Ghrelin (human/mouse/rat) EIA Kit (BN00716)

(Catalog # BN00716, 100 assays, Store at -20°C)

I. Introduction:

Assay Genie's Ghrelin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Ghrelin peptide based on the principle of Competitive Enzyme Immunoassay. The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti- Ghrelin antibody, both biotinylated Ghrelin peptide and peptide standard or targeted peptide in samples interacts competitively with the Ghrelin antibody. Uncompeted (bound) biotinylated Ghrelin peptide then interacts with Streptavidin-horseradish peroxidase (SAHRP), which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Ghrelin peptide in the standard or samples. This is due to the competitive binding of Ghrelin antibody between biotinylated Ghrelin peptide and peptides in standard or samples. A standard curve of known concentration of Ghrelin peptide can be established and the concentration of Ghrelin peptide in the samples can be calculated accordingly. The minimum detectable concentration of Ghrelin is 161 pg/ml or 12.46 pM. The detection range for the kit is 0.1-1,000 ng/ml. The intra-Assay reproducibility is CV<10% & inter-Assay is CV<15%. This EIA kit shows no cross-reactivity with the following cytokines tested: e.g., Nesfatin, Angiotensin II, NPY and APC.

II. Application:

Quantitative protein detection, establishing normal range, validation of antibody array results.

III. Specificity:

The capture antibody provided in this kit recognizes human, mouse & rat Ghrelin.

IV. Sample Type:

- Serum & plasma
- Cell culture media or other sample types

V. Kit Contents:

Components	BN00716	Part No.
Ghrelin Microplate (Item A) coated with secondary antibody, 96 wells	12 stripsx8 wells	BN00716-1
Wash Buffer Concentrate (20x) (Item B)	25 ml	BN00716-2
Lyophilized Standard Ghrelin Peptide (Item C)	2 vials	BN00716-3
Lyophilized anti-Ghrelin Polyclonal antibody (Item N)	2 vials	BN00716-4
5 X Assay Diluent B (Item E)	15 ml	BN00716-5
Lyophilized Biotinylated Ghrelin peptide (Item F)	2 vial	BN00716-6
HRP-Streptavidin Concentrate (Item G), 100x concentrated	600 µl	BN00716-7
Lyophilized Positive Control (Item M)	1 vial	BN00716-8
TMB One-Step Substrate Reagent (Item H) 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution	12 ml	BN00716-9
Stop Solution (Item I), 0.2 M sulfuric acid	8 ml	BN00716-10

VI. User Supplied Reagents and Equipment:

- Microplate reader capable of measuring absorbance at 450 nm.
- Absorbent paper.
- Distilled or deionized water.

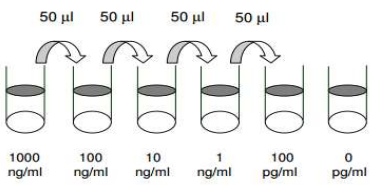
VII. Storage and Handling:

Standard, Biotinylated Ghrelin peptide, and Positive Control should be stored at -20°C after arrival. Avoid multiple freeze thaws. The remaining kit components may be stored at 4°C. Opened Microplate Wells and antibody (Item N) may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

VIII. Reagent and Sample Preparation:

For sample and positive control dilutions, refer to steps 5, 6, 7 and 9 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. **5X Assay Diluent B (Item E)** should be diluted 5-fold with dionized or distilled water.
3. Briefly centrifuge the **GHR Antibody vial (Item N**) and reconstitute with 5 µl of ddH_2O before use. Add 50 µl of 1 X Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.
4. The **antibody concentrate** should then be diluted 100-fold with 1x Assay Diluent B. This is your anti- Ghrelin antibody working solution, which will be used in step 2 of the Assay Procedure.
   NOTE: the following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).
5. Briefly centrifuge the vial of **biotinylated Ghrelin peptide (Item F)** and reconstitute with 20 µl of ddH_2O before use. Add 5 µl of Item F to 5 ml 1 X Assay Diluent B. Pipette up and down to mix gently. The final concentration of biotinylated Ghrelin will be 10 ng/ml. This solution will only be used as the diluent in step 6 of Reagent Preparation.
6. **Preparation of Standards:** Label 6 microtubes with the following concentrations: 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated Ghrelin solution into each tube, except for the 1000 ng/ml (leave this one empty). It is very important to make sure the concentration of biotinylated Ghrelin is 10 ng/ml in all standards.
   a. Briefly centrifuge the vial of Standard Ghrelin peptide (Item C) and reconstitute with 10 µl of ddH_2O. In the tube labeled 1000 ng/ml, pipette 8 µl of Item C and 792 µl of 10 ng/ml



biotinylated Ghrelin solution (prepared in step 5 above). This is your Ghrelin stock solution (1000 ng/ml Ghrelin, 10 ng/ml biotinylated Ghrelin). Mix thoroughly. This solution serves as the first standard.

b. To make the 100 ng/ml standard, pipette 50 µl of Ghrelin stock solution the tube labeled 100 ng/ml. Mix thoroughly.

c. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration. Each time, use 450 µl of biotinylated Ghrelin and 50 µl of the prior concentration until 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.

d. The final tube (0 pg/ml Ghrelin, 10 ng/ml biotinylated Ghrelin) serves as the zero standard (or total binding).

7. Prepare a 10-fold dilution of **Item F**. To do this, add 2 µl of Item F to 18 µl of 1 X Assay Diluent B. This solution will be used in steps 8 and 10.
8. **Positive Control Preparation**: Briefly centrifuge the positive control vial and reconstitute with 100 µl of ddH_2O before use (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent B. Also add 2 µl of 10-fold diluted Item F (prepared in step 7) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample with an expected signal between 10% and 30% of total binding (70-90% of competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Ghrelin is 10 ng/ml.
9. If I**tem B** (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
10. **Sample Preparation**: Use 1 X Assay Diluent B + biotinylated GHR to dilute samples including serum, plasma, cell culture medium and other sample types. Note: It is very important to make sure the final concentration of the biotinylated Ghrelin is 10 ng/ml in every sample. EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5 µl of 10-fold diluted Item F (prepared in step 7), 185 µl of 1 X Assay Diluent B, and 62.5 µl of your sample; mix gently. The total volume is 250 µl, enough for duplicate wells on the microplate. Do not use Item F diluent from Step 4 for sample preparation. If you plan to use undiluted samples, you must still add biotinylated Ghrelin to a final concentration of 10 ng/ml. EXAMPLE: Add 2.5 µl of 10-fold diluted Item F to 247.5 µl of sample.
11. Briefly centrifuge the **HRP-Streptavidin vial (Item G)** before use. The HRP-Streptavidin concentrate should be diluted 100-fold with 1X Assay Diluent B.

IX. Assay Protocol:

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl anti- Ghrelin antibody (see Reagent Preparation step 4) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycles/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1x Wash Buffer (200-300 µl each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µl of each standard (see Reagent Preparation step 6), positive control (see Reagent Preparation step 8) and sample (see Reagent Preparation step 10) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) or overnight at 4°C. Discard the solution and wash 4 times as directed in Step 3.
5. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 11) to each well. Incubate for 45 minutes with gentle shaking at room temperature. It is recommended that incubation time should not be shorter or longer than 45 minutes. Discard the solution and wash 4 times as directed in Step 3.
6. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
7. Add 50 µl of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

X. CALCULATION:

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B – blank OD)/(Bo – blank OD)

where B = OD of sample or standard and

Bo = OD of zero standard (total binding)

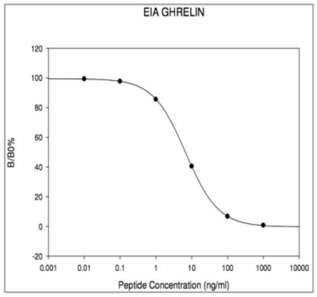


Figure: Standard Curve: These standard curves are for demonstration only. A standard curve must be run with each assay.

FOR RESEARCH USE ONLY! Not to be used on humans.