GHRELIN (Active) RIA KIT
125 TUBES (Cat. # GHRA-88HK)

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I. INTENDED USE

Linco's Ghrelin (Active) Radioimmunoassay (RIA) Kit utilizes an antibody, which is specific for the biologically active form of ghrelin with the octanoyl group on Serine 3. Sensitivity of 7.8 pg/ml can easily be achieved when using a 100µl serum or plasma sample in a two-day, disequilibrium assay (400 µl Total Volume). ***This kit is for research purposes only.***

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 Linco Research, Inc. Ghrelin (Active) assay utilizes ^{125}I-labeled Ghrelin and a Ghrelin antiserum to determine the level of active 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 (Active) Assay Buffer
0.05M Phosphate, 0.025M EDTA, 0.08% Sodium Azide and 0.1% Gelatin, pH 6.85
Quantity: 20 ml/vial
Preparation: Ready to use

B. Ghrelin (Active) Antibody
Guinea Pig anti-Ghrelin Serum in Assay Buffer
Quantity: 13 ml/vial
Preparation: Ready to use

III. REAGENTS SUPPLIED (continued)

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. Hydrate with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing. Freeze remaining label for future use.

D. Ghrelin (Active) Label Hydrating Buffer

Assay Buffer containing 0.025% Triton-X 100 and Normal Guinea Pig IgG as a carrier.
Used to hydrate ^{125}I-Ghrelin
Quantity: 13.5 ml/vial
Preparation: Ready to use

E. Ghrelin (Active) 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 (Active) 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-Guinea Pig 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. 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 there from 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 workstation 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 has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

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
9. 1N HCl (recommended in SPECIMEN COLLECTION AND STORAGE section)
10. Phenylmethylsulfonyl fluoride (PMSF) (recommended in SPECIMEN COLLECTION AND STORAGE section) can be dissolved in 100% methanol or isopropanol.

VII. SPECIMEN COLLECTION AND STORAGE

The active form of the Ghrelin molecule is very unstable and labile in serum/plasma due to the nature of the octanoyl group on serine-3.

Samples should be processed as quickly as possible and kept on ice to retard the breakdown of active Ghrelin. We recommend acidification of the plasma with 50 µl of 1 N HCl and addition of 10 µl of Phenylmethylsulfonyl fluoride (PMSF) per one ml of plasma. Addition of acid may cause a precipitation of some serum proteins but does not affect the assay. This precipitation may be removed by centrifugation if desired.

Note: It is essential to prepare fresh solution of PMSF in 100% methanol or isopropanol at a concentration of 10 mg/ml before addition to serum/plasma.

1. A maximum of 100 µl per assay tube of serum or plasma (plasma is preferred) should 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 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 aliquot and stored at ≤ - 20ºC or below. Multiple freeze/thaw cycles should be avoided since each freeze/thaw cycle will reduce results.
4. Avoid using samples with gross hemolysis or lipemia.

VIII. STANDARD AND QUALITY CONTROLS PREPARATION

Active Ghrelin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using an Eppendorf pipette, reconstitute the Active 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 eight tubes 1, 2, 3, 4, 5, 6, 7 and 8. Add 0.5 mL Assay Buffer to each of the eight 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, mix well and transfer 0.5 mL of tube 6 to tube 7, mix well and transfer 0.5 mL of tube 7 to tube 8 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
7	0.5 mL	0.5 mL of tube 6	X/128
8	0.5 mL	0.5 mL of tube 7	X/256

Active Ghrelin Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using an Eppendorf pipette, reconstitute each of the Active 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. 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	Steps 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 8	100 µl		100 µl		1.0 ml	
9,10	100 µl	100 µl of Tube 7	100 µl		100 µl		1.0 ml	
11,12	100 µl	100 µl of Tube 6	100 µl		100 µl		1.0 ml	
13,14	100 µl	100 µl of Tube 5	100 µl		100 µl		1.0 ml	
15,16	100 µl	100 µl of Tube4	100 µl		100 µl		1.0 ml	
17,18	100 µl	100 µl of Tube 3	100 µl		100 µl		1.0 ml	
19,20	100 µl	100 µl of Tube 2	100 µl		100 µl		1.0 ml	
21,22	100 µl	100 µl of Tube 1	100 µl		100 µl		1.0 ml	
23,24	100 µl	100 µl of Reconstituted Standard	100 µl		100 µl		1.0 ml	
25,26	100 µl	100 µl of QC 1	100 µl		100 µl		1.0 ml	
27,28	100 µl	100 µl of QC 2	100 µl		100 µl		1.0 ml	
29,30	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 7.8 pg/ml (100 µl sample size).

4. The limit of linearity for the Ghrelin assay is 2000 pg/ml (100 µl sample size). Any result greater than 2000 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 7.8 pg/ml when using a 100µl sample size.

B. Performance

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

ED_{80} = 21 ± 5 pg/ml
ED_{50} = 105 ± 5 pg/ml
ED_{20} = 585 ± 20 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 1-10	100 %
Des-Octanoylghrelin	< 0.1 %
Ghrelin 14-28	*
Motilin Related Peptide	*
Leptin	*
Insulin	*
Glucagon	*
GLP-1 (7-36)	*

*-Not detectable

XII. ASSAY CHARACTERISTICS (continued)

D. Precision

Within and Between Assay Variation

Sample No.	Mean pg/ml	Within % CV	Between % CV
1	236.76	9.5	13.7
2	292.81	7.0	14.3
3	313.12	6.5	16.2
4	138.56	6.7	9.6

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	50	114
2	100	96
3	200	83

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
Plasma 1	100 µl	301.73	301.73	100.00
	75 µl	251.50	334.50	110.86
	50 µl	184.18	368.35	122.08
	25 µl	85.42	341.68	113.24
Plasma 2	100 µl	295.34	295.34	100.00
	75 µl	266.69	354.70	120.10
	50 µl	176.94	353.88	119.82
	25 µl	105.22	420.86	142.50
Buffer 3	100 µl	184.10	184.10	100.00
	75 µl	164.98	224.26	121.82
	50 µl	100.15	206.60	112.22
	25 µl	47.23	210.80	114.50

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 Linco Research website www.lincoresearch.com.

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 uCi, 56 kBq)	9088-HK
Ghrelin (Active) Label Hydrating Buffer (13.5 ml)	LHB-88HK
Ghrelin (Active) Standard (lyophilized)	8088-K
Ghrelin (Active) Antibody (13 ml)	1088-HK
Precipitating Reagent (130 ml)	PRUV-HK
Ghrelin (Active) Quality Control 1&2 (lyophilized)	6088-K
Ghrelin (Active) Assay Buffer (20 ml)	AB-88HK

XV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

NOTE: Appropriate license from NRC (or equivalent) must be on file at LINCO before radioactive orders can be shipped.

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: LINCO Research
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is LINCO's policy to sell our products through a network of distributors. To place an order or to obtain additional information about LINCO products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for LINCO Research products may be ordered by fax or phone. See Section A above for details on ordering.

XVI. REFERENCES

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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.