Adrenomedullin (45-92), Pro (Human) Radioactive Immunoassay Kit Protocol

(Cat.# RK-010-06)
(Range: 100-12800 pg/ml)

PHOENIX PHARMACEUTICALS, INC.

Introduction

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

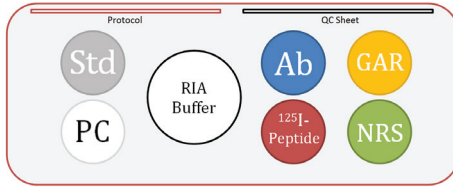
Contents:

1. RIA buffer 50 ml (4x concentrate) (large bottle, silver cap)
2. Standard Peptide 12.8µg
   (lyophilized powder in an eppendorf tube, purple cap)
3. Rabbit antibody specific for the peptide
   (lyophilized powder, blue cap)
4. ^{125}I-peptide, 1.5 µCi
   (lyophilized powder in an eppendorf tube, red cap)
5. Goat Anti-Rabbit IgG Serum (GAR)
   (lyophilized powder, gold cap)
6. Normal Rabbit Serum (NRS)
   (lyophilized powder, green cap)
7. Positive Control (small bottle, silver cap)
   (lyophilized powder in an eppendorf tube)
8. General Protocol, 1 booklet

Materials for extraction are not included.

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.

Packaged RIA Kit



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. All solutions should be used on the same day as rehydration.

General Information

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

Sample Preparation

Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assay 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.

Add standards, samples and antibodies

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Vortex and incubate for 16-24 hours at 4ºC

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Add ^{125}I-peptides

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Vortex and incubate for another 16-24 hours at 4ºC

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Add GAR and NRS (except TC tubes)

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Vortex and incubate at room temperature for 90 minutes

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Add RIA buffer (except TC tubes)

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Vortex and centrifuge for 20 minutes at 3,000 rpm / 1,700 x g

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Aspirate off the supernatant (except TC tubes)

↓

Count assay tubes

↓

Calculation of results

General procedure for utilization of the RIA kit:

1. Dilute the RIA buffer (4X concentrate) (large bottle, silver cap) 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 (purple cap) with 1 ml of RIA buffer. Vortex at least two minutes until ALL the peptide powder is completely dissolved in the eppendorf tube.

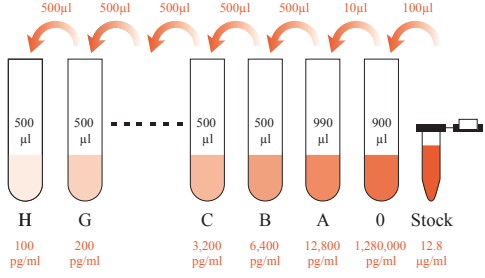
Note: Before adding buffer, carefully examine the eppendorf tube containing the standard. During shipping, part or all of the lyophilized standard may have come loose from the bottom of the tube causing it to stick to the cap or walls of the tube. Gently tap or centrifuge the tube to dislodge powder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

3. Reconstitute the antibody (blue cap) with 13 ml of RIA buffer and vortex.

4. Reconstitute the Positive Control (small bottle, silver cap) with 1ml of RIA buffer and vortex the eppendorph tube.

5. Reconstitute unknown samples with RIA buffer to a concentration that will allow their values to fall within the linear range of the standard curve (we cannot ensure success with other buffers that have not been tested). (Refer to step 7 on page 11).

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



6. Prepare dilutions of the standard as shown in Page 4 and Table 1 below. Vortex each tube and switch each tip between dilutions.

Table 1: Standard Dilutions

Tube	RIA Buffer	Standard	Std. Conc.
Stock	1.0ml	Powder	12.8 µg/ml
0	900 µl	100 µl of Stock	1,280,000 pg/ml
A	990 µl	10 µl of 0	12,800 pg/ml
B	500 µl	500 µl of A	6,400 pg/ml
C	500 µl	500 µl of B	3,200 pg/ml
D	500 µl	500 µl of C	1,600 pg/ml
E	500 µl	500 µl of D	800 pg/ml
F	500 µl	500 µl of E	400 pg/ml
G	500 µl	500 µl of F	200 pg/ml
H	500 µl	500 µl of G	100 pg/ml

7. Set up RIA reactions (see Table 2 on page 6) in up to 125 12 mm 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 unknown 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: The tubes should be prepared in reverse order of serial dilution so that the concentration increases as the number of the tube increases. For example: Begin by pipetting 100 µl of standard H into tubes #7 & #8, then proceed to standard G into #9 & #10...

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

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

Table 2: Contents in Each Tube for Incubation

Tube	Contents	RIA Buffer	Std or Samples	Antibody	Working Tracer Solution (WTS)
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

(After 16-24 hours)

8. a) Add 1 ml RIA buffer into the ^{125}I-peptide in the eppendorf tube (red cap) and vortex. This is the Stock Tracer Solution (STS). Take 10 µl of STS and check its concentration (cpm/µl) using a γ-counter.
   b) Prepare 13 ml RIA buffer in a polystyrene container. Add an adequate amount of STS into this container so that the concentration is 8,000-10,000 cpm/100µl. Confirm the concentration with a γ-counter. This is the Working Tracer Solution (WTS).
   c) Add 100 µl of the WTS to each tube.

9. Vortex the contents in each tube.

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

(After 16-24 hours)

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

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

Note: The Total Count Tubes (TC) are not involved in the following reactions.

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

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

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

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

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

18. 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.

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

Calculations:

1. Calculate the average **NSB** and label it as **NSB** using cpm.

2. Calculate the average **TB** and label it as **TB** using cpm.

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

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

   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:

Table 3: Tabulated Data After Calculation

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	20 pg/ml	3,471	93.3
9,10	G Standard	40 pg/ml	2,287	55.5
▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼	▲ | ▼
21,22	A Standard	2,560 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

Examples of Tabulated Data Continued:

Total Count (Total activity) (cpm/100µl) = 9,000 cpm
NSB = 150 cpm
TB = 4,000 cpm
$\mathbf{B_0}$ = 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 (20-2560 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:
   pg/ml x 1000 ÷ Mol. Wt. = PMole/L

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.

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 WATER DURING DISPOSAL.

Instructions for possession, handling and use of radioactive material

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.
When Handling Radioactive Materials: there should be no drinking, eating or smoking; hands should be covered with gloves, and thoroughly washed after; do not pipette by mouth.
Spills must be quickly and thoroughly cleaned up. Surfaces involved 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:

Radioactive waste should be disposed of in compliance with Federal, State, and Local Government regulations. Agencies that can be consulted include the Environmental Protection Agency (EPA), the Nuclear Regulatory Commission (NRC), the Department of Energy (DOE), and the Department of Transportation.

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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