General Protocol for MRK-031-30

Ghrelin (Human)
Magnetic Bead RIA kit

(range: 25-3200 pg/ml)

Sample volume required is 20µl/tube

Plasma sample extraction is not required

PHOENIX PHARMACEUTICALS, INC.

INTRODUCTION

This kit is designed to measure a specific peptide and its related peptides by a competitive radioimmunoassay using Phoenix Particles. It is intended for in vitro protocols only. The Anti-Peptide Magnetic Beads have been conjugated with antiserum against a synthetic form of the peptide.

CONTENTS:

1. RIA buffer, 100ml
2. Standard peptide, 12.8 µg (lyophilized powder)
3. Anti-peptide Magnetic Beads, 2.6ml (liquid suspension)
4. BSA Magnetic Beads for negative control, 0.2ml (liquid suspension)
5. ^{125}I-peptide, 1.5 µCi (lyophilized powder)
6. 4x Wash Buffer (2 bottles, 50ml each)
7. Positive Control (lyophilized powder)
8. Instructions, 1 booklet and 20 µl pipette tips (12 pieces)

Note: Use 20 µl pipette tips for Magnetic Bead aliquoting

Note: Phoenix Pharmaceuticals, Inc. guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for their particular needs and establish optimum sample concentration.

Storage

This kit contains reagents sufficient for 125 RIA tubes. Refrigerate all reagents (except ^{125}I-peptide) between 2 to 8°C for short term storage. **DO NOT FREEZE** Phoenix anti-peptide Magnetic beads and BSA Magnetic beads. ^{125}I-peptide expires in approximately 6 weeks. Store at -20°C upon receipt. We **strongly recommend** that this kit be used as soon as possible upon receiving it. All solutions should be used on the same day as rehydration.

General Information

The assay is based upon the competition of ^{125}I-peptide and unlabeled peptide (standard or unknown) binding to the limited quantity of antibodies specific for peptide in each reaction mixture. As the quantity of standard or unknown sample in the reaction increases, the amount of ^{125}I-peptide able to bind to the antibody is decreased. By measuring the amount of ^{125}I-peptide bound as a function of the concentration of peptide (in standard reaction mixtures), it is possible to construct a "standard curve" from which the concentration of peptide in the unknown sample can be determined. The assay requires one overnight incubation, so plan accordingly.

Assay Conditions

Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of peptide in the sample is high enough for the sensitivity of the kit to detect.

Blood Collection: See page 10.

Tissue Extraction Method: Visit **www.phoenixpeptide.com** and click on the link, "Sample Preparation," for more information.

General procedure for utilization of the RIA kit:

1. Dilute each bottle of 4x Wash Buffer with 150ml of distilled water. This buffer will be used to wash away non-specific binding for the magnetic beads complex after its incubation with sample and tracer.

2. Reconstitute the standard peptide with 1ml of RIA buffer. Mix well and store on ice.

Note: Before adding buffer, carefully examine the eppendorf tube containing the standard. During shipping, part or all of the lyophilized standard may have come loose from the bottom of the tube causing it to stick to the cap or walls of the tube. Gently tap or centrifuge the tube to dislodge powder from the cap or walls. Carefully open the tube and add buffer.

After adding the RIA buffer, vortex for approximately 10 minutes until ALL the peptide powder is completely dissolved. For hydrophobic and hard-to-dissolve peptides, longer vortexing may be required.

3. Place the anti-peptide Magnetic Bead solution and BSA Control Magnetic Bead solution on a plate shaker for 20 minutes to resuspend (350rpm, room temperature). If homogeneous suspension is not acheived after 20 minutes, place both bottles in an ultrasonic sonicator, then place on shaker for 20 additional minutes. A homogeneous suspension will not exhibit any bead aggregation at the bottom of the bottle. While the bead solution is still on the shaker (at 350rpm), pipette 20 µl of homogeneous solution to the appropriate tubes (see Table 2).

4. Reconstitute the Positive Control with RIA buffer (check QC Data Sheet). Mix well and store on ice.

5. Reconstitute samples with RIA buffer (we cannot ensure success with other buffers since they have not been tested).

6. Prepare dilutions of the standard as in Table 1:

Tube	Sample	RIA Buffer	Std. Conc.
Stock	Powder	1.0ml	12.8 µg/ml
0	10 µl of Stock	990 µl	128,000 pg/ml
A	25 µl of 0	975 µl	3,200 pg/ml
B	500 µl of A	500 µl	1,600 pg/ml
C	500 µl of B	500 µl	800 pg/ml
D	500 µl of C	500 µl	400 pg/ml
E	500 µl of D	500 µl	200 pg/ml
F	500 µl of E	500 µl	100 pg/ml
G	500 µl of F	500 µl	50 pg/ml
H	500 µl of G	500 µl	25 pg/ml

Table 1: Standard Dilutions

7. Set up initial RIA reactions (see Table 2) in 12 x 75 mm polystyrene tubes.

   a) Number tubes TC-1, TC-2, NSB-1, NSB-2, TB-1, TB-2 and #7 - #22 for the standards.
   b) Number tubes #23, #24 for the positive control.
   c) Number tubes #25 up to #125 for the unkown samples.
   d) Pipette 20 µl of RIA buffer and 20 µl of BSA Control Magnetic beads into each NSB tube.
   e) Pipette 20 µl of RIA buffer and 20 µl of anti peptide Magnetic beads into each TB tube.
   f) Pipette 20 µl anti-peptide Magnetic beads and 20 µl of standards H through A into duplicate tubes #7-#22.

Note: Reverse the order of preparation so that the concentration increases as the number of the tube increases. For example: Pipette 20 µl of standard H into tubes #7 & #8.

   g) Pipette 20 µl anti-peptide Magnetic beads and 20 µl of positive control in tubes #23 & #24.
   h) Pipette 20 µl anti-peptide Magnetic beads and 20 µl of unknown sample into duplicate tubes: tube #25 and up.

i) Vortex the contents of each tube.
j) Cover and incubate all tubes for 4 hours at room temperature on an orbital shaker at 350rpm.

8. Reconstitute the ^{125}I-peptide with 6ml of RIA buffer and mix well to make tracer solution. Check the concentration of this tracer solution and adjust it with RIA buffer until the concentration (total activity) is 9,000-14,000 cpm/20 µl.

9. Add 20 µl of the tracer solution to each tube.

10. Vortex the contents in each tube.

11. Cover and incubate all tubes for 16-24 hours at 4°C.

Tube	Contents	RIA Buffer (ADD FIRST)	Magnetic Beads (ADD SECOND)	Std or Samples (ADD THIRD)	^{125}I Peptide (ADD FOURTH)
TC-1 &2	Total Counts				20 µl
NSB-1 & 2	Non-specific binding	20 µl	20 µl BSA		20 µl
TB-1 & 2	Total binding	20 µl	20 µl Anti-peptide		20 µl
7, 8	H Standard		20 µl A-P	20 µl	20 µl
9, 10	G Standard		20 µl A-P	20 µl	20 µl
11, 12	F Standard		20 µl A-P	20 µl	20 µl
13, 14	E Standard		20 µl A-P	20 µl	20 µl
15, 16	D Standard		20 µl A-P	20 µl	20 µl
17, 18	C Standard		20 µl A-P	20 µl	20 µl
19, 20	B Standard		20 µl A-P	20 µl	20 µl
21, 22	A Standard		20 µl A-P	20 µl	20 µl
23, 24	Positive Control		20 µl A-P	20 µl P.C	20 µl
25, 26	Sample 1		20 µl A-P	20 µl	20 µl
27, 28	Sample 2		20 µl A-P	20 µl	20 µl
Etc.	Etc.		20 µl A-P	20 µl	20 µl

Table 2: Contents before Overnight Incubation

12. Shake all tubes for one minute at 350 rpm before adding Wash Buffer.

13. Add 2000 µl of Wash Buffer to each tube except TC tubes. Make sure that beads are suspended in solution; i.e., no pellet is observed at the bottom of any tube. Incubate all tubes at room temperature for 20 minutes. Centrifuge all tubes (except the TC tubes) at 3,000 rpm (approx. 1700 x g) for 20 minutes at 4°C.

14. Carefully aspirate **ALL** the supernatant (without touching the pellet) **immediately** following centrifugation (do not decant as the pellet might be lost or excess liquid could be left). **DO NOT ASPIRATE THE TC TUBES.**

15. Use a γ-counter to count the cpm of the pellets.

16. If results obtained have <15% CV in duplicate tubes, proceed with calculation of results, and skip Steps 17-18.

Note: If results show >15% variance, this indicates presence of high non-specific binding between samples and beads. In this case, proceed with the additional wash procedure detailed in Steps 17-18.

17. If additional wash is necessary, add 1700 µl of Wash Buffer to each tube except TC tubes and vortex the contents in each tube. Incubate all tubes at room temperature for 20 minutes. Centrifuge all tubes (except the TC tubes) at 3,000 rpm (approx. 1700 x g) for 20 minutes at 4°C.

18. Repeat Steps 14-15.

CALCULATIONS:

1. Using cpm, calculate the average **NSB** and label this as **NSB**.

2. Using cpm, calculate the average **TB** and label this as **TB**.

3. To find B_0 use the following equation: $\mathbf{B_0 = TB\text{-}NSB}$

4. To determine the B/B_0 (%) for paired standards and unknown samples use the following calculation:

   a) Example for standard H:

   $$B/B_0\ (\%) = \frac{(\text{Avg. cpm Std. H}) - (\text{NSB})}{B_0} \times 100\%$$

   b) Standards G through A (tubes #9-#22), Positive Control (tubes #23 & #24) and the unknown samples (tubes #25 up to #125) are handled as shown above for standard H.

5. Examples of tabulated data:

Tube	Samples	Peptide	Average cpm	B/B_0 (%)
TC-1,2			8,925	
NSB-1,2			430	
TB-1,2		0 pg/ml	1,836	100
7,8	H Standard	25 pg/ml	1,780	96.0
9,10	G Standard	50 pg/ml	1,597	83.0
▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼
21,22	A Standard	3200 pg/ml	489	4.2
23,24	Positive Control	?	1,386	67.9
25,26	Sample 1	?	918	34.7
27,28	Sample 2	?	?	?

Table 3: Tabulated Data After Calculation

Total Count (Total activity) = 8,925 cpm
NSB = 430 cpm
TB = 1,836 cpm
$\mathbf{B_0}$ = 1,836 cpm - 430 cpm = 1,406 cpm

6. On semi log graph paper, plot B/B_0 (%) (in decimal scale) versus the standard peptide concentrations (in log scale).

    a) Label the concentrations of standard H through A (25-3,200 pg/ml) on the X-axis (log scale).

    b) Label B/B_0 (%) (0 to 100%) on the Y-axis (decimal scale)

    c) Plot B/B_0 (%) for each standard concentration directly above its X-axis designation.

    d) Draw the "Best-Fit" curve.

7. Determination of the concentration of peptide in unknown samples.

    a) Using B/B_0 (%) calculated for each unknown sample, read across the graph to the point of intersection with the "Best-Fit" curve.

    b) The corresponding X-axis coordinate is equivalent to the concentration of peptide (pg/ml) in the assayed sample.

    c) To calculate the amount of peptide in the original sample, multiply the concentration of the assayed sample by any dilution factor used to prepare the sample.

8. Conversion of units:
   100 pg/ml x [1000 ÷ Mol. Wt.] = fmole/ml (PMole/L, pM)

Summary of Assay Protocol

Add anti-peptide Magnetic beads with RIA buffer and samples / standards

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Vortex, then incubate on shaker for 4 hours at room temperature

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Add ^{125}I-peptide and vortex.

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Incubate for 16-24 hours at 4ºC

▼

Vortex/Shake all tubes and add Wash buffer.

▼

Incubate 20 min at room temperature.
Centrifuge 20 min at 1,700 x g

▼

Aspirate off the supernatant (except TC tubes)

▼

Count assay tubes and calculate results.

▼

Continue **ONLY IF NEEDED**. Add Wash buffer.

▼

Incubate 20 min at room temperature. Vortex and centrifuge 20 min at 1,700 x g

▼

Aspirate off the supernatant (except TC tubes)

▼

Count assay tubes and calculate results.

Suggested method for blood plasma collection

Blood Withdrawal:

Collect blood samples into Lavender Vacutainer tubes (Cat. No. VT-6450) which contain EDTA. Each tube can collect 7ml of blood/tube. Gently rock the Lavender Vacutainer tubes several times immediately after collection of blood for anti-coagulation. Transfer the blood from the Lavender Vacutainer tubes to centrifuge tubes containing aprotinin (Cat. No. RK-APRO) (0.6 TIU/ml of blood) and gently rock several times to inhibit the activity of proteinases. Centrifuge the blood at 1600 x g for 15 minutes at 4ºC and collect the plasma. Plasma kept at -70ºC is stable for up to one month.

References:

1. Berson, S.A. and Yalow, R.S. Kinetics of reaction between insulin and insulin binding antibody. J. Clin. Invest 36:873, (1957).

2. Patrono, C. and Peskar, B.A., (eds) Radioimmunoassay in basic and clinical pharmacology. Heidelberg, Springer-Verlag, (1987).

3. Reuter, A., Vrindts-Gevaerts, Y., Meuleman-Gathy, R., Joris, J., Chretien, M. and Franchimont, P. A Radioimmunoassay for Beta-Endorphins. (BETA-END) and (BETA-LPH) in Plasma. Horm Res 25:236, (1987).

4. Dwenger, A. Radioimmunoassay: An Overview, J.Clin. Biochem. 22: 883, (1984)

5. Wang, Y.N., Chou J., Chang, D., Chang, J.K., Avila, C. and Romero, R. Endothelin-1 in Human Plasma and Amniotic Fluid. In Endothelin-Derived Contracting Factors, edited by G. Rubanyi and P. Vanchoutte, Karger, Basel, pg. 143, (1990).

CAUTION: SOME REAGENTS IN THIS KIT CONTAIN SODIUM AZIDE WHICH MAY REACT WITH LEAD AND COPPER PLUMBING TO FORM EXPLOSIVE METAL AZIDES. FLUSH WITH LARGE VOLUMES OF WASTE DURING DISPOSAL.

Instructions for possession, handling and use of radioactive material

This radioactive material shall only be received, acquired, possessed and used by physicians and veterinarians in clinical laboratories or hospitals for in vitro laboratory tests. Its use should not involve internal or external administration of the material and radiation therefrom to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and general license requirements of the U.S. Nuclear Regulatory Commission or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

Precautions in Handling Radioactive Material:

The user should store the by-product material, until used, in the original shipping container or in a container providing equivalent radiation protection. There should be no drinking, eating or smoking while radioactive material is being handled. Hands should be covered with gloves during, and thoroughly washed after the handling of radioactive material. When handling radioactive material do not pipette by mouth. Spills must be quickly and thoroughly cleaned up. Surfaces involves should be washed with an alkali detergent (alconox or the equivalent). Persons under 18 should not be permitted to handle radioactive material or enter radioactive areas.

Disposal:

Used radioactive test solutions must be disposed of by flushing down a laboratory sink drain with copious amounts of water. Radioactive waste should be disposed of in compliance with Federal, State, and Local Government regulations.

THIS PACKAGE CONFORMS TO THE CONDITIONS AND LIMITATIONS SPECIFIED IN 49 CFR173.421 FOR EXCEPTED RADIOACTIVE MATERIAL LIMITED QUANTITY, N.O.S. UN2910.

Notes

Notes

FOR RESEARCH ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

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