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Peptide Radioimmunoassay (RIA) Protocol

CAUTION: INVESTIGATIONAL DEVICE
LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

TECHNICAL HELP and ORDERING INFORMATION
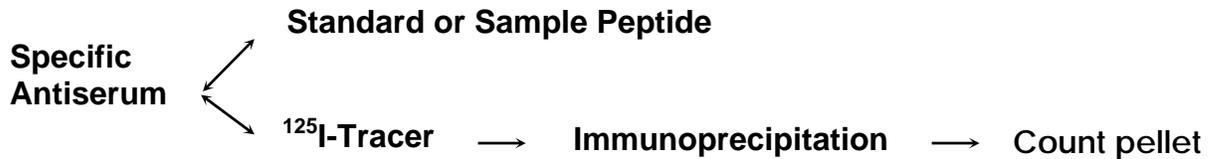
Please e-mail technical questions to techsupport@penlabs.com

To purchase additional kits or other products or to obtain a quote, please email sales@penlabs.com

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RIA KITS - BASIC NOTIONS AND FACTS

Our **RIA** kits are competitive immunoassays whose working principle can be diagrammed as follows:

Specific Antiserum → **Standard or Sample Peptide**

Specific Antiserum → ^{125}**I-Tracer** ⟶ **Immunoprecipitation** ⟶ Count pellet

A constant concentration of ^{125}**I-tracer** (^{125}I radiolabeled tracer) and varying concentrations of unlabeled standard or sample peptide compete for binding specifically to the antiserum. Immunocomplexes are precipitated by adding an excess of non-specific serum and secondary polyclonal antiserum followed by centrifugation. Unbound reagents are removed and the pellet is counted in a gamma counter.

The **sequence of the standard** peptide is shown on the datasheet (note that large protein sequences are usually not shown).

The standard is used to make a **standard curve** in the **range** specified in the kit's **datasheet**. Standard curves are **S-shaped** (on a **semi-log plot**) but for a few kits they appear to be almost linear over the kit's range. The **measuring range** is the range of standard concentrations near the middle or near the **IC$_{50}$** of the standard curve. Typical standard curves can be obtained via e-mail (please see contact information on front cover). Unknown sample concentrations are measured by comparing its cpm with the standard curve.

We include sufficient reagents for **125 RIA test tubes**.

VARIATION • ACCURACY • EXTRACTION • CROSSREACTIVITY

The kit's IC_{50}, or the shape of the standard curve, may show some variation but this will not affect the kit's accuracy in the measuring range. The kit accurately measures sample peptides if the following conditions are met.

A) The kit's antiserum must not cross-react appreciably with other factors present in the sample. Cross-reactivity tables are included with each kit.   The user may wish to test the cross-reactivity with other peptides.

B) The sample peptides must be identical to the kit's standard. Ideally the kit's synthetic standard mimics the natural peptide perfectly. Sometimes, however, natural peptides exist as families of species related by a common or similar sequence. In addition, natural peptides may be enzymatically or spontaneously modified, may exist in complexes, and may assume alternative structural forms. In these cases the kit might not measure the exact concentration of a particular natural peptide species, but, it may still be used for relative average measurements.

C) Serum samples must be extracted. Factors present in serum can bind to kit components and affect the measurements. Thus, it is advisable to extract peptides from serum before measuring them. Extraction may not be as important if the sample is diluted before measuring or if it is in tissue culture medium, but, in general, one may expect low sensitivity or high background problems if extraction is not used. Some antigens can be measured directly in un-extracted serum. If so, a specially formulated **extraction-free** kit may be available in our catalog or as a custom product, which can be used without extraction for human or rat serum.

D) Both samples and standards must be measured under the same conditions.

SAFETY PRECAUTIONS

The ^{125}I radioisotope produces 35.5 keV gamma radiation. It has a half-life of 60.1 days, and a biological half-life of 120 to 138 days. The amount of exposure from 1 mCi ^{125}I is 1.5R/Hr at 1cm. The critical organ for uptake is the thyroid gland. Please use shielding and equipment designed for ^{125}I or gamma radiation.

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 there from to 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.

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 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 liquid scintillation counter to detect ^{125}I. Conduct periodic thyroid counts to determine an internal dose. Isolate waste in sealed, labeled 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.

Some of the kit components (including the RIA buffer) contain sodium azide. Please handle and dispose of according to established safety regulations.

MATERIALS PROVIDED AND STORAGE CONDITIONS

- **RIA buffer 4x concentrate** (50 ml). Store at **4°C** before and after dilution.
- **Standard peptide** lyophilized powder. Store at **-20°C**.
- **Specific rabbit antiserum** lyophilized powder. Store at **-20°C**.
- **^{125}I-tracer**: at least 1.5 μCi (on preparation day). Store at **-20°C**.
- **Secondary polyclonal antiserum** lyophilized powder, goat anti rabbit (GARGG) antiserum. Store at **4°C** before and after re-hydration.
- **Normal rabbit serum** lyophilized powder. Store at **4°C** before and after re-hydration.
- Datasheet.
- ***Standard diluent (8 ml)*** *of peptide-free stripped frozen serum (for extraction-free kits only). Store at* ***-20°C.***

MATERIALS NOT PROVIDED

- 12 x 75 mm polystyrene tubes
- Gamma counter for the above tubes
- Centrifuge for the above tubes
- Curve fitting software (optional)
- Various other standard laboratory items

ASSAY PROTOCOL

We recommend using the same basic 3-day RIA protocol shown below for all our RIA kits. Depending on the kit, the details of the protocol differ with respect to three parameters: **extraction, standard curve,** and **antiserum type**. Please refer to the **enclosed datasheet** to determine these parameters and proceed as required for each step of the protocol.

GENERAL RIA 3-DAY PROTOCOL

We strongly recommend duplication of assays

Please refer to the following pages for detailed instructions for steps 1 –16

Steps:

Step	Day	Description
1	Day 1	Dilute RIA buffer to 200 ml
2	Day 1	Extract Samples (skip for extraction-free)
3	Day 1	Make standard dilutions
4	Day 1	Reconstitute antiserum (13 ml)
5	Day 1	Mix standards / samples with antiserum
6	Day 1	Vortex & incubate O/N 4°C
7	Day 2	Reconstitute ^{125}I-Tracer ~10 K cpm/100µl
8	Day 2	Add ^{125}I-Tracer
9	Day 2	Vortex & incubate O/N 4°C
10	Day 3	Hydrate secondary pAb / Add secondary pAb
11	Day 3	Hydrate norm. ser. / Add norm. ser.
12	Day 3	Vortex & incubate 90 minutes at RT
13	Day 3	Add buffer / Vortex
14	Day 3	Centrifuge 20 minutes 1,700 x g
15	Day 3	Aspirate supernat.
16	Day 3	Count, plot and analyze data

Step 5 — Mix standards / samples with antiserum and volumes for steps 8, 10, 11, 13, 15:

	Step 5 Buffer	Step 5 Std diluent	Step 5 Std or sample	Step 5 Anti-serum	8	10	11	13	15
TC	100 µl	100 µl	---	---	100µl	100µl	100µl	500µl	skip
NSB	100 µl	100 µl	---	---	100µl	100µl	100µl	500µl	aspirt.
TB	---	100 µl	---	100µl	100µl	100µl	100µl	500µl	aspirt.
H	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
G	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
F	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
E	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
D	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
C	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
B	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
A	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.
xyz	---	---	100 µl	100µl	100µl	100µl	100µl	500µl	aspirt.

TC (Total cpm) NSB (non-specific binding) A-H (standards) TB (max. binding) xyz (unknown samples)

DAY 1
1 - Dilute the RIA buffer concentrate to 200 ml.

2 - **Sample extraction** is recommended especially for serum samples. It may not be as important for some tissue culture samples. See "Suggested Protocol for Sample Extraction" below for details. The kit may still be used without extraction but this may cause unexpected results due to the possible binding between serum proteins and kit components.

If you purchased an <u>Extraction-Free Kit,</u> you may use it for the measurement of human or rat serum or plasma (according to its designation) without performing an extraction.

Sample concentration. The concentration of the target molecule must be within the measuring range of the kit (in a region around the IC_{50}). If you cannot estimate the concentration range of your sample you can prepare it at different concentrations such that one of the samples may be within the measuring range.

3 - Reconstitute the standard in 1ml RIA buffer and make serial dilutions covering the kit's range as stated in the enclosed datasheet.
Caution: please note carefully the kit's range. The table on the next page is included to facilitate the serial dilution process.

If you purchased an <u>Extraction-Free Kit,</u> you should use the included standard diluent (8 ml of peptide-free stripped frozen human or rat serum) to make the dilutions.

Note: the standards and the samples should be in the same diluent. If extraction was done the diluent should be RIA buffer. Otherwise, for extraction-free kits use your own diluent if possible or use the included standard diluent for standards to match the serum or plasma samples.

Standard Vial: **12.8 µg** dissolved in 1 ml

	0.001 - 0.64 ng/ml				0.01 - 1.28 ng/ml				0.1 - 12.8 ng/ml			
	µl	of	µl dil	ng/ml	µl	of	µl dil	ng/ml	µl	of	µl dil	ng/ml
stock	----	----	----	12800	----	----	----	12800	----	----	----	12800
O	10	stock	990	128	10	stock	990	128	100	stock	900	1280
A	5	O	995	0.64	10	O	990	1.28	10	O	990	12.8
B	500	A	500	0.32	500	A	500	0.64	500	A	500	6.4
C	500	B	500	0.16	500	B	500	0.32	500	B	500	3.2
D	500	C	500	0.08	500	C	500	0.16	500	C	500	1.6
E	500	D	500	0.04	500	D	500	0.08	500	D	500	0.8
F	500	E	500	0.02	500	E	500	0.04	500	E	500	0.4
G	500	F	500	0.01	500	F	500	0.02	500	F	500	0.2
H	100	G	900	0.001	500	G	500	0.01	500	G	500	0.1

4 - **Reconstitute the antiserum in 13 ml RIA buffer.**

5- Mix 100 µl standards or samples with 100 µl antiserum.
   Add no standard or sample to controls TC, NSB, and TB.
   Add only 100 µl diluent and 100 µl antiserum to TB.
   Add 100 µl diluent plus 100 µl RIA buffer to TC and NSB.

6 - **Vortex and incubate overnight (16-24 hrs.) at 4°C.**

7- Prepare (a) the tracer stock and (b) the diluted tracer solutions.
   a) Tracer stock. Add 1 ml of RIA buffer to the shipped ^{125}I-tracer vial and mix thoroughly.
   b) Diluted tracer. From the tracer stock make a working **diluted tracer** solution in RIA buffer at **10,000 to 12,000 cpm / 100 μl**. The kit will be most sensitive with the least amount of tracer that will still provide accurate counts at the lower end of the assay range. We have determined empirically that 10,000 to 12,000 cpm per assay tube (TC cpm) works well. If you need help in making the correct dilution of tracer please do the following:

   - **N** = number of assays and standards (plus ~ 10%)
   - **V** = **N** x 0.1 ml (volume of needed RIA buffer in ml)
   - **To** = **N** x 2 μl (initial volume of tracer stock in μl)
   - Mix **V** ml RIA buffer with **To** μl tracer stock and count 100 μl
      - **C$_{100}$** = cpm in 100 μl this initial dilution
   - **Tadd** = additional μl tracer that must be added to reach 10,000 cpm/100μl

$$Tadd = (10,000 - C_{100})\frac{To}{C_{100}}$$

   If you require a higher specific activity than 10,000 cpm / 100 μl simply enter a number greater than 10,000 in the equation above. If you require less, enter a lower number and also start with a lower **To**. If **C$_{100}$** exceeds the desired cpm dilute the initial solution in RIA buffer.

The unused tracer stock solution can be stored at 4°C for no more than 1 day to avoid freezing and thawing. If storing for a longer period of time, create aliquots of the tracer to prevent multiple freezing and thawing and store at -20°C or lower for maximum stability.

8- Add 100 μl diluted ^{125}I tracer (e.g. ~10,000 cpm) to all tubes.

9- Vortex and incubate overnight (16-24 hrs.) at 4°C

DAY 3

10, 11 - Add secondary Ab and normal serum

a) Reconstitute the secondary antibody (GARGG) with 13 ml of RIA Buffer.

b) Reconstitute the normal serum (NRS) with 13 ml of RIA Buffer.

c) Add 100 μl of secondary antibody and 100 μl of normal serum to each tube and vortex.

12 - Incubate at room temperature for 90 minutes

13 - Add 500 µl of RIA Buffer to each tube and vortex.

14 - Centrifuge for 20 minutes at 1700 x g.

15 - Set aside the TC tubes. DO NOT ASPIRATE THE TC TUBES. Aspirate all other tubes being careful not to disturb the pellet.

16 - Count, plot and analyze the cpm data.
Use a calibrated gamma counter and collect the cpm data for all tubes.

DATA ANALYSIS

Should you need help for the plotting and analysis of your data we recommend the following procedure:

Set up a spreadsheet as shown below (note that the values on the spreadsheet are merely illustrative and are not necessarily typical for this particular kit). Please e-mail us (contact information on front cover) and we will be happy to send you the actual working Excel spreadsheet shown below.

For tubes labeled TC, NSB, TB, H, G, F, ..., A, enter the cpm from the duplicate tubes and average the values

Calculate all B/Bo values with the following formula:

$$\frac{B}{Bo} = \frac{Average\ sample\ cpm - NSB}{TB - NSB}$$

Plot a standard curve y = f(x) on a semi-log scale where y is B/Bo and x are the standard concentrations in ng/ml.

We recommend that you use a good automatic data fitting computer program, but you may also obtain excellent results by fitting a four-parameter curve to the standard readings on a Excel sheet as shown here. Use the following equation to calculate the values on the "FIT" column. Then change the parameters a, b, c, and d, until you are satisfied that fit is good.

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

Next calculate the average of your sample readings and from that calculate B/Bo for all your samples. Finally, you may use the "reverse" of the equation above to calculate the concentrations in ng/ml for all your samples.

$$x = c\left(\frac{y - a}{d - y}\right)^{1/b}$$

Tube	Duplicate cpm readings		Average
TC	11836	11732	11784
NSB	310	229	270

(TB - NSB)
Bo = 7069

Fit parameters

a (max)	1.012
b (slope)	1.461
c (IC50)	0.035
d (min)	0.025

Fit equaton

$$y = ((a-d)/(1 + (x/c)^b))+d$$

STANDARD CURVE

	Average		B Av - NSB	known ng/ml	B/Bo	FIT
TB	7423	7254	7339	7069	0.001	1.000	1.007
H	6424	6652	6538	6269	0.010	0.887	0.876
G	5215	5231	5223	4954	0.020	0.701	0.710
F	3650	3461	3556	3286	0.040	0.465	0.471
E	2082	1962	2022	1753	0.080	0.248	0.252
D	1319	1048	1184	914	0.160	0.129	0.122
C	905	682	794	524	0.320	0.074	0.062
B	432	549	491	221	0.640	0.031	0.039
A	533	348	441	171	1.280	0.024	0.030

Scatter plot of data
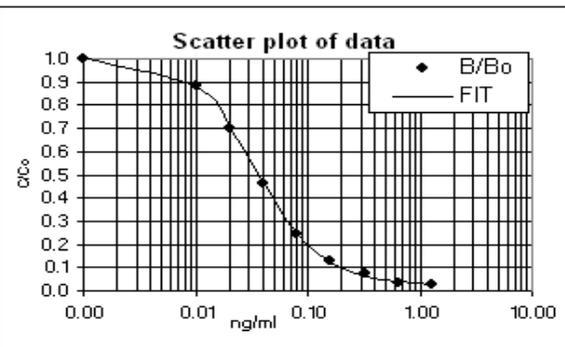
SAMPLES

$x=c*((y-a)/(d-y))^(1/b)$

	tube 1	tube 2	Average	B Av - NSB	calc. ng/ml	B/Bo
X1	2250	2300	2275	2006	0.071	0.284
X2	5200	5100	5150	4881	0.021	0.690
etc...						

Caution
- In the example above we set the TB known ng/ml value at 0.001 so that the value could be plotted on a semi-log scale, but value is actually **ZERO**
- When calculating sample concentrations using the "reverse" equation if your B/Bo value happens to be the same number as the parameter "d" you will get a error because of a division by zero
- If your B/Bo value is above or below the asymptotes obviously the reading is off range and the calculation won't work

SUGGESTED PROTOCOL FOR SAMPLE EXTRACTION

We have provided an excess amount of standard that you may use to determine if extraction is required. For example, if you are working with serum, you may spike it with known amounts of standard and check if they are accurately determined by the assay with and without extraction. Extraction eliminates potentially interfering substances, such as albumin. Extraction may also be necessary to concentrate the sample to within the measuring range. As with any purification technique, recovery of the desired substance is likely to be incomplete. Therefore, both optimization and quantification of the extraction procedure are recommended for more accurate determinations. While we cannot provide you with extraction optimization and quantification protocols, we have included enough standard in the kit should you wish to use it for this purpose.

C18 Sep-Column Extraction Method

The following generic protocol is meant to help users with little experience in extracting their samples. It should be applicable to different biological fluids but should not be thought of as an optimized protocol for any particular antigen.

Required Materials

- SEP-COLUMN containing 200 mg of C18 (Cat. No. Y-1000).
- Buffer A (BUFF-A): 1% trifluoroacetic acid (TFA, HPLC Grade). (Acidifies plasma sample to remove interfering proteins such as albumin) (Cat. No. Y-1040).
- Buffer B (BUFF-B): 60% acetonitrile (HPLC Grade), 1% TFA, and 39% distilled water. (Elutes peptide from column). (Cat. No. Y-1045).
- You may also consider purchasing Extraction kits (Cat. No. S-5000), which include SEP-columns and buffers.

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) as an anticoagulant and Aprotinin (500 KIU/ml of blood) as a protease inhibitor at 4°C. Do not use heparinized tubes as they may interfere with the assay. Vacutainers with EDTA are acceptable.
- Centrifuge blood at 1,600xg for 15 minutes at 4°C.
- Collect the top (plasma) layer.
- Proceed to extraction immediately or freeze at -70°C for later use.

Extraction Procedure

- Add an equal amount of Buffer A to the plasma.
- Centrifuge at 6,000xg to 17,000xg for 20 minutes at 4°C.
- Transfer supernatant to a new tube discarding any pellet that may be present.
- Equilibrate a SEP-COLUMN by washing with 1 ml Buffer B followed by 3 X 3 ml Buffer A.
- Load the plasma solution onto the equilibrated SEP-Column.
- Slowly wash the column with Buffer A (3 ml, twice) and discard the wash. A light vacuum (10 sec/drop) may be applied to the column.
- Elute the peptide slowly with Buffer B (3 ml, once) and collect eluant in a polypropylene tube. A light vacuum may be applied as in previous step.
- Freeze-dry eluant to dryness using a dry ice/methanol bath to freeze the sample and a centrifugal concentrator to evaporate it.
- Dissolve the residue in a suitable volume of RIA buffer such that the concentration of the substance of interest will fall close to the IC_{50} (within the measuring range).

TROUBLESHOOTING

Most problems are caused by poor technique or by alterations of the protocol. What follows are possibly less obvious causes of problems.

Poor precision (high standard deviations) may be caused by
- Poor mixing
- Kit reagents not equilibrated to room temperature before use
- Cross-contamination of samples
- Insufficient centrifugation time or g force

Possible causes of low binding
B_o or "total specific binding" is obtained by subtracting the non-specific binding cpm (**NSB**) from the max binding cpm (**TB**): **B_o = TB - NSB**.
"Total binding" is the percent B_o relative to "total cpm" (**TC**)

$$\text{Total Binding} = 100 \times B_o / TC$$

"Low binding" occurs if "total binding" is below 25%. This leads to shallow curves and poor precision.
Low binding can be caused by:
- Degraded tracer or antiserum due to incorrect storage or excessive freeze-thawing
- Reduced incubation time

Possible causes of nonspecific binding
- Tracer may be sticking more than expected to a particular test-tube. Make sure you add 500 μl RIA buffer before vortexing and centrifuging. Also, test different types of tubes
- High incubation temperature may cause higher sticking
- Contamination with antiserum

Possible causes for higher than expected IC_{50}
- Note that the IC_{50} reported with each of our products is based on the concentration of the prepared standards before they are added to the assay solution
- A difference by a factor of two or three may be normal
- In cases where the standard curves are almost rectilinear, accurate IC_{50} values cannot be calculated with the 4-parameter equation shown above. In these cases it is best to estimate the IC_{50} visually as the x value that results in a B/Bo of 0.50

- Excessive amounts of antiserum or tracer, or using a degraded standard may all raise the IC_{50}

Poor standard curves may be caused by
- Loss of precipitate - use appropriate g force and time for centrifuge
- Aspirate immediately after centrifuging
- Do not leave excessive liquid behind
- Many other trivial explanations: bad pipettes, etc.

Unexpected results may be obtained if
- Standards and samples are in different solvents or conditions
- Antiserum binds to another antigenically similar peptide
- Antigen was lost during extraction or extraction did not eliminate interfering factors (not applicable to extraction-free kits)

Tracer Decay
- Tracers should be used as soon as possible
- The specific activity of the ^{125}I-tracer will decay with a half-life of 60.1 days (for example in 1 month the tracer will lose 30% of its radioactivity)

REFERENCES
1. Patrono, C. and Peskar, B.A. (eds) Radioimmunoassay in Basic and Clinical Pharmacology. Heidelberg, Springer-Verlag, 1987.
2. Dwenger, A. Radioimmunoassay: An Overview. J Clin Biochem 22:883, 1984.

GUARANTEE AND LIMITATION OF REMEDY