KRIBIOLISA™ Anti-Glargine (LANTUS) ELISA (Qualitative)

REF: KBI9001

Ver 2.1

RUO

Immunoassay for Qualitative determination of antibodies to Insulin Glargine 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 KBI9001 Σ 96 tests

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Introduction:

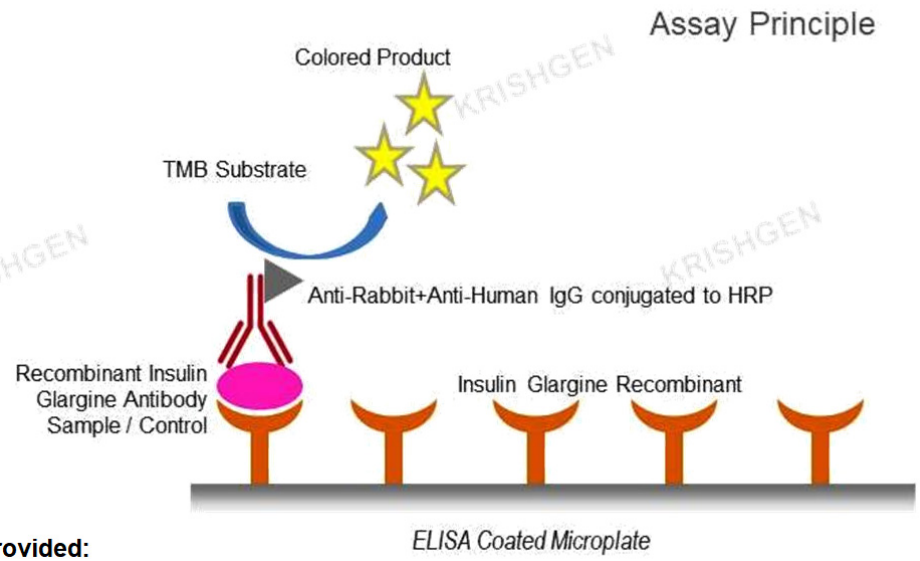
Insulin Glargine is produced by recombinant DNA technology. It is an analogue of human insulin made by replacing the asparagine residue at position A21 of the A-chain with glycine and adding two arginine to the C-terminus (positions B31 and 32) of the B-chain. The resulting protein is soluble at pH 4 and forms micro-precipitates at physiological pH 7.4. Small amounts of insulin Glargine are slowly released from micro-precipitates giving the drug a long duration of action (up to 24 hours) and no pronounced peak concentration.

Intended Use:

The KRIBIOLISA™ Anti-Glargine (LANTUS) ELISA is used as an analytical tool for Qualitative determination of antibodies to Insulin Glargine in Human serum and plasma.

Principle:

The method employs the qualitative sandwich enzyme immunoassay technique. Insulin Glargine is coated into microwells. If antibodies to insulin Glargine are present in the samples or Control, it will form complex with Insulin Glargine, which is pre-coated onto microwells. In the second step, Anti-Human IgG: HRP + Anti – Rabbit IgG HRP conjugate is pipetted and incubated. Free HRP conjugate will be removed by a washing step. In the third step, TMB substrate is added to microwells and color develops proportionally to the amount of Anti-Insulin Glargine present in the samples or controls. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Insulin Glargine Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Insulin Glargine.	1 x 96 wells
Positive Control	Recombinant Anti-Insulin Glargine Positive Control Buffered protein base with preservative thiomersol <0.01%	2ml
Negative Control	Buffered protein base with preservative thiomersol <0.01% and diluted with 1:1000 Human serum	2ml
Anti-Human IgG :HRP + Anti – Rabbit IgG :HRP Conjugate concentrated	Anti-Human IgG: HRP + Anti –Rabbit IgG HRP conjugated to horseradish peroxidase concentrated. (1mg/ml)	1 vial

Part	Description	Qty
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
Sample Diluent	Buffered protein base with preservative thiomersol <0.01%	12 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. 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 sample dilutions.
7. Timer.
8. Absorbent paper.

Storage Information:

1. Store main kit components at 2-8°C.

2. Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

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.

Preparation Before Use:

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

Test Sample preparation - Samples have to be diluted 1:1000 (v/v), e.g. 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.

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 Anti-Insulin Glargine. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Insulin Glargine 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 anti-Insulin Glargine 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 (NaN3), as it could destroy the HRP activity resulting in under-estimation of the amount of Anti-Insulin Glargine.
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 compromisation 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.

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 25ml of 20X Wash Buffer in 475ml of DI water.
4. Anti – Insulin Glargine Positive Control: Ready to use solution.
5. Negative Control: Ready to use solution.
6. **Working Anti-Human IgG:HRP + Anti–Rabbit IgG:HRP Conjugate - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

Assay Procedure:

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all controls and samples be run in duplicate or triplicate.

2. Pipette **100 ul** of **Controls** or **diluted Samples** into the respective wells.

3. Cover the plate and incubate for 60 minutes at room temperature.

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. Add **100 ul** of **Working Anti-Human IgG:HRP + Anti–Rabbit:IgG HRP Conjugate** into each well.

6. Cover the plate and incubate for 60 minutes at room temperature.

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 Room Temperature 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 for Cut-Off Values

Read the sample and negative control wells on microtitre plate reader at 450nm. The OD (Optical Density) of NC (Negative Control) in duplicate should be used for calculating the mean and standard deviation. This is the Negative mean.

The cut-off for Positives is equal to a value greater than (Negative mean + 3*Standard Deviation).

Formula:
Positive Sample Value = OD > (Negative mean + 3*SD)

Typical example –

Sample Type	Absorbance #1	Absorbance #2	Mean
Negative	0.131	0.128	0.129

Therefore Cut-off = Mean + 3*SD (Classen et al. 1987)
= 0.129 + 3* 0.0021
= 0.129 + 0.0042
= 0.133

* The cutoff value is based on validation using recombinant antibodies in the assay. Users may set up their own cutoff values based on different patient serum panels from different geographic locations or ethnic backgrounds.

Interpretation of Results:

Positive Samples	> Cut Off
Negative Samples	<= Cut Off
Unequivocal/Grey Zone Samples *	<Cut Off - >(Cut Off - 0.50)

Note:
1. In case your samples show false positive or false negative results, we recommend to increase the cut-off to 5*SD. (Classen et al. 1987).
2. In case your samples report absorbance close to the cut-off absorbance, we recommend to report such samples as unequivocal samples in absence of clinical interpretation.

Validity of the Test:
The use of controls allows validation of the test. The test is valid if the following conditions are met,

Positive Control Value: Absorbance > 0.5
Negative Control Value: Absorbance < 0.3

In case the control value is out of range, we recommend you to repeat the assay.

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 Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 0.8 ug/ml.

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 0.9 ug/ml.

Note: The sensitivity has been measured in experiments using calibrated standards although the kit is a qualitative kit.

Specificity:
The capture protein used in the kit is specific for glargine. The peptide has been calibrated against the therapeutic product reference Lantus™. The control used in the kit is antibody specific to Insulin Glargine. The control has expressed crossed reactivity to recombinant insulin and other insulin analogs.

Cross Reactivity:
The standard used in the kit is an antibody that has demonstrated cross reactivity with recombinant insulin and other insulin analogs.

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 (10 ng/ml), medium (80 ng/ml) and high (640 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	<12%	<12%
Medium	<12%	<10%
High	<10%	<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 rat 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.

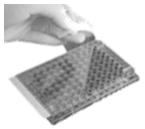
Components (2-8ºC) → 30 mins RT Before Use → 

→ 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) → AVOID → COOL (2-8ºC) → NO CHANGE IN RESULTS

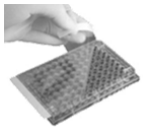
3. Pipette **100 ul Controls** / **diluted Samples** into each well.

4. Cover plate  and incubate for 

at RT.

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

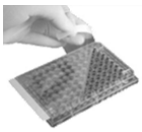
6. Pipette **100 ul Working Anti-Human IgG :HRP + Anti –Rabbit IgG HRP Conjugate** into each well.

7. Cover plate  and incubate for 

at RT.

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 RT.

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

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

of stopping reaction.

LIMITED WARRANTY

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SYMBOLS KEY

MTP	Insulin Glargine Coated Microtiter Plate (12 x 8 wells)
PC	Anti – Insulin Glargine Positive Control
NC	Negative Control
HRP | CONJ	Conjugate Horseradish Peroxidase concentrated
DETN | DIL	Detection Diluent
SAMP | DIL	Sample 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