KRIBIOLISA
Anti-Glargine ELISA

REF : KBI9001

Ver1.0

RUO

Immunoassay for qualitative screening of antibodies to Glargine in 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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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:

This Anti-Glargine ELISA is used as an analytical tool for qualitative laboratory screening of presence or absence of antibodies to Glargine in serum and plasma.

Principle:

The method employs the qualitative sandwich enzyme immunoassay technique. A commercially available Glargine is pre-coated onto microwells. Samples and Controls are pipetted into microwells and antibodies to human Glargine present in the sample and controls are bound by Glargine. Then, a HRP (horseradish peroxidase) conjugated Glargine is pipette and incubated simultaneously with samples. 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 antibodies to Glargine in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:

1. Glargine coated Microtiter plate (96 wells) - 1 no
2. Positive Controls - 0.5ml
3. Negative Control - 0.5ml
4. Glargine:HRP- 12ml
5. Assay Diluent (5X) - 10ml
6. Wash Buffer (20X) - 25ml
7. TMB Substrate - 12ml
8. Stop Solution - 12ml
9. Instruction Manual

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 25µl to 1000µl
3. Deionised (DI) water
4. Wash bottle or automated microplate washer

5. Timer

6. Absorbent Paper

Handling/Storage:

1. All reagents should be stored at 2^0C to 8^0C for stability.

2. Before using, bring all components to room temperature (18-25 ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.

3. 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 or Manufacturing 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.

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

Note: Patient samples have to be diluted 1 + 100 (v/v), e.g. 10µl sample + 1ml 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.

Specimen Collection and Handling:

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material. Samples should be assayed at a number of dilutions to ensure accurate quantitation.

Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x *g*. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles.

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 50ml of 20X Wash Buffer in 950ml of DI water.

4. To make Assay Diluent (1X); dilute 10ml of 5X Assay Diluent in 40ml of DI water.

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-Glargine. High Dose Hook Effect is due to excess of antibody for very high concentrations of anti-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-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 (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of anti-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.

Assay Procedure:

1. Bring all reagents to room temperature prior to use. 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 room temperature (RT).

2. Pipette out 50µl of **Controls** and **Samples** into the respective wells as mentioned in the work list.

3. Cover the plate and incubate it on a plate shaker at 180rpm for 60 minutes at room temperature, 22°C±4°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 without delay in the same order 100µl of **Glargine:HRP Conjugate** into each well.

6. Cover the plate and incubate it on a plate shaker at 180 rpm for 60 minutes at room temperature, 22°C±4°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 100µl of **TMB Substrate** in each well.

9. Incubate the plate at room temperature for 15 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 100µl of **Stop Solution**. Wells should turn from blue to yellow in color.

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

Interpretation of Results:

Results are interpreted qualitatively by calculating a cut-off value. The same maybe done as under –

Read the Sample and Negative Control wells on microtiter plate reader at 450nm. The OD (Optical Density) of NC (Negative Control) in triplicate should be used for calculating the mean and standard deviation. This is the $Blank_{mean}$. The cut-off for Positives is equal to a value greater than ($Blank_{mean}$ + 2*Standard Deviation).

Formula:

Positive Sample Value = OD > (Blank $_{mean}$ + 1 or 2 * SD)

1= If samples are in single wells
2= If samples are in duplicates
3= If samples are in triplicates

For Example –

Sample Type	*Absorbance #1*	*Absorbance #2*	*Absorbance #3*	*Mean*
Negative	*0.200*	*0.219*	*0.221*	*0.213*
Standard Deviation	*0.200-0.213 = -0.013*	*0.219-0.213 = 0.006*	*0.221-0.213 = 0.008*	

Mean Standard Deviation = $\sqrt{(-0.013)^2 + (0.006)^2 + (0.008)^2}$ */ n-1 = 0.0082*

Therefore Cut-Off = Mean + 2 * SD
= 0.213 + 2 * 0.0082
= 0.213 + 0.0164
= 0.229 say 0.23

Typical Reference Values would then be :

Anti- Glargine	Cut-Off Values
Negative	<=0.23
Positive	> 0.23

Note:
The standard deviation (SD) provides an estimate of the reproducibility of replicate data points and can provide confidence levels for assessing if one value is truly different from another. Whatever the measured value, a certain percentage of the values obtained are contained within the standard deviation. For instance, one SD on either side of the mean contains 68% of the values under the curve of that distribution. Approximately two SD (actually 1.96 SD) on either side of the mean contains 95% of all of the values and approximately three SD (actually 2.58 SD) contains 99% of all values. Thus if a value that is greater than three SD different from the mean of a set of samples is obtained, one can be 99% confident that it is truly different from the first set of samples. Mathematically, the SD is the square root of the sum of the variances squared divided by the number of samples minus one.

Precautions:

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

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.

A typical Assay Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	N.C.	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39	S43
B	N.C.	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40	S44
C	P.C.	S4	S8	S12	S16	S20	S24	S8	S32	S36	S40	S44
D	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37	S41	S45
E	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37	S41	S45
F	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38	S42	N.C.
G	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38	S42	P.C.
H	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39	S43	P.C.

* All controls and samples are run in duplicates

Blank – Blank wells
P.C – Positive control wells
N.C – Negative control wells
S. – Sample extract wells

Safety Precautions :

• **This kit is for in vitro 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 reconstituted 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 at 2 - 8 °C before use in the original shipping container.

• Some of the reagents contain small amounts (< 0.1 % w/w) sodium azide 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.

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