General Protocol

Peptide Radioimmunoassay

Note: In order to choose the correct standard curve range to use with this kit, please refer to the accompanying data sheet.

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Introduction

The purpose of this kit is to quantify a specific peptide via 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.

Principle of the Assay

This assay is based upon the competitive binding between ^{125}I-labeled and unlabeled peptide (either standard or unknown) to a limited quantity of peptide specific antibody. When the concentration of the unlabeled peptide increases, the amount of ^{125}I-peptide which binds to the antibody is reduced. By measuring the amount of ^{125}I-peptide bound as a function of the concentration of the unlabeled peptide in standard reaction mixtures, it is possible to construct a "standard curve" from which the concentration of the peptide in unknown samples can be determined. The assay requires two overnight incubations. This kit contains reagents for 125 RIA test tubes.

Note: We strongly recommend doing an extraction on all samples. An extraction procedure for plasma is provided for your reference (materials for extraction not included). Peninsula Laboratories, Inc. guarantees this product to perform as expected provided the purchaser follows the enclosed protocol and accompanying data sheet. The purchaser must determine the suitability of the product for his/her particular research use.

Materials Provided

- RIA buffer concentrate (4x), 50 ml.
- Standard peptide; 12.8 μg lyophilized powder .
- Rabbit antiserum specific for the peptide; lyophilized powder for 13 ml.
- ^{125}I- peptide; liquid form or lyophilized powder (1.5 μCi).
- Goat anti-rabbit IgG serum (GARGG); lyophilized powder for 13 ml.
- Normal rabbit serum (NRS); lyophilized powder for 13 ml.
- Instructions/flow sheet for RIA protocol and guidelines for calculating the results.
- Data sheet.

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.

Assay Procedure

Day 1

A) Dilute the RIA buffer concentrate with distilled water to a final volume of 200 ml. Mix vigorously (vortex) at room temperature. This buffer will be used to reconstitute all of the other components in this kit and should be used for the dilution of samples, if needed.

B) Reconstitute the standard peptide with 1 ml of RIA buffer.

Note: For short-term storage, store at 4 °C to prevent repeated freezing and thawing. For long-term storage or subsequent usage, aliquot to prevent repeated freezing or thawing, and store at –20 °C for maximum stability.

C) Reconstitute the rabbit anti-peptide serum with 13 ml of RIA buffer.

Note: The remaining reagents are not required at this time and should be stored at –20 °C until needed.

D) Reconstitute the samples with the RIA buffer (we cannot guarantee success using other buffers). Prepare a dilution series of the standard as illustrated in Table 1, Table 2 or Table 3 for ranges 0.1-64 pg/tube, 1-128 pg/tube or 10-1280 pg/tube, respectively.. Please refer to the datasheet for the appropriate range.

Table 1: Standard Peptide Dilution Series (0.1-64 pg/tube)

Tube	Sample	RIA Buffer	Amount of Standard Peptide in the RIA Reaction (100 µl/tube)
Stock	Powder	1.0 ml	---
O	10 µl stock	990 µl	---
A	10 µl O	990 µl	---
B	500 µl A	500 µl	64 pg/tube
C	500 µl B	500 µl	32 pg/tube
D	500 µl C	500 µl	16 pg/tube
E	500 µl D	500 µl	8 pg/tube
F	500 µl E	500 µl	4 pg/tube
G	500 µl F	500 µl	2 pg/tube
H	500 µl G	500 µl	1 pg/tube
I	100 µl H	900 µl	0.1 pg/tube

Table 2: Standard Peptide Dilution Series (1-128 pg/tube)

Tube	Sample	RIA Buffer	Amount of Standard Peptide in the RIA Reaction (100 µl/tube)
Stock	Powder	1.0 ml	---
O	10 µl stock	990 µl	---
A	10 µl O	990 µl	128 pg/tube
B	500 µl A	500 µl	64 pg/tube
C	500 µl B	500 µl	32 pg/tube
D	500 µl C	500 µl	16 pg/tube
E	500 µl D	500 µl	8 pg/tube
F	500 µl E	500 µl	4 pg/tube
G	500 µl F	500 µl	2 pg/tube
H	500 µl G	500 µl	1 pg/tube

Table 3: Standard Peptide Dilution Series (10-1,280 pg/tube)

Tube	Sample	RIA Buffer	Amount of Standard Peptide in the RIA Reaction (100 µl/tube)
Stock	Powder	1.0 ml	---
O	100 µl stock	900 µl	---
A	10 µl O	990 µl	1,280 pg/tube
B	500 µl A	500 µl	640 pg/tube
C	500 µl B	500 µl	320 pg/tube
D	500 µl C	500 µl	160 pg/tube
E	500 µl D	500 µl	80 pg/tube
F	500 µl E	500 µl	40 pg/tube
G	500 µl F	500 µl	20 pg/tube
H	500 µl G	500 µl	10 pg/tube

E) Set up the initial RIA reactions (see RIA Test Tube Summary, page 7) in 12x75 mm polystyrene tubes.

1. Label the tubes: TC-1, TC-2, NSB-1, NSB-2, TB-1, TB-2 and #7-22 for the standards (where TC is total counts, NSB is non-specific binding, and TB is total binding).
2. Label tubes #23-125 for the unknown samples.

3. Pipette 200 µl of RIA buffer into the TC and NSB tubes.
4. Pipette 100 µl of RIA buffer into the TB tubes.
5. Pipette 100 µl of the standards (as stated on their respective table) into duplicate tubes #7-22. Begin from the lowest concentration to the highest concentration of standards. You do not need to change pipette tips after each transfer.
6. Pipette 100 µl of unknown sample into duplicate tubes: Tube #23 and higher.
7. Pipette 100 µl of the primary antibody (rabbit anti-peptide serum) into TB-1, TB-2 tubes, #7-22 tubes, and tubes #23 and higher. **DO NOT ADD ANTIBODY TO THE TC AND NSB TUBES**
8. Mix (vortex) the contents of each tube.
9. Cover and incubate overnight (16-24 hrs.) at 4 °C.

F) Cover and store all rehydrated solutions at 4 °C, except for the standard peptide solution, which should be kept frozen at –20 °C, or lower, for maximum stability.

Day 2

A) Reconstitute the ^{125}I-peptide with 1 ml of RIA buffer. Mix thoroughly (this is your **stock solution**). To compensate for efficiency differences between various gamma counters, the quantity of tracer can be optimized as follows. Remove and count 5 µl (count volume) of rehydrated tracer in the scintillation vial gamma counter. Use the following formula to determine the amount of concentrated tracer (X µl), and the amount of RIA buffer (Y ml), needed to perform the RIA:

$$X\ \mu l = \frac{(5\ \mu l)\ (\text{desired cpm value})\ (\#\ \text{of tube in assay} + 10)}{(\text{cpm value from } 5\ \mu l \text{ stock solution})}$$

$$Y\ ml = (0.1\ ml)\ (\text{number of tubes} + 10)$$

B) Combine X µl of ^{125}I-peptide with Y ml of RIA buffer. Mix well. Count a 100 µl sample in the gamma counter. **The desired range per 100 µl for the assay is between 10,000 and 15,000 cpm.** If necessary, adjust the concentration until the desired cpm value is reached.

C) Add 100 µl of the tracer to each solution test tube in the assay.

D) Vortex each test tube.

E) Cover and incubate overnight (16-24 hrs.) at 4 °C.

Note: For short-term storage (1-2 days), the unused tracer stock solution can be stored at 4 °C , to avoid freezing and thawing. If storing for a longer period of time or for subsequent usage, create aliquots of the tracer to prevent multiple freezing and thawing and store at –20 °C or lower for maximum stability.

RIA Test Tube Setup Summary

Tube	Sample	RIA Buffer	Volume STD	Volume AB	Volume ^{125}I-Peptide	Tube
TC-1,2	Total counts	200 µl	---	---	100 µl	TC-1,2
NSB-1,2	Nonspecific binding	200 µl	---	---	100 µl	NSB-1,2
TB-1,2	Total binding	100 µl	---	100 µl	100 µl	TB-1,2
7,8	Standard	---	100 µl	100 µl	100 µl	7,8
9,10	Standard	---	100 µl	100 µl	100 µl	9,10
11,12	Standard	---	100 µl	100 µl	100 µl	11,12
13,14	Standard	---	100 µl	100 µl	100 µl	13,14
15,16	Standard	---	100 µl	100 µl	100 µl	15,16
17,18	Standard	---	100 µl	100µl	100µl	17,18
19,20	Standard	---	100 µl	100 µl	100 µl	19,20
21,22	Standard	---	100 µl	100 µl	100 µl	21,22
23,24	Sample 1	---	100 µl	100 µl	100 µl	23,24
25,26	Sample 2	---	100 µl	100 µl	100 µl	25,26
27,28	Sample 3	---	100 µl	100 µl	100 µl	27,28
etc.	etc.					etc.

Note: Standards 7 through 22 are arranged from lowest to highest concentration as stated on Day 1 , step E5.

Day 3

A) Reconstitute the goat anti-rabbit IgG serum (GARGG) with 13 ml of RIA buffer.
B) Reconstitute the normal rabbit serum (NRS) with 13 ml of RIA buffer.
C) Add 100 µl of GARGG to every test tube.
D) Add 100 µl of NRS to every test tube.
E) Mix (vortex) each tube and incubate at room temperature for 90 minutes.
F) Add 500 µl of RIA buffer to each tube and mix.
G) Centrifuge the samples at 3,000 rpm (approx. 1,700 *g*) for 20 minutes at 4 °C.
H) Set aside the TC tubes. **DO NOT ASPIRATE THE TC TUBES.**
I) From the remaining tubes, carefully aspirate the supernatant (do not decant since the pellet may be lost or excess liquid may be left in the test tube).

Summary of Assay Protocol

1. Add sample or standard and antiserum.
2. Mix and incubate overnight (16-24 hrs.) at 4 °C.
3. Add ^{125}I-peptide.
4. Vortex and incubate overnight (16-24 hrs.) at 4 °C.
5. Add GARGG and NRS.
6. Mix and incubate at room temperature for 90 minutes.
7. Add RIA buffer.
8. Vortex and centrifuge for 20 minutes at 1,700 *g*
9. Aspirate off the supernatant (**except TC tubes**).
10. Count assay tubes.

Total assay time is 3 days.

This procedure is not intended to replace the preparation steps, precautions and procedures shown in this booklet. Please consult those sections for complete directions.

Calculations of Results

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

   NSB.

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

   TB.

3. To find $\mathbf{B_o}$ use the following equation:

   $B_0 =$ **TB -NSB**

4. To determine the $\%B/B_o$ for the duplicate standards and unknown samples use the following calculation:
   a. Example for Standard H:

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

   b. All other standards (tubes # 9-22) and unknown samples (tubes # 23-125) are also calculated as above.

5. Examples of tabulated data (using 1-128 pg/tube range): Total cpm/100 µl=10,000 cpm

Tube	Sample	Peptide	Avg. cpm	$\%B/B_o$
TC-1,2			10,000	
NSB-1,2			165	
TB-1,2		0 pg/tube	3,960	100
7,8	H Standard	1 pg/tube	3,789	95.5
9,10	G Standard	2 pg/tube	3,489	87.6
---	---	---	---	---
---	---	---	---	---
---	---	---	---	---
21,22	A Standard	128 pg/tube	343	4.7
23,24	Sample 1	?	1,432	33.4
25,26	Sample 2	?	913	19.7
27,28	Sample 3	?	2,624	64.8

B_0 = 3,960-165 = 3,795

Generating Standard Curve

For manual plot: on semilog graph paper, plot %B/B_o versus the log of the standard concentrations of the peptide.

a. Label the concentration of standards H through A (1-128 pg/100 µl) on the X-axis.
b. Label % B/B_o (0 to 100%) on the Y-axis.
c. Plot % B/B_o for each standard directly above its X-axis designation. Draw the "best fit" curve.

Determination of the Sample Concentration

a. Using %B/B_o 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/100 µl) in the assayed sample.
c. To calculate the amount of peptide in the original sample, multiply the concentration of the assayed sample by the dilution factor used to prepare the sample.

^{125}I Technical Information

^{125}I radioisotope produces a gamma ray photon that has a half-life of 60 days, a biological half-life of 138 days, and a principle photon of 0.0355 MeV. The amount of exposure from a 1.5 µCi tracer is 0.3 µR/Hr at contact. The critical organ for uptake is the thyroid gland. Please use shielding and equipment designed for gamma photon radiation.

Notes and Warnings

This radioactive material shall be received, acquired, possessed and used by licensed investigators in laboratories or hospitals for *in vitro* laboratory tests only. Its use shall not involve internal or external administration of the material and radiation of human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and 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 designate an area for handling ^{125}I and clearly label all containers. There should be no drinking, eating, or smoking where ^{125}I is being handled. Use disposable transfer pipettes, spill trays, and absorbent coverings to confine contamination. All users should wear disposable lab coats, gloves, and wrist guards as a secondary precaution. Regularly monitor and promptly decontaminate gloves and surfaces to maintain contamination control. Use a NaI (TI) detector or a liquid scintillation counter to detect ^{125}I. Conduct periodic thyroid counts to determine an internal dose. Isolate waste in sealed, labelled containers.
Establish surface contamination, air concentration, and thyroid burden action levels below maximum limits and investigate any causes that threaten these levels to be exceeded. Persons under 18 years old 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.

THIS PACKAGE CONFORMS TO THE CONDITIONS AND LIMITATIONS SPECIFIED IN TITLE 49 CFR173.421 FOR RADIOACTIVE MATERIAL.

Appendix 1: Sample Preparation and Extraction

Measurement of Peptides in Biological Fluids

This kit is designed to quantify the peptide in the supplied RIA buffer. It may not give accurate results when the peptide is suspended in a different assay matrix such as plasma, serum or urine. This problem is traditionally addressed by extracting the peptide from the biological fluid.
A good extraction procedure serves two purposes:

1. It separates the peptide from potentially interfering substances, such as albumin.
2. It allows the investigator to concentrate the sample.

Unfortunately, as with any purification technique, a recovery of the analyte is never 100%. Therefore, it is recommended that the extraction procedure is optimized on the individual basis . The analyte recovery can best be determined by evaluating the extraction of control samples paired with the same samples spiked with known quantities of the peptide of interest. The difference in the measured quantity of peptide between spiked and non-spiked samples will allow the user to determine the recovery of an extraction procedure.

In the following pages we describe a generic plasma extraction procedure to help researchers get started. This generic procedure can also be used on other type of samples. If a better extraction method is found in the literature, we encourage the researcher to use it.

C18 Sep-Column Extraction Method

Blood Withdrawal and Preparation of Plasma:

Collect blood samples (2 - 6 ml) into a chilled syringe and transfer into a polypropylene tube containing EDTA (1 mg/ml of blood) and aprotinin (500 KIU/ml of blood)[1] at 0 °C. Centrifuge blood at 1,600xg for 15 minutes at 0 °C. Store unused portion at -70 °C.

- No heparinized tubes - they may interfere in assay. Vacutainers with EDTA are acceptable.
- Purpose of EDTA: anticoagulant.
- Purpose of aprotinin: protease inhibitor.
- If collecting samples which will not be run immediately, store them below 0 °C.

Required Chromatography Solvents:

1. Buffer A (Y-1040): 1% trifluoroacetic acid (TFA, HPLC grade). Acidifies plasma sample to remove interfering proteins such as albumin.
2. Buffer B (Y-1045): 60% acetonitrile (HPLC grade), 1% TFA, and 39% distilled water. Eludes peptide from C_{18} column.

[1] **1 TIU = 900 KIU**

Extraction Procedure:

1. Add an equal volume of buffer A to the plasma sample. Centrifuge at 6,000 to 17,000 *g* for 20 minutes at 4 °C. Discard any pellet that may be present.
2. Equilibrate a SEP-column containing 200 mg of C_{18} (Cat No. Y-1000) by washing with buffer B or buffer D (100% acetonitrile) (1 ml, once) followed by buffer A (3 ml, three times).
3. Load the plasma solution onto the pre-treated C_{18} SEP-column.

Note: For steps 4 and 5, a light vacuum (10 sec/drop) may be applied to the column.

4. Slowly wash the column with buffer A (3 ml, twice) and discard the wash.
5. Elute the peptide slowly with buffer B (3 ml, once) and collect eluate in a polypropylene tube.
6. Evaporate eluate to dryness using a centrifugal concentrator or by a suitable method.
   Ex: Lyophilizer.(It is recommended to freeze eluate with dry ice/methanol to speed up the process .
7. Dissolve the residue in RIA buffer for radioimmunoassay as follows: For a normal subject, dissolve in 250 µl RIA buffer for a two-tube assay. Aliquot 100 µl into each tube (50 µl is left over). If each tube is found to contain 3.963 pg/tube, then the total level of peptide in plasma sample = 3.962 pg/tube x 2.5 tube = 9.9 pg. If upon assaying, the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the samples accordingly.
8. The total time for extraction should be 0.5 days for extraction and 0.5 days for lyophilization. Once the sample is lyophilized, it can be stored at –70 °C before assaying, but should be assayed as soon as possible.

Troubleshooting Guide

Poor Precision

Possible causes and actions:

1. **Incomplete mixing of sample** - try vortex mixing or centrifuging the samples before dispensing.
2. **Kit reagents were not allowed to reach room temperature before use** - ensure that reagents are allowed to reach the room temperature before use. Large volumes may take longer time to reach the ambient temperature.
3. **Carry-over of analyte** - use fresh pipette tips for each transfer.
4. I**nsufficient centrifugation time or g force** - check the protocol and follow the instruction.
5. **Skipping procedure step** - adhere to the recommended procedure. Be sure to add the final 500 µl of RIA buffer to each tube and mix well before centrifuging.

Low Binding

Possible causes and actions:

1. **Cross-contamination of one reagent by another** - check that the dispensing equipment is cleaned thoroughly and replace the reagents. Add one reagent at a time and use fresh tips for each transfer.
2. I**ncorrect storage of reagents** - follow the storage instruction on the package insert.
3. **Reduced incubation time** - check the protocol and follow the instruction.

High Nonspecific Binding

Possible causes and actions:

1. **Tracer adheres to test tube surface** - add 500 µl of RIA buffer to each tube and mix before centrifuging or try using different types of tubes, to find one that causes less interference.
2. **Incubation temperature was too high** - check the protocol and follow the instruction.
3. **Contamination with antiserum** - repeat the assay.

High IC_{50}

Possible causes and actions:

1. **The kit was near or past its expiration** - always use fresh tracer.
2. **Excess liquid was left in test tube** - use the appropriate equipment (e.g. vacuum pump) to aspirate the supernatant. Ensure the pellet is dry.
3. **Incorrect incubation time and/or temperature** - use recommended incubation time and temperature.
4. **Repeated freezing and thawing of kit components** - tracer and standard should be aliquoted and stored at – 20 °C

Poor Standard Curve

Possible causes and actions:

1. **Improper preparation of standards** - ensure use of the appropriate diluent to reconstitute standards. Reconstitute with the volume recommended in the kit insert.
2. **Incorrect incubation temperature and/or time** - check the protocol and follow the instruction.
3. **Unequal volume of standards were added** - check pipette function. Ensure the pipette tips are securely attached.
4. **Loss of the precipitate** - use appropriate g force and time for centrifuge. Aspirate immediately after centrifuging.

Unexpected Results

Possible causes and actions:

1. **Interfering factors in samples** - remove interfering proteins and other components from samples by doing a sample peptide extraction before the assay.
2. **High sample values caused by an extraction problem** - increase the wash volume and/or number of washes with 1% TFA buffer. Use charcoal stripped serum (removes proteins from serum) together with the extracted samples The value of analyte detected in the charcoal stripped serum is then subtracted from the value obtained from the sample. This helps eliminate one source of background (false positive) from the assay readings.

References

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Heidelberg, Springer-Verlag (1987)
2. A. Dwenger
Radioimmunoassay: An Overview.
J. Clin. Chem. Clin. Biochem. **22**, 883-894 (1984)

Ordering Information

For additional kits or our most current catalog products, please visit our web site at www.bachem.com or call us at:

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Technical Support

If you need technical information or assistance with assay procedures, please call our Technical Service Department at (800) 922-1516 or (650) 592-5392. Our staff will be happy to answer your questions about this or any other products.

Guarantee and Limitation of Remedy

Peninsula Laboratories, Inc. makes no guarantee of any kind, expressed or implied, which extends beyond the description of the material in this kit, except that these materials and this kit will meet our specifications at the time of delivery. Customer's remedy and Peninsula Laboratories, Inc.'s sole liabilities hereunder are limited at Peninsula Laboratories, Inc.'s option to either refund the purchase price or to replace the material that does not meet our specifications. By the acceptance of our products, the customer indemnifies and holds Peninsula Laboratories, Inc. harmless against, and assumes all liability for, the consequences of its use or misuse by the customer, its employees or others.