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User's Manual

Human Ghrelin (total) ELISA Kit

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This kit is used for the non-radioactive measurement of total human ghrelin (both intact and des-octanoyl forms) in serum and plasma.

General Description

Ghrelin is a 28 amino acid hunger-stimulating peptide and hormone that is produced mainly by P/D1 cells lining the fundus of the

human stomach and epsilon cells of the pancreas. Ghrelin levels increase before meals and decrease after meals. It is considered the counterpart of the hormone leptin, produced by adipose tissue, which induces satiation when present at higher levels. In some bariatric procedures, the level of ghrelin is reduced in patients, thus causing satiation before it would normally occur. Ghrelin is a potent stimulator of growth hormone from the anterior pituitary gland. The ghrelin receptor is a G proteincoupled receptor, known as the growth hormone secretagogue receptor. Ghrelin binds to the GHSR1a splice-variant of this receptor which is present in high density in the hypothalamus, pituitary as well as vagal afferent cell bodies and vagal afferent endings throughout the gastro-intestinal tract Ghrelin plays a significant role in neurotrophy, particularly in the hippocampus, and is essential for cognitive adaptation to changing environments and the process of learning. Recently, ghrelin has been shown to activate the endothelial isoform of nitric oxide synthase in a pathway that depends on various kinases including Akt.

Principles of Testing

1) capture of human ghrelin molecules (both active and des-octanoyl forms) 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 total human ghrelin in the unknown sample, the concentration of total 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 And Materials Provided

1. Microtiter Plate: Coated with pre-titered anchor antibodies. Quantity: 1 Strip Plate; Preparation: Ready to use.
2. Note: Unused strips should be resealed in the foil pouch with the dessicant provided and stored at 2-8 °C.
3. Adhesive Plate Sealer: Quantity: 2 sheets. Preparation: Ready to use.
4. 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.

5. Human Ghrelin (Total) Standard: Human Ghrelin (total) reference standard, lyophilized. Quantity: 1 bottle, 2 mL/bottle upon

hydration. Preparation: Hydrate thoroughly in distilled or de-ionized water immediately before use. Please refer to the analysis

sheet for exact concentration.

6. Human Ghrelin (Total) Quality Controls 1 and 2: One vial each, lyophilized, containing human ghrelin (total) 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.
7. Human Ghrelin (Total) Matrix: Processed serum matrix containing 0.08% Sodium Azide. Quantity: 1 mL/vial. Preparation:

Ready to use.

8. 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.

9. Human Ghrelin (Total) Capture Antibody: Pre-titered capture antibody solution in buffer. Quantity: 3 mL/vial.
10. Human Ghrelin (Total) Detection Antibody: Pre-titered detection antibody solution in buffer. Quantity: 3 mL/vial
11. Enzyme Solution: Pre-titered streptavidin-horseradish peroxidase conjugate in buffer. Quantity: 12 mL/vial.
12. Substrate: 3, 3',5,5'-tetramethylbenzidine in buffer. Quantity: 12 mL/vial.
13. Stop Solution: 0.3 M HCl; Caution: Corrosive Solution. Quantity: 12 mL/vial

Materials Required But Not Supplied

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 use in Sample Collection and Storage.
10. 5 N HCl, recommended for Sample Collection and Storage.

Storage

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.

Specimen Collection And Preparation

1. To prepare serum, whole blood is directly drawn into a Vacutainer® serum tube that contains no anti-coagulant. Immediately

add enough aebsf to a final concentration of 1 mg/mL. Let blood clot at room temperature for 30 min. Promptly centrifuge the

clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2 °C. 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. Use freshly prepared serum or store samples at –20 ± 5 °C for later use. Avoid multiple (> 5) freeze/thaw cycles.

2. To prepare plasma sample, whole blood should be collected into EDTA-plasma tubes and treated with 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. 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. Avoid using samples with gross hemolysis or lipemia.

Reagent Preparation

Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Human Ghrelin (Total). Standard with 2mL of deionized water. Please refer to the analysis sheet for exact concentration. Invert and mix gently until completely in solution. Label five tubes 1, 2, 3, 4, and 5.
2. Add Assay Buffer to each of the five 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.

Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Reconstitute each Human Ghrelin (Total) 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.

Preparation of Capture and Detection Antibody Mixture

Prior to use, combine the entire contents of Human Ghrelin (Total) Capture Antibody (3 mL) and Human Ghrelin (Total)

Detection Antibody (3 mL) at a 1:1 ratio and invert to mix thoroughly.

Assay Procedure

1. Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire contents of both buffer bottles with 900 mL deionized 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.
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/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 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.

Calculation

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.

Detection Range

100 - 5000 pg/mL

Sensitivity

30 pg/mL