This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

1 INTENDED USE

This kit is used for the non-radioactive quantification of human ghrelin (active) in serum and plasma. There is no cross reactivity to des-octanoyl-ghrelin. Circulating ghrelin is a multifunctional hormone produced primarily by the stomach. It consists of 28 amino acids and the n-octanoylation of serine3 position in the molecule is necessary for its bioactivity. Originally found as an endogenous ligand for the growth hormone secretagogue receptor in the pituitary gland, it distinguishes itself from the hypothalamic growth hormone-releasing hormone as another potent stimulator for growth hormone secretion. It is also an important orexigenic hormone in the regulation of energy homeostasis.

One kit is sufficient to measure 38 unknown samples in duplicate.

This kit is for research purpose only.

2 PRINCIPLES OF ASSAY

This assay is a Sandwich ELISA based on:
1) capture of human ghrelin molecules (active form) in the sample by anti-human ghrelin IgG and immobilization of the resulting complex to the wells of a microtiter plate coated by a pre-titered amount of anchor antibodies,
2) and the simultaneous binding of a second biotinylated antibody to ghrelin,
3) wash away of unbound materials, followed by conjugation of horseradish peroxidase to the immobilized biotinylated antibodies,
4) wash away of free enzyme, and
5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human ghrelin (active form) in the unknown sample, the concentration of active ghrelin can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human ghrelin.

3 REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well plate and contains the following reagents:

1. **Microtiter Plate**
   Coated with pre-titered anchor antibodies.
   Quantity: 1 Strip Plate
   Preparation: Ready to use.
   Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C.
2. **Adhesive Plate Sealer**
   Quantity: 2 sheets
   Preparation: Ready to use.

3. **10X HRP Wash Buffer Concentrate**
   10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
   Quantity: 2 bottles containing 50 ml each
   Preparation: Dilute 1:10 with distilled or de-ionized water.

4. **Human Ghrelin (Active) Standard**
   Human Ghrelin (active) reference standard, lyophilized
   Quantity: 2ml/vial upon hydration.
   Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis sheet for exact concentration.
   After hydration dilute with Assay Buffer according to § 8.1.

5. **Quality Controls 1 and 2**
   One vial each, lyophilized, containing human ghrelin (active) at two different levels.
   Quantity: 0.5 ml/vial upon hydration.
   Preparation: Reconstitute each vial with 0.5 ml de-ionized water immediately before use. Aliquot unused portion in smaller quantity and freeze at -20°C for later use. Avoid further freeze and thaw.

6. **Matrix Solution**
   Processed serum matrix containing 0.08% Sodium Azide
   Quantity: 1 ml/vial
   Preparation: Ready to use.

7. **Assay Buffer**
   0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.05 % Triton X-100, 0.08% sodium azide, and 0.1% BSA.
   Quantity: 15 ml/vial
   Preparation: Ready to use.

8. **Human Ghrelin (Active) Capture Antibody**
   Pre-titered capture antibody solution in buffer
   Quantity: 3 ml/vial
   Preparation: Mix 1:1 with Human Ghrelin (Active) Detection Antibody before use according to § 8.3.

9. **Human Ghrelin (Active) Detection Antibody**
   Pre-titered detection antibody solution in buffer
   Quantity: 3 ml/vial
   Preparation: Mix 1:1 with Human Ghrelin (Active) Capture Antibody before use according to § 8.3.

10. **Enzyme Solution**
    Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
    Quantity: 12 ml/vial
    Preparation: Ready to use

11. **Substrate**
    3, 3',5,5'-tetramethylbenzidine in buffer.
    Quantity: 12 ml/vial
    Preparation: Ready to use. Minimize the exposure to light.

12. **Stop Solution**
    0.3 M HCl; [Caution: Corrosive Solution]
    Quantity: 12 ml/vial
    Preparation: Ready to use.

4 STORAGE AND STABILITY

All components of the kit should be stored at 4°C.

Prepare and use standard/QC solutions within a day after reconstitution and aliquot in smaller quantity and store at -20°C for later use, if necessary. Avoid further freeze/thaw cycles.

Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

5 REAGENT PRECAUTIONS

1. Sodium Azide

Sodium azide has been added to certain reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, sodium azide may react with lead and copper pluming to form explosive metal azides. On disposal, flush with large volume of water to prevent azide build up.

2. Hydrochloric Acid

Hydrochloric acid is corrosive, can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

6 MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipettes and pipette tips: 10 µL ~ 20 µL or 20 µL ~ 100 µL
2. Multi-channel Pipettes and pipette tips: 5 ~ 50 µL and 50 ~ 300 µL
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. De-ionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
7. Orbital Microtiter Plate Shaker
8. Absorbent Paper or Cloth
9. Pefabloc or AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoreide], 100 mg/ml aqueous stock solution (store at -20°C, minimize multiple freeze/thaw cycles) is recommended for Sample Collection and Storage.
10. 5 N HCl, recommended for Sample Collection and Storage.

7 SAMPLE COLLECTION AND STORAGE

The active ghrelin molecule is extremely unstable in serum/plasma and should be rigorously protected during blood sample collection.

Ideally all samples should be processed as quickly as possible and kept on ice to retard the breakdown of active ghrelin.

For maximum protection, we recommend addition of Pefabloc or AEBSF and acidification of all samples.

Acidification will result in noticeable protein precipitation but does not affect the assay. However, if the presence of precipitates interferes with the sample pipetting accuracy, the sample should be centrifuged and the supernatant used for assay.

1. **To prepare serum**, whole blood is directly drawn into a Vacutainer® serum tube that contains no anti-coagulant. Immediately add enough Pefabloc or AEBSF to a final concentration of 1 mg/ml. Let blood clot at room temperature for 30 min.

2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C.
3. Transfer serum samples in separate tubes and acidify with HCl to a final concentration of 0.05 N. Aliquot acidified serum in small quantities. Date and identify each sample.
4. Use freshly prepared serum or store samples at –20 ± 5°C for later use. Avoid multiple (> 5) freeze/thaw cycles.
5. **To prepare plasma sample**, whole blood should be collected into Vacutainer® EDTA-plasma tubes and treated with Pefabloc or AEBSF as described for serum, followed by immediate centrifugation. Acidify plasma samples with HCl to a final concentration of 0.05 N. Observe same precautions in the preparation of serum samples.
6. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
7. Avoid using samples with gross hemolysis or lipemia.

8 REAGENT PREPARATION

8.1 Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Ghrelin (Active) Standard with 2ml of deionized water. . Please refer to the analysis sheet for exact concentration. Invert and mix gently until completely in solution.
2. Label six tubes 1, 2, 3, 4, 5, and 6.
   Add Assay Buffer to each of the six tubes according to the volumes outlined in the chart below.
   Dilute the reconstituted standard stock according to the chart below.
   Vortex each tube briefly to ensure complete mixing.

   Note: Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of standard should be stored in small aliquots at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration pg/mL
2 mL	0	X (refer to analysis sheet For exact concentration)

Tube #	**Volume of Assay Buffer to Add**	**Volume of Standard to Add**	**Standard Concentration (pg/mL)**
1	500 µL	500 µL of reconstituted Standard	X/2
2	500 µL	500 µL of Tube 1	X/4
3	500 µL	500 µL of Tube 2	X/8
4	500 µL	500 µL of Tube 3	X/16
5	500 µL	500 µL of Tube 4	X/32
6	500 µL	500 µL of Tube 5	X/64

8.2 Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials.

Reconstitute each Human Ghrelin (Active) Quality Control 1 and Quality Control 2 with 0.5 ml distilled or deionized water and gently invert to ensure complete hydration.

Unused portions of the reconstituted Quality Controls should be stored in small aliquots at ≤ -20°C. Avoid further freeze/thaw cycles.

8.3 Preparation of Capture and Detection Antibody Mixture

Prior to use, combine the entire contents of Human Ghrelin (Active) Capture Antibody (3 ml) and Human Ghrelin (Active) Detection Antibody (3 ml) at a 1:1 ratio and invert to mix thoroughly.

9 HUMAN GHRELIN (ACTIVE) ELISA ASSAY PROCEDURE

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 ml de-ionized or glass distilled water.
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C.
   Assemble the strips in an empty plate holder and fill each well with 300 µL diluted Wash Buffer. Decant wash buffer and remove the residual amount by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate using this procedure 2 additional times. **Do not let wells dry before proceeding to the next step.**
   If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 20 µL Matrix Solution to Blank, Standards and Quality Control wells (refer to § 10. for suggested well orientations).
4. Add 30 µL assay buffer to each of the Blank and sample wells.
5. Add 10 µL assay buffer to each of the Standard and Quality Control wells.
6. Add in duplicate 20 µl Ghrelin Standards in the order of ascending concentrations to the appropriate wells.
7. Add in duplicate 20 µL QC1 and 20 µL QC2 to the appropriate wells.
8. Add sequentially 20 µL of the unknown samples in duplicate to the remaining wells.
9. Transfer the Antibody Solution Mixture (1:1 mixture of capture and detection antibody) to a buffer or reagent reservoir and add 50 µl to each well with a multi-channel pipette.
10. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
12. Wash wells 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.
13. Add 100 µl Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 min on the micro-titer plate shaker.
14. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.

15. Wash wells 6 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.
16. Add 100 µl of Substrate solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5-20 minutes.

(**Note:** Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time

Blue color should be formed in wells of Ghrelin standards with intensity proportional to increasing concentrations of Ghrelin. Remove sealer and add 100 µl stop solution **[CAUTION: CORROSIVE SOLUTION]** and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Read absorbance at 450 nm and 590nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well.

Assay Procedure for Human Ghrelin (Active) ELISA Kit

	Step 1	Step 2	Step 3	Step 4-5	Step 6-8	Step 9	Step 10-12	Step 13	Step 14-15	Step 16		Step 17	
Well #	Dilute both bottles of 10X HRP Wash Buffer with 900 ml de-ionized water.	Wash plate 3X with 300 µl diluted HRP wash buffer. Remove residual buffer by tapping smartly on absorbent towels	Matrix Solution	Assay Buffer	Standards/QCs/ Samples	Capture/ Detection Ab. Mixture	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer.	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 6X with 300 µl Wash Buffer.	Substrate	Seal, Agitate, Incubate 12-15 minutes at Room Temperature.	Stop Solution	Read Absorbance at 450 nm and 590 nm.
A1, B1			20 µl	30 µl	--	50 µl		100 µl		100 µl		100 µl	
C1, D1			20 µl	10 µl	20 µl of Tube 6 Std								
E1, F1			20 µl	10 µl	20 µl of Tube 5 Std								
G1, H1			20 µl	10 µl	20 µl of Tube 4 Std								
A2, B2			20 µl	10 µl	20 µl of Tube 3 Std								
C2, D2			20 µl	10 µl	20 µl of Tube 2 Std								
E2, F2			20 µl	10 µl	20 µl of Tube 1 Std								
G2, H2			20 µl	10 µl	20 µl of reconstituted Standard								
A3, B3			20 µl	10 µl	20 µl of QC 1								
C3, D3			20 µl	10 µl	20 µl of QC 2								
E3, F3			--	30 µl	20 µl of Sample 1								
G3, H3 Etc.			--	30 µl	20 µl of sample 2								

10 MICROTITER PLATE ARRANGEMENT

Human Ghrelin (Active) ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Tube 3 Std	QC1	Etc.								
B	Blank	Tube 3 Std	QC1	Etc.								
	Tube 6 Std	Tube 2 Std	QC2									
D	Tube 6 Std	Tube 2 Std	QC2									
E	Tube 5 Std	Tube 1 Std	Sample 1									
F	Tube 5 Std	Tube 1 Std	Sample 1									
G	Tube 4 Std	Reconstituted Standard	Sample 2									
H	Tube 4 Std	Reconstituted Standard	Sample 2									

11 CALCULATIONS

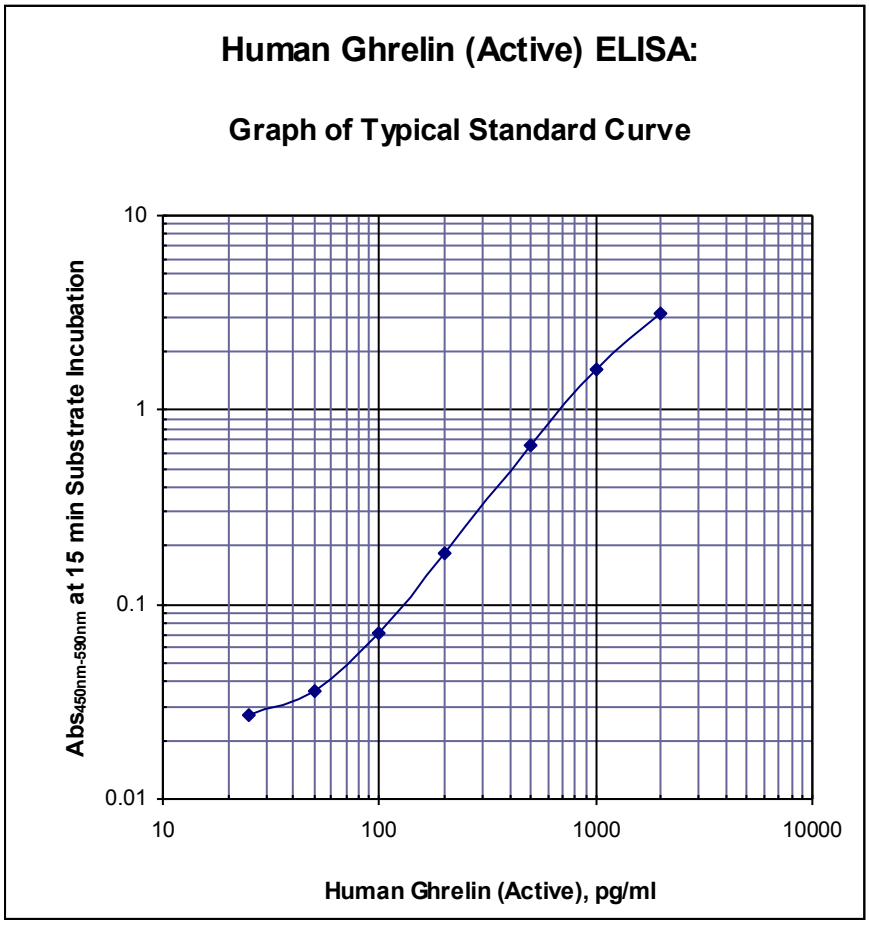
Graph a reference curve by plotting the absorbance unit of 450 nm, less unit at 590 nm, on the Y-axis against the concentrations of Ghrelin standard on the X-axis.

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation.

The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µl, compensate the volume deficit with matrix solution.

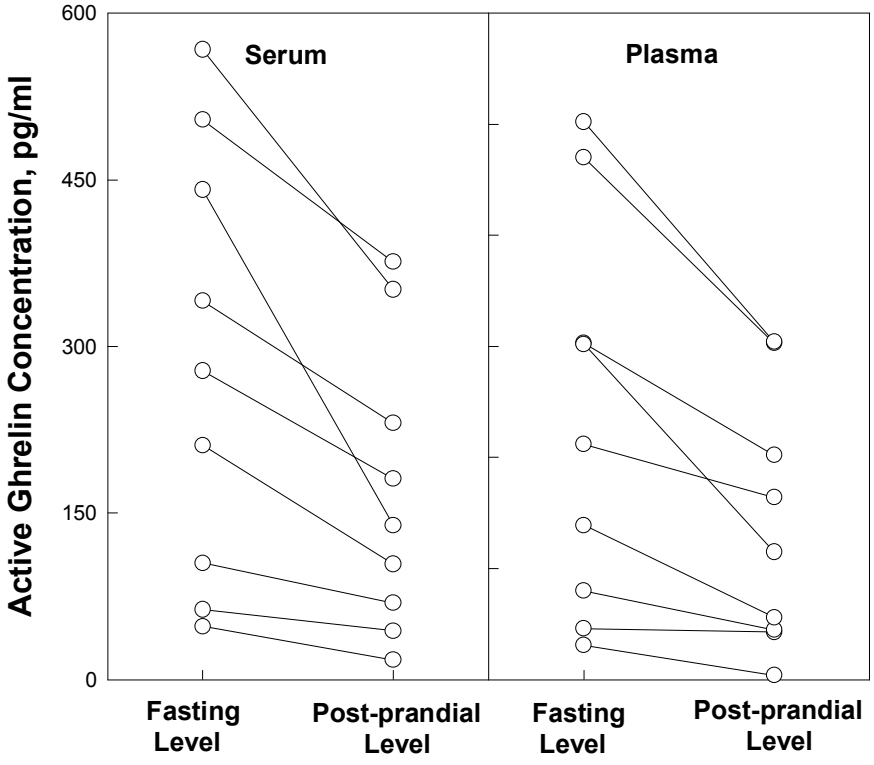
12 GRAPH OF TYPICAL REFERENCE CURVE



For Demonstration Only – Do not use for calculations

13 POST-PRANDIAL ATTENUATION OF ACTIVE GHRELIN IN BLOOD

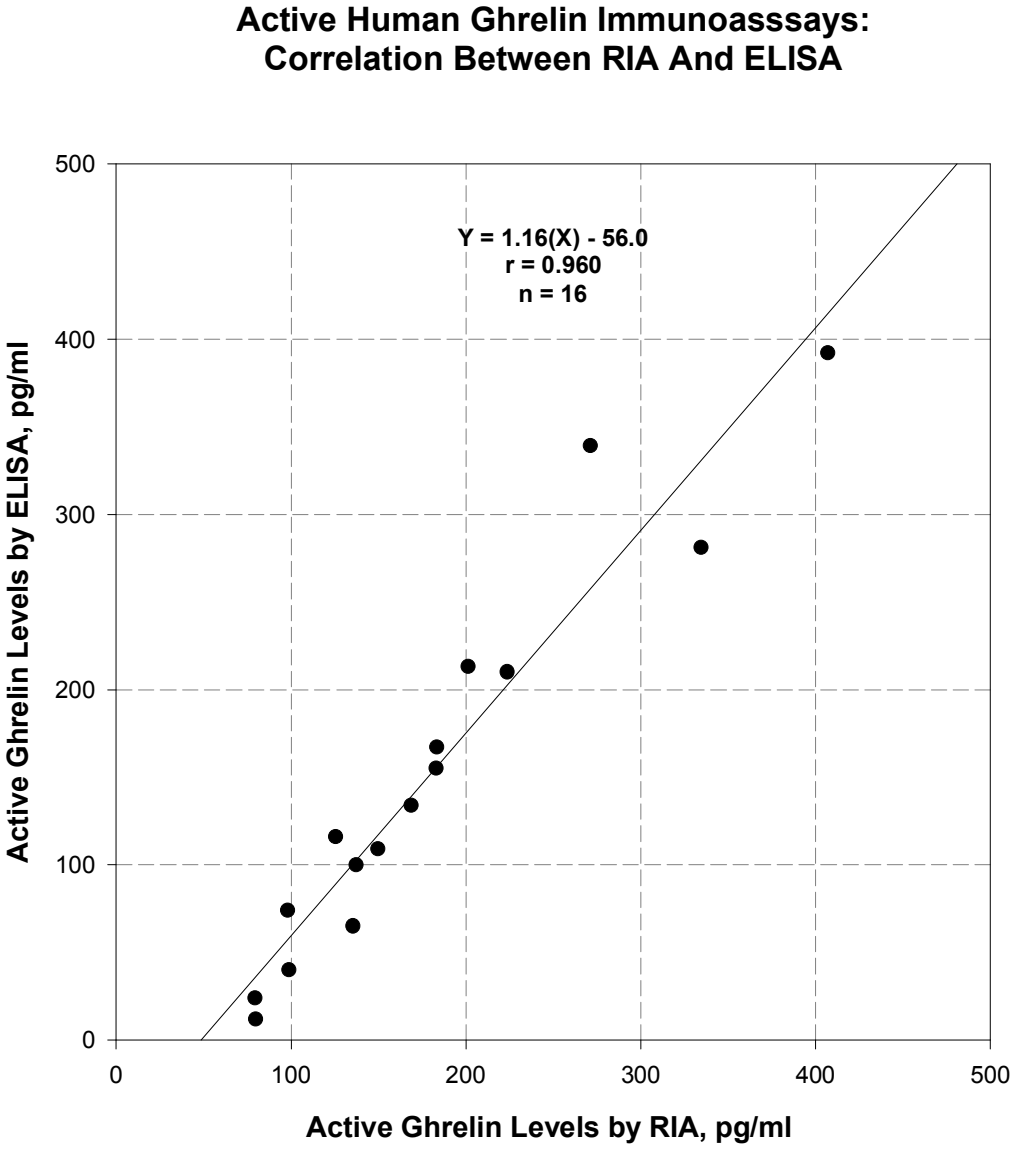
Post-meal Attenuation of Active Ghrelin Level in Blood



Fasting and 1-hour postprandial serum and plasma from 9 individuals were assayed for active ghrelin by ELISA.

14 CORRELATION GRAPH

DRG RIA REF RIA-3966 vs DRG ELISA REF EIA-4710



Fasting and postprandial serum/plasma samples from 4 individuals were assayed for active ghrelin by RIA and ELISA. Paired results are analyzed by linear regression analysis.

15 QUALITY CONTROLS

The ranges for Quality Control 1 and 2 are provided on the card insert.

16 TROUBLESHOOTING GUIDE

1. To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure all reagents and samples are added to the bottom of each well.
6. Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample or 2) inadequate washing of wells with HRP Wash Buffer or 3) overexposure to light after substrate has been added.

17 ORDERING INFORMATION

17.1 Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to human or animals.

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