User Guide

Human Ghrelin (Active) ELISA Kit

96-Well Plate

EZGRA-88K
EZGRA-88BK

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 Use Only. Not for use in Diagnostic Procedures.

Principles of Assay

This assay is a Sandwich ELISA based, sequentially, on:

- 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
- Binding of a second biotinylated antibody to the captured molecules
- Washing of unbound materials from samples
- Binding of streptavidin-horseradish peroxidase conjugate to the immobilized biotinylated antibodies
- Washing of excess free enzyme conjugates
- Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3’,5,5’-tetramethylbenzidine

The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, 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.

Reagents Supplied

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

Note: Store all reagents at 2-8 °C

Reagents Supplied	Volume	Quantity	Cat. No.
Microtiter Plate with 2 plate sealers coated with pre-titered anchor antibodies. **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2–8 °C	-	1 plate 2 sealers	EPRAM
Human Ghrelin (Active) Standard	2 mL Lyophilized	1 bottle	E8088-K
Quality Controls 1 and 2	0.5 mL Lyophilized	1 vial each	E6088-K
Matrix Solution	1 mL	1 vial	EMTX-GA
Assay Buffer	15 mL	1 vial	EABGR
10X HRP Wash Buffer Concentrate	50 mL	2 bottles	EWB-HRP
Human Ghrelin (Active) Detection Antibody	3 mL	1 bottle	E1088-D
Human Ghrelin (Active) Capture Antibody	3 mL	1 bottle	E1088-C
Enzyme Solution	12 mL	1 bottle	EHRP-88
Substrate Solution **Note:** Minimize light exposure.	12 mL	1 bottle	ESS-TMB2
Stop Solution **Caution:** Corrosive Solution	12 mL	1 bottle	ET-TMB

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Reconstituted standards and controls can be frozen for future use but repeated freeze/thaw cycles should be avoided. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Reagent Precautions

Sodium Azide

Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, Sodium azide and Proclin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Hydrochloric Acid

Hydrochloric acid is corrosive and 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.

Note: See full labels of Hazardous Components on next page.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
Human Ghrelin (Active) Capture / Detection Antibody	E1088-C		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses,if present and easy to do. Continue rinsing.
Human Ghrelin (Active) Detection Antibody	E1088-D		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Human Ghrelin (Active) Quality Controls 1 & 2	E6088-K	 	**Danger:** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Human Ghrelin (Active) Standard 2	E8088-K	 	**Danger:** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Assay Buffer	EABGR		**Warning:** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Ingredient	Cat. No.	Full Label	
Stop Solution	ET-TMB		**Warning:** May be corrosive to metals.
10X HRP Wash Buffer Concentrate	EWB-HRP		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Materials Required (Not Provided)

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

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 °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.

Reagent Preparation

Human Ghrelin (Active) Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Ghrelin (Active) Standard with 2 mL 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 reconstituted 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 Stock Concentration
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

Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Quality Control 1 and Quality Control 2 with 0.5 mL distilled or de-ionized 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.

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), or at a 1:1 ratio, and invert to mix thoroughly.

Human Ghrelin (Active) ELISA Assay Procedure

Warm all reagents to room temperature before setting up the assay.

1. Dilute 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. Add 300 µL diluted Wash Buffer to each well of the plate. Decant Wash Buffer and remove the residual volume by inverting the plate and tapping it smartly onto absorbent towels several times. Repeat wash 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 of appropriate Matrix Solution to Blank, Standards and Quality Control wells (refer to Microtiter Plate Arrangement).

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 order or 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. Blue color should be formed in wells of Ghrelin standards with intensity proportional to increasing concentrations of Ghrelin.

    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.

17. 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. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there are 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		
Well #			Matrix Solution	Assay Buffer	Standards/ QCs/Samples	Capture/Detect. Ab Mixture		Enzyme Solution		Substrate		Stop Solution
A1, B1	Dilute both bottles of 10X HRP Wash Buffer with 900 mL de-ionized water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	20 µL	30 µL	-	50 µL	Seal, Agitate, Incubate 2 hours at Room Temperature on a plate shaker. Wash 3X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 30 minutes at Room Temperature on a plate shaker. Wash 6X with 300 µL Wash Buffer.	100 µL	Seal, Agitate, Incubate 5-20 minutes at Room Temperature on a plate shaker.	100 µL	Read absorbance at 450 nm and 590 nm.
C1, D1			20 µL	10 µL	20 µL of Tube 6							
E1, F1			20 µL	10 µL	20 µL of Tube 5							
G1, H1			20 µL	10 µL	20 µL of Tube 4							
A2, B2			20 µL	10 µL	20 µL of Tube 3							
C2, D2			20 µL	10 µL	20 µL of Tube 2							
E2, F2			20 µL	10 µL	20 µL of Tube 1							
G2, H2			20 µL	10 µL	20 µL Recons Std							
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							

Microtiter Plate Arrangement

Human Ghrelin (Active) ELISA

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

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.

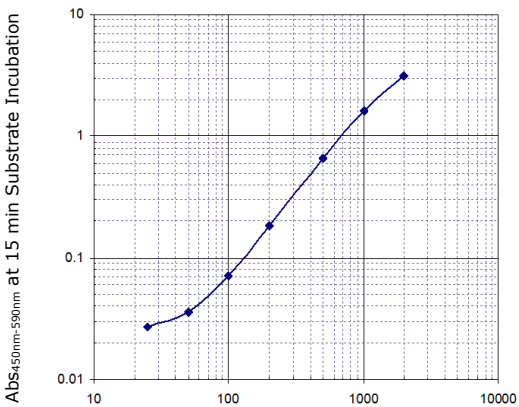
Note: When sample volumes assayed differ from 20 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (for example, if 10 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 20 µL, compensate the volume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

Interpretation

- The assay will be considered accepted when all Quality Control values fall within the calculated QC range. If any QCs fall outside of the control range, review results with a supervisor.
- If the difference between duplicate results of a sample is > 15% CV, repeat the sample.
- The theoretical minimal detecting concentration of this assay is 15 pg/mL Active Ghrelin (20 µL sample size).
- The appropriate range of this assay is 25 pg/mL to 2,000 pg/mL Active Ghrelin (20 µL sample size). Any result greater than 2,000 pg/mL in a 20 µL sample should be diluted using matrix solution and the assay repeated until the results fall within range.

Graph of Typical Reference Curve

Human Ghrelin (Active) ELISA



For Demonstration Only—Do not use for calculations

Assay Characteristics

Sensitivity

The lowest level of Active Ghrelin that can be detected by this assay is 15 pg/mL when using a 20 µL sample size.

Specificity

* Purified Ghrelin only. This kit should not be used for ghrelin assay in rat/mouse serum or plasma.

Human Ghrelin (Active)	100%
Des-Octanoyl Human Ghrelin	0%
Canine Ghrelin (Active)	106%
Porcine Ghrelin (Active)	57%
Motilin Related Peptide (Human, Rat/Mouse)	0%
PYY 3-36 (Human, Mouse, Porcine)	0%
NPY (Human/Rat)	0%
Pancreatic Polypeptide (Human, Rat)	0%
Human GIP (1-42)	0%
Human GIP (3-42)	0%
Human Insulin	0%
Human Leptin	0%
Human GLP-1	0%
Human C-peptide	0%
Human Amylin	0%
Glucagon	0%
Rat/Mouse Ghrelin (Active)	100%*
Des-Octanoyl Rat/Mouse Ghrelin	0%

Precision

Intra- and Inter-Assay Variation

Samples	Active Ghrelin (pg/mL) Mean, n =6	Intra-Assay %CV	Inter-Assay %CV
Serum #1	65.2	3.63	3.55
Serum #2	333.2	1.70	5.50
Serum #3	548.2	1.62	6.58
Plasma #1	40.7	7.53	12.9
Plasma #2	259.8	0.88	7.54
Plasma #3	397.3	3.17	9.30

The assay variations of Human Ghrelin (Active) ELISA kits were studied on three fasting human serum and plasma samples with varying concentrations of endogenous ghrelin. Intra-assay variations were calculated from results of six duplicate determinations in one assay. Inter-assay variations were calculated from results of six separate assays with duplicate samples in each assay.

Spike Recovery of Human Ghrelin (Active) in Assay Samples

Sample I.D.	Active Ghrelin Spiked (pg/mL)	Serum Ghrelin		Plasma Ghrelin	
		pg/mL	Recovery Rate	pg/mL	Recovery Rate
1	0 (Basal)	325	-	239	-
	100	416	91%	338	99%
	1,000	1,203	88%	1,204	97%
2	0 (Basal)	139	-	90	-
	100	227	88%	187	97%
	1,000	1,041	90%	1,061	97%
3	0 (Basal)	438	-	393	-
	100	545	107%	496	103%
	1,000	1,321	88%	1,386	99%
4	0 (Basal)	143	-	125	-
	100	231	88%	225	100%
	1,000	1,004	86%	1,153	103%
5	0 (Basal)	75	-	45	-
	100	143	68%	120	75%
	1,000	770	70%	821	78%
Mean ± S.D. (n = 5)	100	-	88.4 % ± 13.8 %	-	94.8 % ± 11.3%
	1,000	-	84.4 % ± 8.4 %	-	94.7 % ± 9.9 %

Varying amounts of active human ghrelin were added to 5 fasting human serum and plasma samples and the ghrelin content of each sample was assayed by Human Ghrelin (Active) ELISA. The recovery rate = [(Observed ghrelin concentration after spike – Basal ghrelin level) / spiked ghrelin concentration] x 100%.

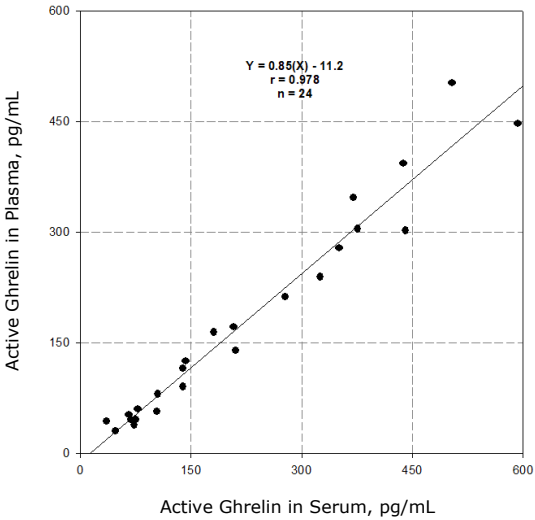
Linearity of Sample Dilution

Sample I.D.	Volume Assayed	Serum Ghrelin		Plasma Ghrelin	
		pg/mL	% of Expected	pg/mL	% of Expected
1	20 µL	325	100%	239	100%
	10 µL	170	105%	144	95%
	5 µL	90	111%	57	95%
2	20 µL	139	100%	90	100%
	10 µL	64	92%	39	87%
	5 µL	33	95%	21	93%
3	20 µL	438	100%	393	100%
	10 µL	226	103%	192	98%
	5 µL	113	103%	93	95%
4	20 µL	143	100%	125	100%
	10 µL	69	97%	56	90%
	5 µL	37	104%	26	83%
5	20 µL	75	100%	45	100%
	10 µL	39	104%	18	80%
	5 µL	26	139%	8	71%
Mean ± S.D. (n = 5)	20 µL		100%		100%
	10 µL		100.1 ± 5.5%		89.9 ± 7.1%
	5 µL		110.2 ± 16.9%		87.5 ± 10.4%

Fasting serum and plasma samples from 5 individuals were assayed at 20, 10 and 5 µL each for active ghrelin by ELISA. Measured ghrelin levels are corrected for various dilution factors and then divided by levels found at 20 µL sample size to obtain the % of expected values.

Normal Range of Active Ghrelin Levels in Human Blood

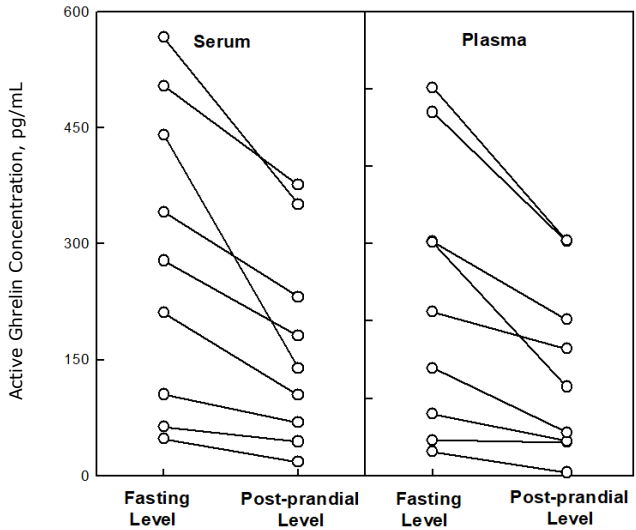
Human Ghrelin (Active) ELISA: Serum vs Plasma Sampling



Thirteen pairs of fasting human serum and plasma samples and 7 pairs of postprandial serum and plasma samples were assayed for active ghrelin by ELISA. The paired results are analyzed by linear regression analysis.

Post Prandial Attenuation of Active Ghrelin in Blood

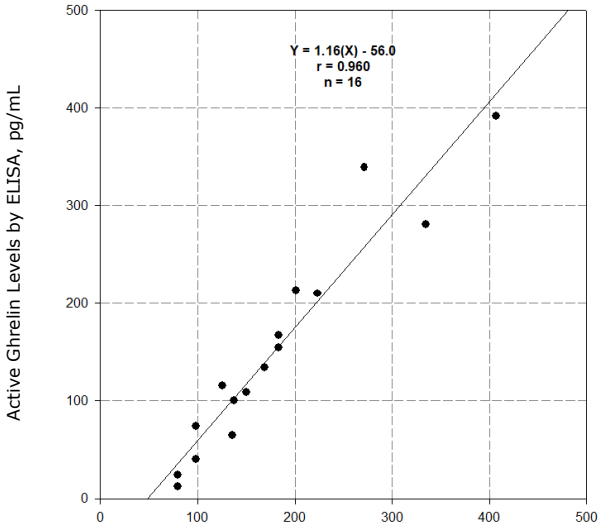
Post-meal Attenuation of Active Ghrelin Level in Blood



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

RIA Cat. No. GHRA-88HK vs. ELISA Cat. No. EZRGRA-99K

Active Human Ghrelin Immunoassays: Correlation Between RIA and ELISA



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.

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

- 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.
- Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
- 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.
- Avoid cross contamination of any reagents or samples to be used in the assay.
- Make sure all reagents and samples are added to the bottom of each well.
- 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.
- Remove any air bubbles formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
- High absorbance in background or blank wells could be due to
    - cross well contamination by standard solution or sample, or
    - inadequate washing of wells with Wash Buffer, or
    - overexposure to light after substrate has been added.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
Microtiter Plate	EPRAM
10X HRP Wash Buffer Concentrate	EWB-HPR
Human Ghrelin (Active) Standard	E8088-K
Human Ghrelin (Active) Quality Controls 1 & 2	E6088-K
Matrix Solution	EMTX-GA
Assay Buffer	EABGR
Human Ghrelin (Active) Capture Antibody	E1088-C
Human Ghrelin (Active) Detection Antibody	E1088-D
Enzyme Solution	EHRP-88
Substrate	ESS-TMB2
Stop Solution	ET-TMB
10-pack of Human Ghrelin (Active) ELISA kits	EZGRA-88BK

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