General Protocol for RK-003-02

Leptin (57-92) (Human) - RIA Kit

(range: 10-1280 pg/ml)

PHOENIX PHARMACEUTICALS, INC.

INTRODUCTION

This kit is designed to measure a specific peptide and its related peptides by a competitive radioimmunoassay. It is intended for in vitro protocols only. The antiserum used for this assay was raised against a synthetic form of the peptide.

CONTENTS:

1. RIA buffer, 50ml (4x concentrate)
2. Standard peptide, 12.8 µg (lyophilized powder)
3. Rabbit antiserum specific for the peptide, 13 ml (lyophilized powder)
4. ^{125}I-peptide, 1.5 µCi (lyophilized powder)
5. Goat Anti-Rabbit IgG Serum (GAR), 13 ml (lyophilized powder)
6. Normal Rabbit Serum (NRS), 13ml (lyophilized powder)
7. Positive Control (lyophilized powder)
8. Instructions, 1 booklet

Materials for extraction are not included.
If needed, extraction procedure for plasma is provided.

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. ^{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 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 two overnight incubations, so plan accordingly.

Assay Conditions

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

Blood Collection: See page 10.

Plasma Extraction: Extraction is strongly recommended but not required. It is up to the discretion of the paper reviewers. 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 the RIA buffer (4X concentrate) with 150ml of distilled water. This buffer will be used to reconstitute all of the other compounds in this kit and should be used for dilution of samples if needed.

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 2 minutes until ALL the peptide powder is completely dissolved. For hydrophobic and hard-to-dissolve peptides, longer vortexing may be required.

3. Reconstitute the rabbit anti-peptide serum with 13ml of RIA buffer, mix well and store on ice.

Note: The remaining reagents are not required at this time and should be stored in their lyophilized state until needed.

4. Reconstitute the Positive Controls with 1ml of RIA buffer. 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 below:

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	10 µl of 0	990 µl	1,280 pg/ml
B	500 µl of A	500 µl	640 pg/ml
C	500 µl of B	500 µl	320 pg/ml
D	500 µl of C	500 µl	160 pg/ml
E	500 µl of D	500 µl	80 pg/ml
F	500 µl of E	500 µl	40 pg/ml
G	500 µl of F	500 µl	20 pg/ml
H	500 µl of G	500 µl	10 pg/ml

Table 1: Standard Dilutions

7. Set up initial RIA reactions (see diagram on page 5) 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 controls.
   c) Number tubes #25 up to #125 for the unkown samples.
   d) Pipette 200 µl of RIA buffer into each NSB tube.
   e) Pipette 100 µl of RIA buffer into each TB tube.
   f) Pipette 100 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 100 µl of standard H into tubes #7 & #8.

   g) Pipette 100 µl of positive control in tubes #23 & #24.
   h) Pipette 100 µl of unknown sample into duplicate tubes: tube #25 and up.

i) Pipette 100 µl of primary antibody (rabbit anti-peptide serum) into all tubes EXCEPT TC AND NSB TUBES.
j) Vortex the contents of each tube.
k) Cover and incubate all tubes for 16-24 hours at 4°C.

8. Reconstitute the ^{125}I-peptide with 13ml of RIA buffer and mix well to make tracer solution. Please check the concentration of this tracer solution and adjust it with RIA buffer until the concentration (total activity) is 8,000-10,000 cpm/100 µl.

9. Add 100 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	STD or Samples	Primary Antibody	^{125}I Peptide
TC-1 &2	Total Counts				100 µl
NSB-1 & 2	Non-specific binding	200 µl			100 µl
TB-1 & 2	Total binding	100 µl		100 µl	100 µl
7, 8	H Standard		100 µl	100 µl	100 µl
9, 10	G Standard		100 µl	100 µl	100 µl
11, 12	F Standard		100 µl	100 µl	100 µl
13, 14	E Standard		100 µl	100 µl	100 µl
15, 16	D Standard		100 µl	100 µl	100 µl
17, 18	C Standard		100 µl	100 µl	100 µl
19, 20	B Standard		100 µl	100 µl	100 µl
21, 22	A Standard		100 µl	100 µl	100 µl
23, 24	Positive Control		100 µl P.C	100 µl	100 µl
25, 26	Sample 1		100 µl	100 µl	100 µl
27, 28	Sample 2		100 µl	100 µl	100 µl
Etc.	Etc.			100 µl	100 µl

Table 2: Contents before Incubation

12. Reconstitute the Goat Anti-Rabbit IgG serum (GAR) with 13ml of RIA buffer

13. Reconstitute the Normal Rabbit Serum(NRS) with 13ml of RIA buffer.

14. Add 100 µl of GAR to each tube except the TC tubes.

15. Add 100 µl of NRS to each tube except the TC tubes.

16. Vortex the contents of each tube. Incubate all tubes at room temperature for 90 minutes

17. Add 500 µl of RIA buffer to each tube except the TC tubes and vortex.

18. Centrifuge all tubes (except the TC tubes) at 3,000 rpm (approx. 1700 x g) for 20 minutes at 4°C.

19. 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.**

Note: For best results, the supernatant should be immediately aspirated after centrifugation. If the pellet sits for more than 15-30 minutes, it may become detached and make aspiration difficult. Do not aspirate any solids.

20. Use a γ-counter to count the cpm of the pellet.

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 Controls (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			9,000	
NSB-1,2			150	
TB-1,2		0 pg/ml	4,000	100
7,8	H Standard	10 pg/ml	3,471	93.3
9,10	G Standard	20 pg/ml	2,287	55.5
▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼
21,22	A Standard	1280 pg/ml	420	7.0
23,24	Positive Control	?	2,171	52.5
25,26	Sample 1	?	976	21.5
27,28	Sample 2	?	1,383	32.0

Table 3: Tabulated Data After Calculation

Total Count (Total activity) (cpm/100µl) = 9,000 cpm
NSB = 150 cpm
TB = 4,000 cpm
B_0 = 4,000 cpm - 150 cpm = 3850 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 (10-1280 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 sample or standard and antibody

▼

Vortex and incubate 16-24 hours at 4ºC

▼

Add ^{125}I-peptide

▼

Vortex and incubate for 16-24 hours at 4ºC

▼

Add GAR and NRS

▼

Vortex and incubate at room temperature for 90 minutes

▼

Add RIA buffer

▼

Vortex and centrifuge for 20 minutes at 1,700 x g

▼

Aspirate off the supernatant (except TC tubes)

▼

Count assay tubes

▼

Calculation of results

Suggested method for the extraction of Peptides from plasma

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.

Elution Solvents:

1. Buffer A (Cat. No. RK-BA-1)

2. Buffer B (Cat. No. RK-BB-1)

Extraction of Peptides from Plasma:

1. Acidify the plasma with an equal amount of buffer A. For example, if you are using 1ml of plasma, add 1ml of buffer A. Mix and centrifuge at 6,000 to 17,000 x g for 20 minutes at 4ºC. Keep the supernatant.

2. Equilibrate the SEP-COLUMN containing 200mg of C18 (Cat. No. RK-SEPCOL-1) by washing with buffer B (1ml, once) followed by buffer A (3ml, 3 times)

Note: From steps 3-5, no pressure should be applied to the column.

3. Load the acidified plasma solution onto the pre-treated C-18 SEP-COLUMN.

4. Slowly wash the column with buffer A (3ml, twice) and discard the wash.

5. Elute the peptide slowly with buffer B (3ml, once) and collect eluant into a polystyrene tube.

6. Evaporate eluant to dryness in a centrifugal concentrator or by a suitable substitute method

7. Dissolve the residue in RIA buffer for radioimmunoassay as follows: For normal subjects, dissolve in 250µl of RIA buffer for a two-tube assay. Aliquot 100µl into each tube (50µl is left over). Please note, at this point the sample has been concentrated by 4. If each tube is found to contain 100pg/ml of the peptide, then the total level of peptide in plasma = 100pg/ml ÷ 4 = 25pg/ml (where the concentration factor is 4). If upon assay the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the sample accordingly.

Tips for extraction of plasma:

When using SEP-COLUMN for the first time, use the enclosed bulb to apply pressure to the column after addition of 1ml of buffer B to facilitate flow. From steps 3-5, no pressure should be applied.

Ensure there is a constant flow for all solutions during the extraction procedure. Do not allow air bubbles to enter the C-18 matrix for optimal sample processing and recovery.

Drying Sample After Extraction:

A combination of centrifugal concentrator (i.e Speedvac) and a lyophilizer (freeze-dryer) produces the best results for drying the sample after extraction. First, use a Speedvac to dry sample for approximately 15 minutes to remove the organic layer. Then snap-freeze the remaining sample, and freeze-dry overnight using a lyophilizer. This two-step procedure produces a more consistent fluffy powder that is easier to rehydrate than a sample dried only with a centrifugal concentrator. However, if a centrifugal concentrator is not accessible, freeze-drying overnight using a lyophilizer will be sufficient.

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.

FOR RESEARCH ONLY

NOT FOR USE IN DIAGNOSTIC

PROCEDURES

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