GHRELIN (Total)

125 Tubes

Cat. # GHRT-89HK

GHRELIN (Total) RIA KIT
125 TUBES (Cat. # GHRT-89HK)

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GHRELIN (Total) RIA KIT
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I. INTENDED USE

EMD Millipore's Ghrelin (Total) Radioimmunoassay (RIA) Kit utilizes an antibody which is specific for total ghrelin and does not require the presence of the octonyl group on Serine 3. Sensitivity of 93 pg/mL can easily be achieved when using a 100 µL serum or plasma sample in a two-day, disequilibrium assay (400 µL Total Volume). ***For Research Use Only. Not for Use in Diagnostic Procedures.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The EMD Millipore Ghrelin (Total) assay utilizes ^{125}I-labeled Ghrelin and a Ghrelin antiserum to determine the level of Total Ghrelin in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents.

A. Ghrelin (Total) Assay Buffer
0.01M Phosphate, 0.01M EDTA, 0.08% Sodium Azide and 0.1% Gelatin, pH 8.5
Quantity: 20 mL/vial
Preparation: Ready to use

B. Ghrelin (Total) Antibody
Rabbit anti-Ghrelin Serum in Assay Buffer
Quantity: 13 mL/vial
Preparation: Ready to use

C. ^{125}I-Ghrelin
^{125}I-Ghrelin Label, HPLC purified (specific activity 302 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains <1.5 µCi (56 kBq), calibrated to the 1st Monday of each month.
Quantity: 13.5 mL/vial upon hydration
Preparation: Contents Lyophilized. On the day the tracer is added to the assay, hydrate with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing. Immediately freeze remaining label for future use.

D. Ghrelin (Total) Label Hydrating Buffer
Assay Buffer containing 0.025% Triton-X 100 and Normal Rabbit IgG as a carrier. Used to hydrate ^{125}I-Ghrelin
Quantity: 13.5 mL/vial
Preparation: Ready to use

E. Ghrelin (Total) Standard (lyophilized)
Lyophilized standard containing Ghrelin in sodium phosphate buffer containing a non-mercury preservative.
Preparation: Contents Lyophilized. Reconstitute with 2 mL distilled or deionized water. The actual concentration of Ghrelin present in the vial will be lot-dependent. Please refer to the analysis sheet for exact Ghrelin concentration present in a specific lot.

F. Ghrelin (Total) Quality Controls 1 and 2 (lyophilized)
One vial each, lyophilized, containing Ghrelin at two different levels.
Preparation: Contents Lyophilized. Reconstitute with 1 mL distilled or deionized water.

G. Precipitating Reagent
Goat anti-Rabbit IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide
Quantity: 130 mL/vial
Preparation: Ready to use; chill to 4°C

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8°C for short term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number and are unopened. Unused reconstituted last Standard and Quality Controls should be aliquotted and stored at ≤ -20°C.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation thereof to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.

2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.

3. Monitor hands, shoes, and clothing and immediate area surrounding the work station for contamination after each procedure and before leaving the area.

4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.

5. Never pipette radioactive material by mouth.

6. Dispose of radioactive waste in accordance with NRC rules and regulations.

7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.

8. Use absorbent pads for containing and easily disposing of small amounts of contamination.

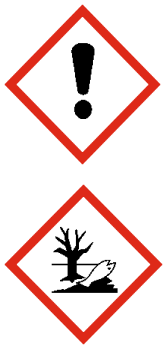
9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

B. Sodium Azide

Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide 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.

V. REAGENT PRECAUTIONS (continued)

Full labels of hazardous components in this kit:

Ingredient, Cat #		Full Label	
RB Anti Ghrelin (Total) Antibody	1089-HK		**Warning.** Flammable liquid and vapour. Causes serious eye irritation. May cause drowsiness or dizziness. Keep away from heat. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Ghrelin (Total) Quality Controls 1 & 2	6089-K		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Ghrelin (Total) Kit Standard	8089-K		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Precipitating Reagent	PR-81HK		**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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. 100 µL pipette with disposable tips
3. 10 µL, 100 µL & 1.0 mL repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets is not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A maximum of 100 µL per assay tube of serum or plasma (plasma is preferred) can be used, although, 50 µL per assay tube is adequate for most applications. Tissue culture and other media may also be used.

2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

3. Specimens can be stored at 4ºC if they will be tested within 4 hours. For longer storage, specimens should be aliquoted and stored at ≤ - 20ºC or below. Multiple freeze/thaw cycles should be avoided since each freeze/thaw may reduce results.

4. Avoid using samples with gross hemolysis or lipemia.

VIII. STANDARD AND QUALITY CONTROLS PREPARATION

Total Ghrelin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the Total Ghrelin Standard with 2 mL distilled or deionized water to give a concentration prescribed in the analysis sheet. Invert and mix gently, let sit for 5 minutes or until completely dissolved then mix well.

2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.5 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.5 mL of the reconstituted standard to tube 1, mix well and transfer 0.5 mL of tube 1 to tube 2, mix well and transfer 0.5 mL of tube 2 to tube 3, mix well and transfer 0.5 mL of tube 3 to tube 4, mix well and transfer 0.5 mL of tube 4 to tube 5, mix well and transfer 0.5 mL of tube 5 to tube 6 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. **Unused portions of the reconstituted standard should be aliquotted and stored at ≤ -20°C.** Avoid multiple freeze/thaw cycles.

Volume of Deionized Water to Add	Volume of Standard to Add	Standard Concentration pg/mL
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	0.5 mL	0.5 mL of reconstituted standard	X/2
2	0.5 mL	0.5 mL of tube 1	X/4
3	0.5 mL	0.5 mL of tube 2	X/8
4	0.5 mL	0.5 mL of tube 3	X/16
5	0.5 mL	0.5 mL of tube 4	X/32
6	0.5 mL	0.5 mL of tube 5	X/64

Total Ghrelin Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the Total Ghrelin Quality Control 1 and Quality Control 2 with 1 mL distilled or deionized water. Invert and mix gently, let sit for 5 minutes then mix well.

Note: For exact concentration of Quality Control 1 and 2, refer to Analysis Sheet. **Unused portions of the reconstituted Quality Controls should be stored at ≤ -20°C**. Avoid multiple freeze/thaw cycles.

IX. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

Day One

1. Pipette 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 µL of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 100 µL of Assay Buffer to tubes seven through the end of the assay.

2. Pipette 100 µL of Standards and Quality Controls in duplicate (see assay flow chart).

3. Pipette 100 µL of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Ghrelin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 µL (e.g., when using 50 µL of sample, add 50 µL of Assay Buffer). Refer to Section IX for calculation modification.

4. Pipette 100 µL of Ghrelin Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).

5. Vortex, cover, and incubate overnight (20-24 hours) at 4ºC.

Day Two

6. Hydrate the ^{125}I-Ghrelin tracer with 13.5 mL of Label Hydrating Buffer. Gently mix. Pipette 100 µL of ^{125}I-Ghrelin to all tubes.

7. Vortex, cover and incubate overnight (22-24 hours) at 4ºC.

Day Three

8. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).

9. Vortex and incubate 20 minutes at 4°C.

10. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000 xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:

    $xg = (1.12 \times 10^{-5})\ (r)\ (rpm)^2$

    r = radial distance in cm (from axis of rotation to the bottom of the tube)

    rpm = revolutions per minute

11. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 15-60 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

Assay Procedure Flow Chart

Day One					Day Two		Day Three	
Set-up	Step 1	Step 2 & 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9 - 11
Tube Number	Add Assay Buffer	Add Standard/QC Sample	Add Ghrelin Antibody	**Vortex, Cover, and Incubate 20-24 hrs at 4°C**	Add I-125 Ghrelin Tracer	**Vortex, Cover and Incubate 22-24 hrs at 4°C**	Add Precipitating Reagent	**Incubate 20 min. at 4°C, Centrifuge at 4°C for 20 min Decant and Count**
1,2	-	-	-		100 µL		-	
3,4	300 µL	-	-		100 µL		1.0 mL	
5,6	200 µL	-	100 µL		100 µL		1.0 mL	
7,8	100 µL	100 µL of Tube 6	100 µL		100 µL		1.0 mL	
9,10	100 µL	100 µL of Tube 5	100 µL		100 µL		1.0 mL	
11,12	100 µL	100 µL of Tube 4	100 µL		100 µL		1.0 mL	
13,14	100 µL	100 µL of Tube 3	100 µL		100 µL		1.0 mL	
15,16	100 µL	100 µL of Tube 2	100 µL		100 µL		1.0 mL	
17,18	100 µL	100 µL of Tube 1	100 µL		100 µL		1.0 mL	
19,20	100 µL	100 µL of Reconstituted	100 µL		100 µL		1.0 mL	
21,22	100 µL	100 µL of QC 1	100 µL		100 µL		1.0 mL	
23,24	100 µL	100 µL of QC 2	100 µL		100 µL		1.0 mL	
25,26	100 µL	100 µL of unknown	100 µL		100 µL		1.0 mL	

X. CALCULATIONS

A. Explanation

The calculations for Ghrelin can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data. [NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.]

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.

2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.

3. Calculate the percentage of tracer bound
   (Total Binding Counts/Total Counts) X 100
   This should be 35-50%.

4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
   %B/Bo = (Sample or Standard/Total Binding) X 100

5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.

6. Construct the reference curve by joining the points with a smooth curve.

7. Determine the pg/mL of Ghrelin in the unknown samples and controls by interpolation of the reference curve.

   [**Note:** When sample volumes assayed differ from 100 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 µL of sample is used, then calculated data must be multiplied by 2).]

XI. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.

2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

3. The limit of sensitivity for the Ghrelin assay is 93 pg/mL (100 µL sample size).

4. The limit of linearity for the Ghrelin assay is 6,000 pg/mL (100 µL sample size). Any result greater than 6,000 pg/mL should be repeated on dilution using Assay Buffer as a diluent.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Ghrelin that can be detected by this assay is 93 pg/mL when using a 100 µL sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED_{80} = 258 ± 62 pg/mL

ED_{50} = 945 ± 251 pg/mL

ED_{20} = 3739 ± 1141 pg/mL

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Human Ghrelin	100 %
Rat Ghrelin	100 %
Canine Ghrelin	100 %
Ghrelin 14-28	100 %
Des-Octonylghrelin	100 %
Ghrelin 1-10	*
Motilin Related Peptide	*
Glucagon	*
Glp-1 (7-36)	*
Human Leptin	*
Human Insulin	*

*-not detectable

D. Precision

Within and Between Assay Variation

Sample No.	Mean pg/mL	Within % CV	Between % CV
1	1000	10.0	14.7
2	1500	3.3	17.8
3	2000	7.9	16.0
4	3000	4.4	16.7

Within and between assay variations were performed on four human plasma samples containing varying concentrations of Human Ghrelin. Data (mean and %CV) shown are from five duplicate determinations of each plasma sample in six separate assays.

E. Recovery

Spike & Recovery of Ghrelin in Human Plasma

Sample No.	Ghrelin Added pg/mL	% Recovery
1	500	96
2	1000	90
3	2000	91

Varying concentrations of Human Ghrelin were added to three different human plasma samples and the Ghrelin content was determined by RIA. Mean of the observed levels from three duplicate determinations in three separate assays are shown. Percent recovery was calculated on the observed vs. expected.

XII. ASSAY CHARACTERISTICS (continued)

F. Linearity

Effect of Plasma Dilution

Sample No.	Volume Sampled	Observed pg/mL	Expected pg/mL	% Of Expected
1	100 µL	2676	2676	100
	75 µL	1988	2644	99
	50 µL	1542	3083	115
	25 µL	748	2991	112
2	100 µL	1457	1457	100
	75 µL	1096	1457	100
	50 µL	814	1629	112
	25 µL	500	1999	137
3	100 µL	1660	1660	100
	75 µL	1330	1769	107
	50 µL	934	1868	113
	25 µL	607	2430	146

Aliquots of pooled Human Plasma containing varying concentrations of Ghrelin were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2 and 4 representing 100 µL, 75 µL, 50 µL, and 25 µL, respectively, were applied in calculating observed concentrations. Mean Ghrelin levels and percent of expected for three separate assays are shown.

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website emdmillipore.com using the catalog number as the keyword..

Recommended batch analysis decision using two controls (Westgard Rules[4]):

1. When both controls are within ±2 SD.
   Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD.
   Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

   Technician check of systems:
   1. Check for calculation errors
   2. Repeat standards and controls
   3. Check reagent solutions
   4. Check instrument

XIV. REPLACEMENT REAGENTS

Reagent	Cat #
^{125}I-Ghrelin (<1.5 µCi, 56 kBq)	9088-HK
Ghrelin (Total) Label Hydrating (T) Buffer (13.5 mL)	LHB-89HK
Ghrelin (Total) Standard (lyophilized)	8089-K
Ghrelin (Total) Antibody (13 mL)	1089-HK
Precipitating Reagent (130 mL)	PR-81HK
Ghrelin (Total) Quality Control 1 & 2 (lyophilized)	6089-K
Ghrelin (Total) Assay Buffer (20 mL)	AB-89HK

XV. ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at emdmillipore.com/msds.

XVI. REFERENCES

1. Morgan, C.R. and Lazarow, A. Immunoassay of Insulin: Two antibody system. Plasma insulin levels in normal, Subdiabetic, and diabetic rats. Diabetes 12:115-126, 1963.
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4. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.