RIA ASSAY PROTOCOL
(Range: 1-128pg/tube)

INTRODUCTION:

This kit is designed to measure a specific peptide and its related peptide 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. The amino acid sequence of this peptide is depicted in the accompanying data sheet.

CAUTION:

Investigational device. Limited by law to investigational use. For research use only. Not for use in diagnostic procedures.

CONTENTS of Kit:

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

Note: Phoenix Pharmaceuticals guarantees that its products conform to the information contained in this publication. **The purchaser must determine the suitability of the product for its particular use**. ***Extraction procedure for plasma is provided for your information.*** (Materials for extraction not included).

STORAGE:

This kit contains sufficient reagents for 125 RIA tubes. The ^{125}I-peptide will expire in approximately 6 weeks. Store at -20°C upon receipt. However, we ***strongly recommend*** that this kit be used as early as possible after receiving and all solutions be used on the same day of rehydration.

GENERAL INFORMATION:

The assay is based upon the competition of ^{125}I-peptide and peptide (either standard or unknown) binding to the limited quantity of antibodies specific for peptide in each reaction mixture. As the quantity of standard or unknown 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 unknown samples can be determined. The assay requires two overnight incubations, so plan accordingly.

ASSAY CONDITIONS:

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

Blood Collection: See page 5 below.

PLASMA EXTRACTION:

Extraction is strongly recommended but not required. It is up to the discretion of the paper reviewers. For plasma extraction protocol see page 5 below

TISSUE EXTRACTION METHOD:

Please visit our web site at www.PhoenixPeptide.com under “RIA, EIA kits & Protocols”

GENERAL PROCEDURE FOR UTILIZATION OF THE RIA KIT:

1. Dilute the RIA buffer (concentrate) with 150 ml 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 1 ml 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 the entire lyophilized peptide may have come loose from the bottom of the tube and sticking to the cap or walls of the tube.*
   a. *Gently tap or centrifuge the tube to dislodge powder from the cap or walls.*
   b. *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 samples with RIA buffer (we cannot assure success with other buffers since they have not been tested).
5. Prepare peptide standard solutions as follows:

Tube	Sample	RIA Buffer	Amount of Standard Peptide in RIA reaction
Stock	Powder	1.0 ml	N/A
0	10µl stock	990µl	N/A
A	10µl 0	990µl	128pg/tube
B	500µl A	500µl	64pg/tube
C	500µl B	500µl	32pg/tube
D	500µl C	500µl	16pg/tube
E	500µl D	500µl	8pg/tube
F	500µl E	500µl	4pg/tube
G	500µl F	500µl	2pg/tube
H	500µl G	500µl	1pg/tube

6. Set up initial RIA reactions (see diagram on page 4) in 12 x 75 mm polystyrene tubes.
7. Number tubes TC-1, TC-2, NSB-1, NSB-2, TB-1, TB-2 and #7-#22 for the standards.
8. Number tubes #23 up to #125 for the unknown samples.
9. Pipette 200µl of RIA buffer into each NSB tube.
10. Pipette 100µl of the RIA buffer into each TB tube.
11. Pipette 100µl of the standards H through A into duplicate tubes #7-#22.
    a. Note: *Reverse the order of preparation so that the concentration increases as the number of the tube increases. For example, pipette 100µl of standard H into tubes #7 & #8.*
12. Pipette 100µl of unknown sample into duplicate tubes: tube #23 and up.
13. Pipette 100µl of primary antibody (rabbit anti-peptide serum) into TB-1, TB-2 tubes and up.
    a. DO NOT ADD TO TC AND NSB TUBES.
14. Vortex the contents of each tube.
15. Cover and incubate all tubes for 16-24 hours at 4°C.
16. Reconstitute the 125-I-peptide with 13 ml 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 is 8,000~10,000 cpm/100µl.
17. Add 100µl of the tracer solution to each tube.
18. Vortex the contents in each tube.

19. 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				100µl
NSB-1&2	Non-specific Binding	200µl			100µl
TB-1&2	Total Binding	100µl		100µl	100µl
7,8	H Standard		100µl	100µl	100µl
9,10	G Standard		100µl	100µl	100µl
11,12	F Standard		100µl	100µl	100µl
13,14	E Standard		100µl	100µl	100µl
15,16	D Standard		100µl	100µl	100µl
17,18	C Standard		100µl	100µl	100µl
19,20	B Standard		100µl	100µl	100µl
21,22	A Standard		100µl	100µl	100µl
23,24	Sample 1		100µl	100µl	100µl
25,26	Sample 2		100µl	100µl	100µl
27,28	Sample 3		100µl	100µl	100µl
etc.	etc.		100µl	100µl	100µl
Contents Before Incubation					

20. Reconstitute the Goat Anti-Rabbit IgG Serum (GAR) with 13.0ml of RIA buffer.
21. Reconstitute the Normal Rabbit Serum (NRS) with 13.0ml of RIA buffer.
22. Add 100µl of GAR to each tube except the TC tubes.
23. Add 100µl of NRS to each tube except the TC tubes.
24. Vortex the contents of each tube and Incubate all tubes at room temperature for 90 minutes.
25. Add 500µl of RIA buffer to each tube except the TC tubes and vortex the contents in each tube.
26. Centrifuge all tubes (except the TC tubes) at 3,000rpm (approx. 1700 x g) for 20 minutes at 4°C.
27. Carefully aspirate off ALL the supernatant (without touching the pellet) immediately follow centrifugation (do not decant since the pellet might be lost or excess liquid could be left).
    a. **DO NOT ASPIRATE THE TC TUBES.**
    b. **Note:** *For best results, the supernatant should be immediately aspirated after centrifugation. If the pellet is allowed to sit for more than 15-30min, it may become detached and make aspiration difficult. Do not aspirate any solid.*
28. Use a Gamma-counter to count the CPM of the pellet.

CALCULATIONS:

1. Using cpm, calculate the average NSB and label this as average NSB.
2. Using cpm, calculate the average TB and label this as average TB.
3. To find Bo use the following equation:

   Bo = averageTB - average NSB
4. To determine the B/Bo (%) for paired standards and unknown samples use the following calculation: Example for Standard H:

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

5. Standards G through A (tubes #9-#22) and the unknown samples (tubes #23 up to #125) are handled as shown above for standard H.
6. 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/tube	4,000	100
7,8	H Standard	1 pg/tube	3,741	93.3
9,10	G Standard	2 pg/tube	2,287	55.5
↓	↓	↓	↓	↓
21,22	A Standard	128 pg/tube	420	7.0
23,24	Sample 1	?	2.171	52.5
25,26	Sample 2	?	976	21.5
27,28	Sample 3	?	1,383	32.0
Tabulated Data After Calculation				

7. Total Count (cpm/100µl)=9,000cpm
   NSB=150cpm
   TB=4,000cpm
   B_0=4,000cpm-150cpm=3,850cpm
8. On semilog graph paper, plot B/B_0(%) (in decimal scale) versus the standard peptide concentrations (in long scale).
9. Label the concentrations of standard H through A (1-128pg/tube) on the X-axis (log scale).
10. Label B/Bo (%) (0 to 100%) on the Y-axis (decimal scale).
11. Plot B/Bo (%) for each standard concentration directly above its X-axis designation.
12. Draw the "Best fit" curve.

Determination of the concentrations of peptide in unknown samples.

13. Using B/Bo (%) calculated for each unknown sample read, across the graph to the point of intersection with the "Best fit" curve.
14. The corresponding X-axis coordinate is equivalent to the concentration of peptide (pg/100 µl) in the assayed sample.
15. 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.

SUMMARY OF ASSAY PROTOCOL:

1. Add sample of standard and antibody.
2. Vortex and incubate 16-24 hours at 4°C.
3. Add ^{125}I-peptide.
4. Vortex and incubate 16-24 hours at 4°C.
5. Add GAR and NRS.
6. Vortex and incubate at room temperature for 90 minutes.
7. Add RIA buffer.
8. Vortex and centrifuge for 20 minutes at 1,700 x g
9. Aspirate of the supernatant (except TC tubes)
10. Count assay tubes
11. Calculation of results

SUGGESTED METHOD FROM THE EXTRACTION OF PEPTIDES FROM PLASMA:

Blood Withdrawal:

1. Collect blood samples into the Lavender Vacutaner tubes (#VT6450), which contain EDTA and can collect 7ml blood/tube.

2. Gently rock the Lavender Vacutaner tubes several times immediately after collection of blood for anti-coagulation.
3. Transfer the blood from the Lavender Vacutaner tubes to centrifuge tubes containing aprotinin (0.6TIU/ml of blood) and gently rock for several times to inhibit the activity of proteinase.
4. Centrifuge the blood at 1,600 x g for 15 minutes at 4°C and collect the plasma.
   a. Plasma kept at -70°C may be stable for one month.

Elution Solvents:
Buffer A (Code RK-BA-1): 1% trifluoroacetic acid (TFA, HPLC Grade) in H2O.
Buffer B (Code RK-BB-1): 60% acetonitrile (HPLC Grade) in 1% TFA.

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.
2. Mix and centrifuge at 6,000 to 17,000 x g for 20 minutes at 4°C. Keep supernatant.
3. Equilibrate a SEP-COLUMN containing 200mg of C18 (Code RK-SEPCOL-1) by washing with buffer B (1ml, once) followed by buffer A (3ml, 3 times).
4. NOTE: From steps 3-5, no pressure should be applied to the column.
5. Load the acidified plasma solution onto the pre-treated C18 SEP-COLUMN.
6. Slowly wash the column with buffer A (3ml, twice) and discard the wash.
7. Elute the peptide slowly with buffer B (3ml, once) and collect eluent in a polypropylene tube.
8. Evaporate eluent to dryness in a centrifugal concentrator or by a suitable substitute method.

Dissolve the residue in RIA buffer for radioimmunoassay as follows:
1. For a normal subject, dissolve in 250 µl RIA buffer for a two-tube assay.
2. Aliquot 100 µl into each tube (50 µl is left over).
   a. If each tube is found to contain 10 pg of the peptide, then the total level of peptide in plasma = 10 pg/tube x 2.5 tubes = 25 pg/ml.
   b. If upon assay the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the samples 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 the flow through the column. From step 3-5, no pressure should be applied.

Drying Sample After Extraction:
Ideally, using combination of centrifugal concentrator (i.e. Speedvac) and lyophilization (freeze dryer) produce best results. Fist, use a Speedvac to dry sample for approximately 15 min to remove the organic layer, then freeze the remaining sample, freeze-drying overnight using a lyophilizer. This produces a more consistent and fluffy powder that is easier to rehydrate than the sample dried with a Speedvac.

REFERENCES:

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Patrono, C. and Peskar, B.A. (eds) Radioimmunoassay in basic and clinical pharmacology. Heidelberg, Springer-Verlag, 1987.
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.
Dwenger, A. Radioimmunoassay: an overview. J Clin Biochem 22:883, 1984.
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, pp. 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 WATER DURING DISPOSAL.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 WATER 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 being 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 and the surfaces involved shall 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.

CAUTION:

INVESTIGATIONAL DEVICE
LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE
FOR RESEARCH ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES