Human Ghrelin (total) ELISA Kit

Enzyme Immunoassay for the quantification of Ghrelin (total) in serum and plasma samples

Catalog number: ARG82773

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INTRODUCTION

GHRL gene encodes the ghrelin-obestatin preproprotein that is cleaved to yield two peptides, ghrelin and obestatin. Ghrelin is a powerful appetite stimulant and plays an important role in energy homeostasis. Its secretion is initiated when the stomach is empty, whereupon it binds to the growth hormone secretagogue receptor in the hypothalamus which results in the secretion of growth hormone (somatotropin). Ghrelin is thought to regulate multiple activities, including hunger, reward perception via the mesolimbic pathway, gastric acid secretion, gastrointestinal motility, and pancreatic glucose-stimulated insulin secretion. It was initially proposed that obestatin plays an opposing role to ghrelin by promoting satiety and thus decreasing food intake, but this action is still debated. Recent reports suggest multiple metabolic roles for obestatin, including regulating adipocyte function and glucose metabolism. Alternative splicing results in multiple transcript variants. In addition, antisense transcripts for this gene have been identified and may potentially regulate ghrelin-obestatin preproprotein expression. [provided by RefSeq, Nov 2014]
Ghrelin is the ligand for growth hormone secretagogue receptor type 1 (GHSR). Induces the release of growth hormone from the pituitary. Has an appetite-stimulating effect, induces adiposity and stimulates gastric acid secretion. Involved in growth regulation.
Obestatin may be the ligand for GPR39. May have an appetite-reducing effect resulting in decreased food intake. May reduce gastric emptying activity and jejunal motility (By similarity). [UniProt]

PRINCIPLE OF THE ASSAY

This is an Enzyme Immunoassay for the quantification Ghrelin (total) in human serum and EDTA-plasma samples. (This kit is designed for serum and plasma samples but it may be used for other samples when the expression level is fall within the linear range.) This assay employs the competitive quantitative enzyme immunoassay technique. A secondary antibody has been pre-coated onto a microtiter plate. The secondary antibody can bind to the Fc fragment of the primary antibody which recognizes Ghrelin. The primary antibodies in the kit will be competitively bound by biotinylated-Ghrelin peptides and Ghrelin peptides in standards or targeted Ghrelin peptides in samples. The wells are washed and then incubated with Streptavidin-HRP reagent. The biotinylated peptide interacts with streptavidin-horseradish peroxidase to form a complex. After washing away any unbound Streptavidin-HRP reagent, a substrate solution (TMB) is added to the wells and color develops in inverse-proportion to the amount of Ghrelin peptide present in the samples. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of 450nm±2 nm. The concentration of Ghrelin peptide in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Secondary antibody coated microplate	12 x 8 wells	4°C
20X Wash Buffer	50 ml	4°C
Primary antibody	1 vial	4°C
Biotinylated peptide	1 vial	4°C
Standard	1 vial	4°C
1000X Streptavidin-HRP conjugate	30 µl	4°C
TMB substrate	12 ml (Ready-to-use)	4°C (Protect from light)
STOP solution (2N HCl)	15 ml (Ready-to-use)	4°C
Positive Controls (Accept. Range dependent on lot#)	2 vials	4°C
Plate sealer	3 pieces	Room Temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Microplate shaker (300-400rpm)
- Aprotinin (enzyme inhibitor)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Before opening any Eppendorf tubes for reconstitution, briefly centrifuge at ~3,000rpm for 5 seconds to ensure that all the lyophilized material is at the bottom of the tube.
- If crystals are observed in the 20X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that all solutions be used as soon as possible after reconstitution.
- Unused microplate strips should be placed back in the foil pouch with a desiccant and stored at 4°C. Do not allow moisture to enter the wells.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- 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 300-400 rpm is recommended for all incubations.

- It is highly recommended that the standards, samples and controls be assayed in duplicates.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated. This kit is designed for serum and plasma samples but it may be used for other samples when the expression level is fall within the linear range.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1600 x g at 4°C. Collect serum and assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 4°C at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -80 °C up to 1 month. Avoid repeated freeze-thaw cycles.

Note:

We recommended add Aprotinin (enzyme inhibitor) for ALL sample collection to prevent sample degradation. 0.6 TIU or 100 µl of Aprotinin per mL of sample solution.

REAGENT PREPARATION

- 1X Wash Buffer: Dilute 20X Wash Buffer into distilled water to yield 1X Wash buffer. Store the diluted 1X Wash Buffer at 4°C. If crystals appear in 20X Wash Buffer, warm the buffer in 37°C water bath for 30 minutes or until crystals disappear. Mix well before use.
- Primary antibody: Reconstitute the Primary antibody vial with 5 ml of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted antibody at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that antibody should only be frozen-thawed once.
- Biotinylated peptide: Reconstitute the Biotinylated peptide vial with 5 ml of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted peptide at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that peptide should only be frozen-thawed once.
- Positive control: Centrifuge and reconstitute the Positive control vial with 200 µl of 1X Wash Buffer. Allow it to sit for 5 minutes to completely dissolve, mix well and keep rehydrated solution at 4°C before use. Store the reconstituted control at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that control should only be frozen-thawed once. (acceptable range dependent on lot#)

- **1X Streptavidin-HRP conjugate:** 1X Streptavidin-HRP conjugate working solution should be prepared freshly before use. Centrifuge 1000X Streptavidin-HRP conjugate briefly and add 12µl of Streptavidin-HRP to 12ml 1X wash buffer to make a 1X Streptavidin-HRP working solution. Vortex thoroughly.
- **Standard peptide**: Centrifuge and reconstitute the standard with 1 ml of 1X Wash buffer and vortex. The concentration of this stock solution is 1000 ng/ml. Allow the solution to sit for at least 10 minutes at room temperature to completely dissolve. Store the reconstituted standard at 4°C up to a week. For long-term storage, aliquot & store at -20°C for up to 3 months. Avoid repeated freeze-thaw cycles. It is recommended that standard should only be frozen-thawed once. Dilute peptide standard solutions with 1X Wash buffer to 100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml, 0.01 ng/ml as follows:

The example of the dilution of standards

Standard No.	Standard Conc. (ng/ml)	1X Wash Buffer (µl)	Standard (µl)
Stock	1000	-	-
S1	100	900	100 µl of Stock
S2	10	900	100 µl of S1
S3	1	900	100 µl of S2
S4	0.1	900	100 µl of S3
S5	0.01	900	100 µl of S4
S0 (Total binding)	0	900	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-23°C) 30 minutes before opening and starting the assay. Standards, samples, control and blank should be assayed in duplicates.

1. Remove excess microtiter strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl of 1X Wash Buffer** as Total Binding (S0, zero standard). Two empty wells should be left as blank.
3. Add **50 µl of prediluted peptide standards** (add from S5 to S1), **50 µl positive controls** or **50 µl samples** into corresponding wells. It is advisable to assay each condition in duplicates.
4. Add **25 µl of primary antibody** into each well **except the Blank wells**.
5. Add **25 µl of Biotinylated peptide** into each well **except the Blank wells**. It is not recommended to use a multi-channel pipette to load the primary antibody and biotinylated peptide.
6. Seal the microtiter plate with plate sealer. Incubate for **2 hours at RT (20-23°C).** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.
7. Prepare 1X Streptavidin-HRP conjugate working solution: Mix and centrifuge Streptavidin-HRP concentrate vial (3,000-5,000 rpm for 5 seconds) before use. Pipette 12 µl of Streptavidin-HRP concentrate into 12 ml of 1X Wash Buffer to make a Streptavidin-HRP working solution. Vortex thoroughly. Prepare freshly.
8. Remove sealer from plate.
9. Aspirate each well and wash, repeating the process 3 times for a **total 4**

washes. Wash by filling each well with **1× Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.

10. Add **100 µl of diluted (1X) Streptavidin-HRP** working solution into **each well**.
11. Reseal the plate with sealer. Incubate for **1 hour at RT.** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.
12. Remove sealer from plate. **Wash** as according to step 9.
13. Add **100 µl of TMB substrate** solution into **each well**.
14. Reseal the plate with sealer. Incubate for **1 hour at RT in dark.** Orbital shaking on a microplate shaker at 300-400 rpm is recommended.
15. Remove sealer from plate. (**DO NOT** wash or discard the contents of the wells)
16. Add **100 µl of STOP solution (2N HCl)** into **each wells** to stop the reaction. Gently tap the plate to ensure thorough mixing. The color of the solution should change from blue to yellow.
17. Read the OD with a microplate reader at **450 nm** immediately. It is recommended that the wells be read within 20 minutes after adding the Stop Solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. If samples have been diluted prior to the assay, the measured concentration must be multiplied by their respective dilution factors.

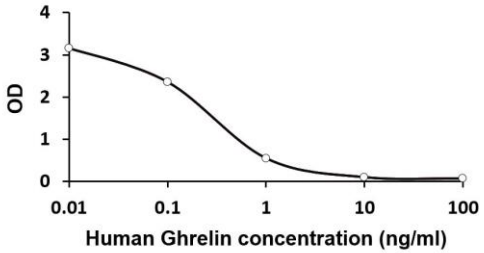
6. Measured Sample Levels:

Human Plasma: ~1.12 ng/ml

Human Serum: ~3.52 ng/ml

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The standard of Ghrelin peptide ranged from 0-100 ng/ml.

The mean MDD was 0.13 ng/ml.

Linear Range

0.13 – 1.34 ng/ml

Precision:

Intra-assay: < 10%

Inter-assay: < 15%

Cross Reactivity

The cross reactivity ratio of the tested peptide as the table:

Peptide	Cross Reactivity (%)
Human Ghrelin	100
Rat Ghrelin	100
Human Ghrelin 1-27 (O-n-Octanoyl-Ser3)	100
Human Ghrelin 1-27 (O-n-Decanoyl-Ser3)	100
Human Ghrelin (Des-Octanoyl-Ser3)	100
Rat Ghrelin (Des-Octanoyl-Ser3)	100
C-Terminal of Ghrelin	100
Human Ghrelin (Gln28)	100
Monkey Ghrelin	85
Pig Ghrelin	0
Human Secretin	0
Human VIP	0
Pig VIP	0
Rat VIP	0
Rat Prolactin-Releasing Peptide-31	0
Human Prolactin-Releasing Peptide-31	0
Rat Galanin	0
Human Galanin	0
Rat GH-RF	0
Human GH-RF	0
Human NPY	0
Human Orexin A	0
Bovine Orexin A	0
Mouse Orexin A	0
Rat Orexin A	0
Human Orexin B	0