Insulin (Human)
ELISA KIT PROTOCOL

(Catalog No. EK-035-06)
(range: 0.781-50µIU)

PHOENIX PHARMACEUTICALS, INC.

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INTRODUCTION AND PROTOCOL OVERVIEW

Insulin is composed of two peptide chains linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids. Insulin is normally secreted by the islet cells of the pancreas. The stimulus for insulin secretion is high blood glucose level. Common effects of insulin tend to lower the blood glucose level. In muscle or fat cell, Insulin activates enzymes to store glucose as glycogen for energy. However, people with Type 1 diabetes produce inadequate amounts of insulin, and in Type 2 diabetes the problem is not a lack of insulin output, but an increase in resistance of the body's cells to the effects of insulin.

Phoenix Pharmaceutical's Human Insulin ELISA Kit is designed to measure the concentration of Human Insulin from human serum/plasma, or conditioned medium.

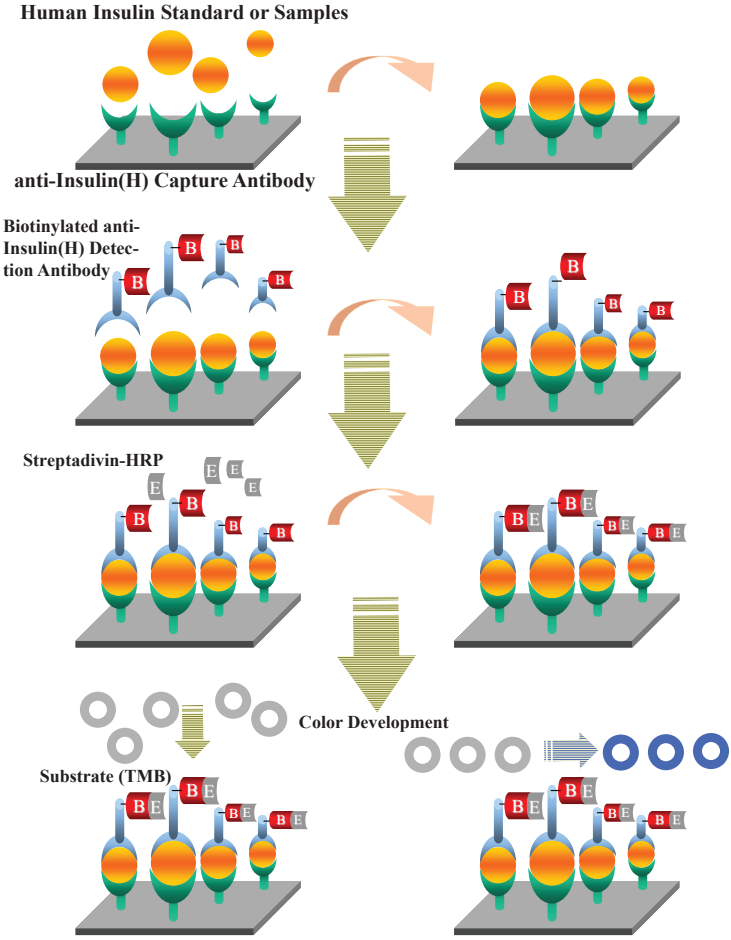
The immunoplate in this kit is precoated with anti-Human Insulin-Capture Antibody and the nonspecific binding sites are blocked. The Human Insulin the sample or in the standard solution can bind to the capture antibody immobilized in the wells. After washing procedure, the Biotinylated anti-Human Insulin Detection Antibody which can bind to the Human Insulin trapped in the wells is added. After washing, the Streptavidin-Horseradish Peroxidase (SA-HRP) which catalyzes the Substrate Solution (TMB) is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the the color is directly proportional to the amount of Human Insulin in the standard solutions or samples. A standard curve of Human Insulin with known concentration can be established accordingly. The Human Insulin with unknown concentration in samples can be determined by extrapolation to this standard curve.

ASSAY CONDITIONS

Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of the sample is high enough the for the sensitivity of the kit to detect it.

CAUTION: Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use and establish optimum sample concentrations.

Assay Principle



LIST OF COMPONENTS

Store all components at 4°C. DO NOT FREEZE.

1. **20x** Assay Buffer Concentrate *(50ml)*.....**Catalog No. EK-BUF**

2. 96 Well anti-Human Insulin..... **Catalog No. EK-Plate-035-06**
   Capture Antibody-Coated Plate *(1 plate)*

3. Human Insulin Standard *(500µIU/vial)*.....**Catalog No. EK-S-035-06**

4. Biotinylated anti-Human Insulin.....**Catalog No. EK-D-035-06**
   Detection Antibody *(1 vial)*

5. Human Insulin Positive Control.....**Catalog No. EK-PC-035-06**
   (2 vials)
6. Streptavidin-Horseradish Peroxidase.....**Catalog No. EK-HRP**
   (SA-HRP) *(30µl)*

7. Substrate Solution (TMB) *(12ml)*.....**Catalog No. EK-SS**

8. Stop Solution 2N HCl *(15ml)*.....**Catalog No. EK-HCl**

9. Acetate Plate Sealer (APS) *(3 pieces)*.....**Catalog No. EK-APS**

10. Assay Diagram *(1 sheet)*

MATERIALS REQUIRED BUT NOT SUPPLIED

- Micropipettor(s) and disposable pipette tips
- Multi-channel pipette capable of dispensing 50-100µl
- Solution Reservoir *(recommended)*
- Microtiter plate washer *(recommended)*
- Orbital plate shaker capable of 300-500 rpm *(recommended)*
- Microtiter plate reader capable of absorbance measurement 450nm
- Well-closed containers (15ml tubes or more in capacity)
- Absorbent material for blotting

REAGENT PREPARATION

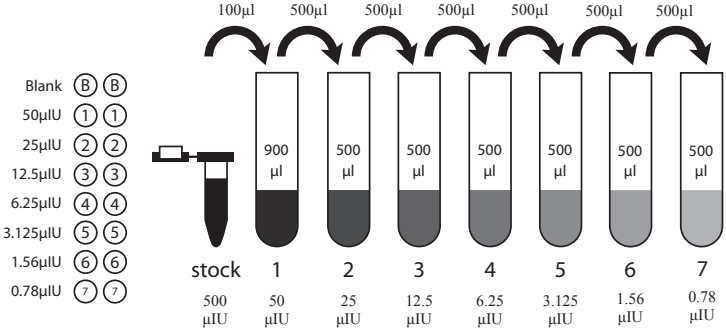
Note: *The kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recom nended that the solutions be used as soon as possible after rehydra tion.*

1. **1x Assay Buffer:** Dilute the **20x** Assay Buffer Concentrate with 950ml of distilled water. This assay buffer will be used to wash the plate and reconstitute all of the other components in this kit. If crystals are observed in the **20x** Assay buffer, warm the bottle in a 37°C water bath for approximately, 30 minutes or until the crystals disappear. After preparation, store **1x** Assay Buffer at 4°C.
2. **Biotinylated anti-Human Insulin Detection Antibody:** Rehydrate Biotinylated anti-Human Insulin Detection Antibody with 100µl of **1x** Assay Buffer, vortex (centrifuge the tube to dislodge powder from the cap or walls). Dilute Biotinylated anti-Human Insulin Detection Antibody to 1:300 and mix thoroughly before use.
3. **Streptavidin-Horseradish Peroxidase (SA-HRP):** Centrifuge the HRP vial (30µl) provided in this kit (3,000-5,000 rpm, 5 seconds) and dilute HRP with **1x** Assay Buffer to 1:2000 before use. Vortex thoroughly.
4. **Human Insulin Positive Control:** Rehydrate Human Insulin Positive Control with 250µl of **1x** Assay Buffer (centrifuge the tube to dislodge powder from cap or walls). Vortex thoroughly.

HUMAN INSULIN STANDARD PREPARATION

1. Rehydrate recombinant Human Insulin Standard with 1ml **1x** Assay Buffer, vortex. Allow the solution to sit at least 10 min utes at room temperature (20-23°C) to completely dissolve in solu tion. Vortex and centrifuge before use. The concentration of this stock solution is 500µIU/ml.
2. Prepare Human Insulin Standard solutions as follows:

Standard No.	Std. volume	Assay Buffer	Concentrations
Stock	Powder	1000µl	500µIU/ml
#1	100µl of Stock	900µl	50µIU/ml
#2	500µl of #1	500µl	25µIU/ml
#3	500µl of #2	500µl	12.5µIU/ml
#4	500µl of #3	500µl	6.25µIU/ml
#5	500µl of #4	500µl	3.12µIU/ml
#6	500µl of #5	500µl	1.56µIU/ml
#7	500µl of #6	500µl	0.78µIU/ml



HUMAN INSULIN ELISA PROTOCOL

1. Thorouhgly read this protocol before performing an assay. Allow all reagents to come to room temperature (20-23°C) prior to the start of the assay.
2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove un-needed strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 4°*C*.
3. Wash each well with 300µl of **1x** Assay Buffer. Allow it to sit for at least five minutes. Discard the buffer, invert and blot dry plate. Do not let wells dry before proceeding to the next step.
4. Leave wells A-1 and A-2 empty as **Blank**.

5. Add 100µl of the prepared Human Insulin Standard solu tions from **#7** to **#1** (reverse order of serial dilution) in duplicate to each well.
6. Add 100µl of Human Insulin Positive Control solution in duplicate.
7. Add 100µl diluted samples in duplicate into their designated wells.
8. Seal the immunoplate with acetate plate sealer (APS). Incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400 rpm).
9. Before washing the plate, remove the plate sealer carefuly. Completely discard the liquid from wells. Wash each well with 300-350µl assay buffer four times. At the end of the wash, discard the buffer, invert the plate, and tap on a clean absorbent towel.
10 Add 100µl Biotinylated anti-Human Insulin Detection An tibody into each well. Reseal the immunoplate with plate sealer and incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400rpm).
11. Wash 4 times with the **1x** Assay Buffer as described in step 9.
12. Add 100µl SA-HRP solution into each well. Reseal the im munoplate with plate sealer and incubate the plate for 30 minutes at room temperature (20-23°C) on plate shaker (300-400rpm).
13. Wash 4 times with the **1x** Assay Buffer as described in step 9.
14. Add 100µl Substrate Solution (TMB) provided in this kit into each well. Reseal the plate with plate sealer to protect from light and incubate the plate for 20-30 minutes at room tempera ture (20-23°C) on a plate shaker (300-400 rpm).
15. Add 100µl Stop Solution (2N Hydrochloric Acid) into each well to stop the reaction. The color in the well should change from blue to yellow. If the color change does not appear to be uniform, gently tap the plate to ensure thorough mixing. Proceed to the next step within 20 minutes.
16. Read Absorbance O.D. at 450nm using a Microtiter Plate Reader.

ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

- Reagents of different lot numbers shoud not be mixed.
- Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
- Unused microplate strips should be placed in the foil pouch with a dessicant and stored at 4°C. Do not allow moisture to enter the wells.
- When handling the plate, avoid touching the bottom.
- Manual washing may cause high duplicate coefficient variations. To reduce this factor, liquid from the plate should be removed by inverting and blotting the plate on an absorbent material.
- If the room temperature is not within the suggested range (20-23°C), variations in results may occur.
- The same reservoir for the reagents may be reused if the reservoir is washed well with distilled water before each use.
- Each laboratory must determine the appropriate dilution factors for the samples to be measured to ensure that the samples are within the dynamic range of the standard curve.
- High levels of interfering proteins may cause variations within the sample results, therefore, it is imperative to select the appropriate sample preparation procedure to obtain the optimal results.
- Each time a new tip is used, make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
- Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
- For optimal results, an orbital plate shaker capable of 300-500 rpm is recommended for all incubations.
- Modification of the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage conditions, and kit expiration) may affect the sensitivity and specificity of the test.

SUMMARY OF ASSAY PROTOCOL

Add 100µl/well of Human Insulin Standard, Positive Control or Samples

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Incubate at room temperature (20-23°C) for 2 hours

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Wash immunoplate 4 times with 350µl/well of **1x** Assay Buffer

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Add 100µl/well of Biotinylated anti-Human Insulin Detection Antibody

▼

Incubate at room temperature (20-23°C) for 2 hours

▼

Wash immunoplate 4 times with 350µl/well of **1x** Assay Buffer

▼

Add 100µl/well of SA-HRP solution

▼

Incubate at room temperature (20-23°C) for 30 minutes

▼

Wash immunoplate 4 times with 350µl/well of **1x** Assay Buffer

▼

Add 100µl/well of Substrate Solution (TMB)

▼

Incubate at room temperature (20-23°C) for 20-30 Minutes

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Terminate reaction with 100µl/well of 2N HCl

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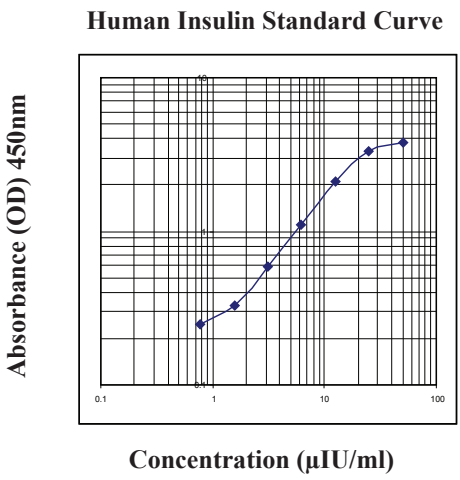
Read absorbance O.D. at 450nm and calculate results

CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentration of Human Insulin Standard and its corresponding O.D. reading is plotted on the log scale (X-axis) and the log scale (Y-axis), respectively. The standard curve shows a correlated relationship between Human Insulin concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the yellow color, and in turn the O.D. absorbance, increases.

The concentration of Human Insulin in a sample is determined by plotting the sample's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point will intersect the X-axis at a coordinate corresponding to the Human Insulin concentration in the unknown sample.

Refer to QC Data sheet for acceptable values of the positive conrtol.



STORAGE

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to room temperature (20-23°C) before assay.
2. Store **1x** Assay Buffer at 4°C.
3. Remove any unneeded strips from Human Insulin Anti body-Coated plate, reseal them in zip-lock foil and keep at 4°C.
4. Keep rehydrated solution of Human Insulin Standard, Bioti nylated anti-Human Insulin Detection Antibody and SA-HRP at 4°C. Prepare only the required amount.

NOTE:

1. It is recommended that the solutions be used on the same day of rehydration.
2. Unextracted serum samples of normal subjects are to be diluted with **1x** Assay Buffer.
3. After adding Stop Solution, read the plate within 20 minutes.

REFERENCES

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4. MacDonald MJ, Gapinski JP. (1989) A rapid ELISA for measuring insulin in a large number of research samples. Metabolism. May; 38(5):450-2.

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