# KRIBIOLISA™ Insulin Glargine ELISA

REF: KBI2001

Ver 1.1

**RUO**

Immunoassay for quantitative determination of 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** **KBI2001** **Σ 96 tests**

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**Introduction:**

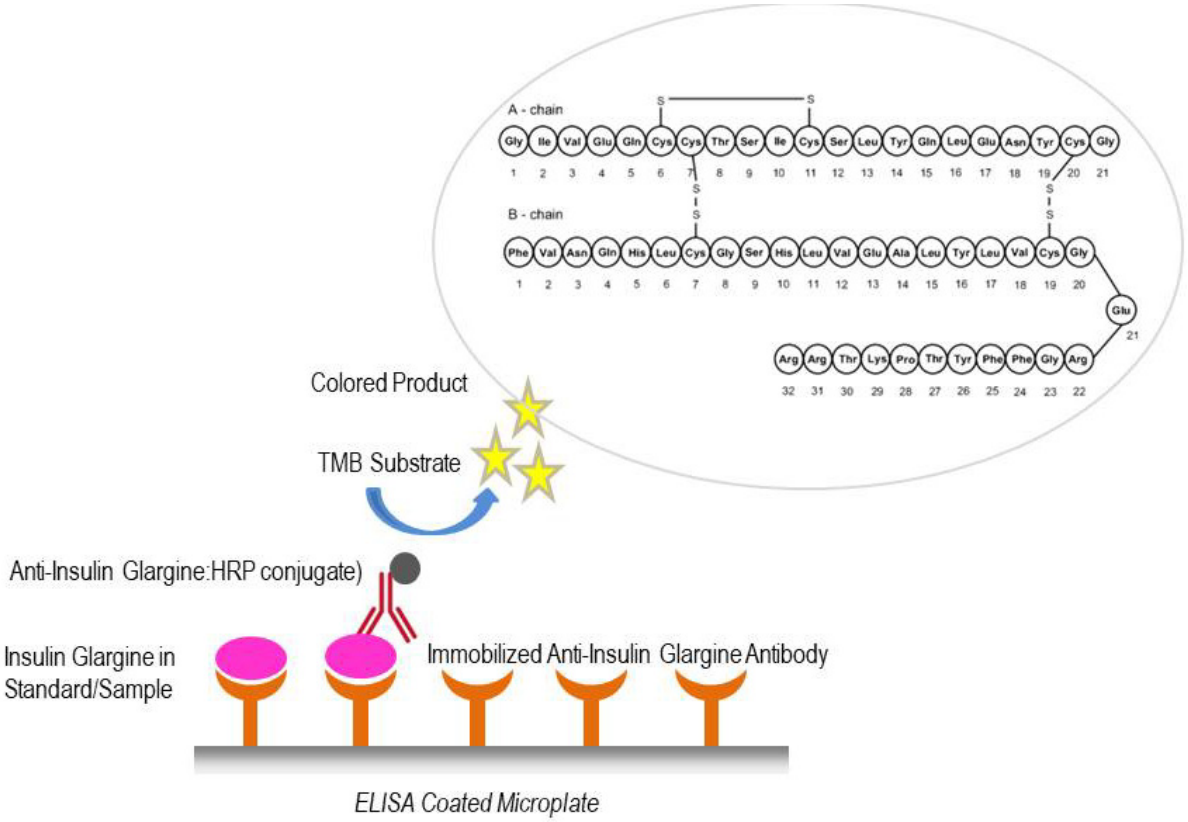
Insulin Glargine is produced by recombinant DNA technology. It is an analogue of human insulin made by replacing the asparagine residue at position A21 of the A-chain with glycine and adding two arginine to the C-terminus (positions B31 and 32) of the B-chain. The resulting protein is soluble at pH 4 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™ Insulin Glargine ELISA is used as an analytical tool for quantitative determination of Insulin Glargine in human serum and plasma.

**Principle:**

The method employs the quantitative sandwich enzyme immunoassay technique. In the first step, samples and standards are pipetted into microwells. If insulin Glargine is present in the samples or standards, it will form complex with Anti-insulin Glargine antibody, which is pre-coated onto microwells. In the second step, anti-Insulin Glargine 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 Insulin Glargine present in the samples or standards. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.




**PRINCIPLE OF THE KRIBIOLISA™ INSULIN GLARGINE ELISA**

**Materials Provided:**

| Part | Description | Qty |
|---|---|---|
| Anti-Insulin Glargine antibody Coated Microtiter Plate | 96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Insulin Glargine antibody. | 1 x 96 wells |
| Recombinant Human Insulin Glargine Standard | Recombinant Human Insulin Glargine Standard (concentrated. 100 IU/ml) | 2 vials |
| (1X) Standard Diluent | Buffered protein base with 1:1000 human serum and with preservative thiomersol <0.01% | 10 ml |
| Anti-Insulin Glargine Antibody:HRP Conjugate | Anti-Insulin Glargine antibody conjugated to horseradish peroxidase 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% | 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 | 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.

**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.
3. After reconstitution of standards, it has to be used immediately and cannot be stored.

**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.

**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 in 1000 (v/v), e.g. 1 µl sample in 999ul of (1X) Sample diluent, prior to assay. The samples may be kept at 2 -8ºC for up to three days. Long-term storage requires -20ºC.

**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 Insulin Glargine. High Dose Hook Effect is due to excess of antibody for very high concentrations of 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 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 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. The Main standard Concentration is 100 IU/ml. Add 175 ul of main standard stock (100 IU/ml) to 75 ul of Standard Diluent to get a concentration of 70 IU/ml. Do further dilutions of the standards Thus, the Human Insulin Glargine standard concentrations are 65 IU/ml. 60 IU/ml, 50 IU/ml and 40 IU/ml. 1X Standard Diluent serves as the zero standard (0 IU/ml).

| Standard Concentration | Standard Vial | Dilution Particulars |
|---|---|---|
| 100 IU/ml | Original Standard | Original Standard provided in the Kit |
| 70 IU/ml | Standard No.5 | 175 ul of Original Standard + 75 ul of Standard Diluent (1X) |
| 65 IU/ml | Standard No.4 | 162.5 ul of Original Standard + 87.5 ul of Standard Diluent (1X) |
| 60 IU/ml | Standard No.3 | 150 ul of Original Standard + 100 ul of Standard Diluent (1X) |
| 50 IU/ml | Standard No.2 | 125 ul of Original Standard + 125 ul of Standard Diluent (1X) |
| 40 IU/ml | Standard No.1 | 100 ul of Original Standard + 150 ul of Standard Diluent (1X) |
| 0 IU/ml | Standard No.0 | Only Standard Diluent |

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

**Assay Procedure:**

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. Pipette **100 ul** of **prepared Standards** or **Samples** into the respective wells.

3. Cover the plate and incubate for 120 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 **Anti-Insulin Glargine Antibody:HRP Conjugate** into each well.

6. Cover the plate and incubate for 120 minutes at 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 **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 of Results:**

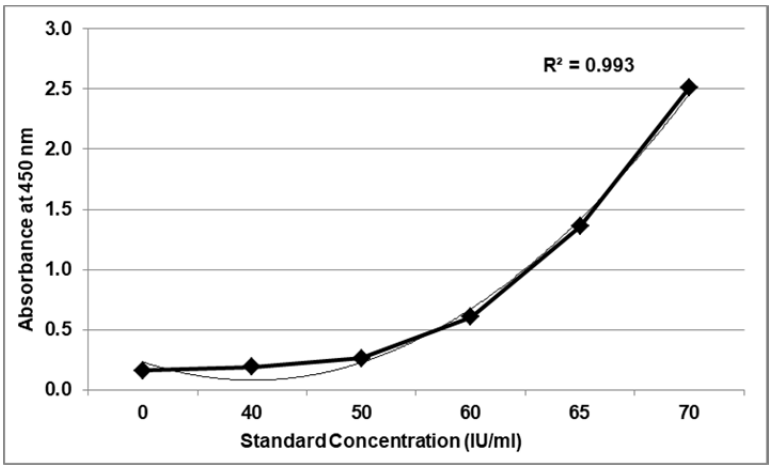
Determine the mean absorbance for each set of duplicate or triplicate standards and samples. Subtract the mean absorbance of the zero standards (background) from each well. Using semi-log graph paper or computer programs, plot the optical densities of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit straight line through the standard points. To determine the unknown Human Insulin Glargine concentrations, find the unknowns 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 Insulin Glargine concentration. If samples were diluted, multiply by the appropriate dilution factor.

Computer based curve-fitting software, 4-PL or cubic spline or polynomial regression ($2^{nd}$ order) may be preferred.

**Typical Data**

| Std Concentration (IU/ml) | Mean Abs | Interpolated Concentration | % Interpolated Concentration against Actual Concentration |
|---|---|---|---|
| 0 | 0.160 | -- | -- |
| 40 | 0.191 | 37.8 | 94.4 |
| 50 | 0.260 | 52.9 | 105.8 |
| 60 | 0.603 | 59.9 | 99.8 |
| 65 | 1.357 | 65.0 | 100.0 |
| 70 | 2.511 | 70.0 | 100.0 |

**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 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 and the LOD was found to be less than 38 IU/ml

**Specificity:**
The antibodies used in the kit are monoclonal antibodies specific for Insulin glargine. The standard / calibrator used in the kit are calibrated against commercially sourced Insulin Glargine (Lantus Injection™)

**Linearity:**
Standards provided in the kit will be used for measuring the linearity range of Insulin Glargine 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 (40 IU/ml), medium (60 IU/ml) and high (70 IU/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 | <10% | <10% |
| High | <10% | <10% |

**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:**

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