 mins at RT and shaking at 200 rpm.

5. 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.

6. Add **100 ul** of **TMB Substrate** in each well.

7. Incubate the plate at RT 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.

8. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

9. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

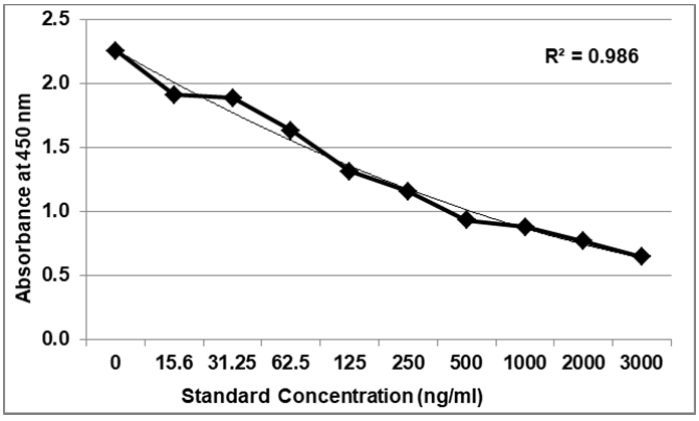
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Leuprolide 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 Leuprolide Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4-PL (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 3000 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	2.258		--
15.6	1.914	21.0	134.7
31.25	1.890	23.3	74.7
62.5	1.634	58.0	92.8
125	1.317	148.7	119.0
250	1.160	241.8	96.7
500	0.933	570.6	114.1
1000	0.883	727.5	72.8
2000	0.773	1458.7	72.9
3000	0.646	3829.1	127.6

Typical Graph



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:

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.

Detection Range:
15.6 ng/ml - 3000 ng/ml.

Sensitivity:
The minimum detectable dose of Leuprolide is <50 ng/ml.

Precision:
Intra-Assay Precision: 3 samples with low, middle and high level human Leuprolide were tested 20 times on one plate, respectively.
Inter-Assay Precision: 3 samples with low, middle and high level human Leuprolide were tested on 3 different plates, 8 replicates in each plate.
CV (%) = SD/mean X 100
Intra-Assay: CV<15%
Inter-Assay: CV<18%

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.

References:

Leuprolide Acetate, a GnRH Agonist, Holds Up Neurodegeneration in an Experimental Glaucoma Model
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Spontaneous dichorionic diamniotic twin pregnancy following a leuprolide acetate trigger and freeze all cycle for OHSS risk: A case report
J White, J Gale, A Jackson - Obstet Gynecol, 2023 - ijcriog.com

Androgen-deprivation therapy with leuprolide increases abdominal adiposity without causing cardiac dysfunction in middle-aged male mice: effect of sildenafil
L Xi, D Kraskauskas, S Muniyan… - American Journal …, 2023 - journals.physiology.org

SCHEMATIC ASSAY PROCEDURE

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

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

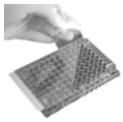
→ Thaw at Room Temperature (18-24ºC) → USE NOW

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 Standard** and **Sample** into respective well.

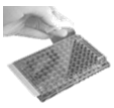
4.  Pipette **100 ul Leuprolide:HRP conjugate** into the respective wells.

5. Cover plate  and incubate for 

at RT and shaking at 200 rpm.

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

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

8. Cover plate  and incubate for 

at RT.

9. 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 Leuprolide
1A 2A	zero standard zero standard			
1B 2B	15.6 ng/ml 15.6 ng/ml			
1C 2C	31.25 ng/ml 31.25 ng/ml			
1D 2D	62.5 ng/ml 62.5 ng/ml			
1E 2E	125 ng/ml 125 ng/ml			
1F 2F	250 ng/ml 250 ng/ml			
1G 2G	500 ng/ml 500 ng/ml			
1H 2H	1000 ng/ml 1000 ng/ml			
3A 4A	2000 ng/ml 2000 ng/ml			
3B 4B	3000 ng/ml 3000 ng/ml			
3C 4C	Sample			

LIMITED WARRANTY

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 product; 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 product 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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SYMBOLS KEY

MTP	**Anti-GnRH Coated Microtiter Plate (12x8 wells)**
STD	**Standard, lyophilized**
HRP CONJ	**Conjugate Horseradish Peroxidase**
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**