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Product Information

Obestatin EIA Kit
for serum, plasma, culture supernatant, and cell lysates

Catalog Number **RAB0208**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Obestatin Enzyme Immunoassay (EIA) Kit is an *in vitro* quantitative assay for detecting Obestatin 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- Obestatin antibody, both biotinylated Obestatin peptide, and peptide standard or targeted peptide in samples interacts competitively with the Obestatin antibody. Uncompeted (bound) biotinylated Obestatin peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP), 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 Obestatin peptide in the standard or samples. This is due to the competitive binding to Obestatin antibody between biotinylated Obestatin peptide and peptides in standard or samples. A standard curve of known concentration of Obestatin peptide can be established and the concentration of Obestatin peptide in the samples can be calculated accordingly.

Components

1. 96-well plate coated with secondary antibody (Item A) - RAB0208A: 96 wells (12 strips × 8 wells) coated with secondary antibody.
2. 20x Wash Buffer (Item B) - RABWASH3: 25 mL
3. EIA Obestatin Peptide standard (Item C) - RAB0208C: 2 vials, 10 mL/vial
4. Anti-Obestatin Detection Antibody (Item N) - RAB0208F: 2 vials, 5 mL/vial
5. EIA Assay Diluent A (Item D) - RABDIL9: 30 mL, contains 0.09% sodium azide as preservative. Diluent for standards, and serum or plasma samples.
6. EIA 5x Assay Diluent B (Item E) - RABDIL10: 15 mL of 5x concentrated buffer. Diluent for standards and cell culture media or other sample types.
7. Biotinylated Obestatin Peptide (Item F) - RAB0208G: 2 vials, 20 mL/vial
8. HRP-streptavidin (Item G) - RABHRP3: 600 µl of 1,000x concentrated HRP-conjugated Streptavidin.
9. Obestatin Positive Control Sample, Lyophilized (Item M) - RAB0208K: 1 vial, 100 mL
10. TMB Substrate solution (Item H) - RABTMB2: 12 mL of 3,3′,5,5′- tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item I) – RABSTOP3: 8 mL of 0.2 M sulfuric acid.

Reagents and Equipment Required but Not Provided.

1. Microplate reader capable of measuring absorbance at 450nm.
2. Precision pipettes to deliver 2 µL to 1 mL volumes.
3. Adjustable 1-25 mL pipettes for reagent preparation.
4. 100 mL and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models).
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker.
10. Aluminum foil.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8, and 10 of Preparation Instructions.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

3. Briefly centrifuge the Anti-Obestatin Antibody vial (Item N) before use. Add 50 µL of 1x 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 the anti-Obestatin antibody working solution, which will be used in Procedure, step 2.

Note: The following steps may be done during the antibody incubation procedure (Procedure, step 2).

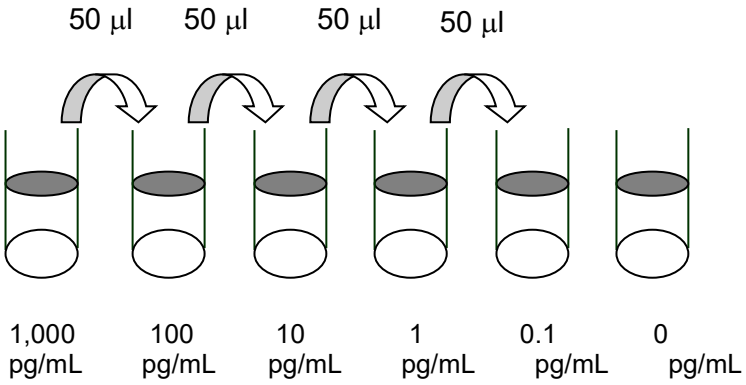
5. Briefly centrifuge the vial of Biotinylated Obestatin (Item F) before use. Add 5 µL of Item F to 5 mL of the appropriate Assay Diluent. Pipette up and down to mix gently. The final concentration of biotinylated Obestatin will be 10 pg/mL. This solution will only be used as the diluent in Preparation, step 6.

6. Preparation of Standards: Label 6 microtubes with the following concentrations: 1,000 pg/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL, 0.1 pg/mL and 0 pg/mL. Pipette 450 µL of biotinylated Obestatin solution into each tube, except for the 1,000 pg/mL (leave this one empty).

   Note: It is very important to make sure the concentration of biotinylated Obestatin is 10 pg/mL in all standards.

   a. Briefly centrifuge the vial of Obestatin (Item C). In the tube labeled 1,000 pg/mL, pipette 8 µL of Item C and 792 µL of 10 pg/mL biotinylated Obestatin solution (prepared in step 5). This is the Obestatin stock solution (1,000 pg/mL Obestatin, 10 pg/mL biotinylated Obestatin). Mix thoroughly. This solution serves as the first standard.
   b. To make the 100 pg/mL standard, pipette 50 µL of Obestatin stock solution the tube labeled 100 pg/mL. Mix thoroughly.
   c. Repeat this step with each successive concentration, preparing a dilution series as shown in Figure 1. Each time, use 450 µL of biotinylated Obestatin and 50 µl of the prior concentration until 0.1 pg/mL is reached. Mix each tube thoroughly before the next transfer.
   d. The final tube (0 pg/mL Obestatin, 10 pg/mL biotinylated Obestatin) serves as the zero standard (or total binding).

Figure 1.
Dilution Series for Standards



7. Prepare a 10-fold dilution of Item F. To do this, add 2 µL of Item F to 18 µL of the appropriate Assay Diluent. This solution will be used in steps 8 and 10.

8. Positive Control Preparation: briefly centrifuge the positive control vial (Item M). To the tube of Item M, add 101 µL of 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 10–30% of total binding (70–90% of competition) if diluted as described. It may be diluted further if desired, but be sure the final concentration of biotinylated Obestatin is 10 pg/mL.

9. If Item 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 Assay Diluent A + biotinylated Obestatin to dilute serum/plasma samples. For cell culture medium and other sample types, use 1x Assay Diluent B + biotinylated Obestatin as the diluent.

    Note: It is very important to make sure the final concentration of the biotinylated Obestatin is 10 pg/mL in every sample.

    For 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 appropriate Assay Diluent, and 62.5 µL of the sample; mix gently. The total volume is 250 µL, enough for duplicate wells on the microplate.

    Do not use Item F diluent from step 5 for sample preparation.

    If undiluted samples are to be used, add biotinylated Obestatin to a final concentration of 10 pg/mL. For 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 1,000-fold with 1x Assay Diluent B.

    Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in step 11.

Storage/Stability

Store the kit at –20 °C. It remains active for up to 1 year. Avoid repeated freeze-thaw cycles.

The reconstituted standard should be stored at –20 °C or –70 °C (–70 °C is recommended). Opened microplate strips or reagents may be store for up to 1 month at 2–8 °C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

Procedure

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 of anti-Obestatin antibody (see Preparation, step 4) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1–2 cycles/sec) or 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 Preparation, step 6), positive control (see Preparation, step 8), and sample (see 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.

5. Discard the solution and wash 4 times as directed in step 3.

6. 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.

7. Discard the solution and wash 4 times as directed in Step 3.

8. 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).

9. Add 50 µL of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

Results

Calculations

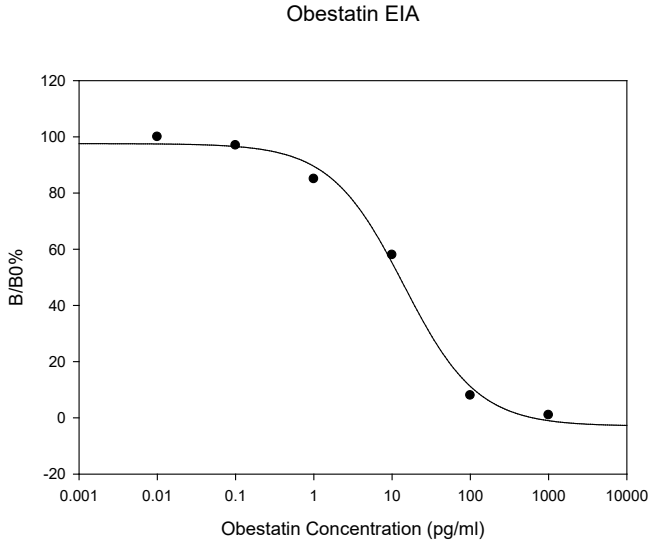
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis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

Percentage absorbance = (B – blank OD)/(B_o – blank OD) where

B = OD of sample or standard and
B_o = OD of zero standard (total binding)

Typical Data

Standard curve(s) is for demonstration only. Standard curve(s) must be run with each assay.



Product Profile

Sensitivity: The minimum detectable concentration of Obestatin is 0.1pg/mL.

Reproducibility:
Intra-Assay: CV <10%
Inter-Assay: CV <15%

Specificity

Cross Reactivity: This kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY, and APC.

References

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2. Gourcerol, G. et al., Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? Regul. Pept., **141**(1–3), 1–7 (2007).
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4. Sjölund, K. et al., Covariation of plasma ghrelin and motilin in irritable bowel syndrome. Peptides, **31**(6), 1109–12 (2010).
5. Qi, X. et al., Circulating obestatin levels in normal subjects and in patients with impaired glucose regulation and type 2 diabetes mellitus. Clin. Endocrinol. (Oxf), **66**(4), 593–7 (2007).
6. Harsch, I.A. et al., Ghrelin and obestatin levels in type 2 diabetic patients with and without delayed gastric emptying. Dig. Dis. Sci., **54**(10), 2161–6 (2009).

Appendix

Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the standard at <–20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light
	Stop solution	Stop solution should be added to each well before measurement.

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