RAT C-PEPTIDE RIA KIT
250 TUBES (Cat. # RCP-21K)

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I. INTENDED USE

Millipore's Rat C-Peptide Radioimmunoassay (RIA) Kit utilizes an antibody made specifically against Rat C-Peptide. Sensitivity of 25 pM can easily be achieved when using a 100 µl serum sample in a two day, disequilibrium assay (400 µL Total Volume). ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The Millipore Rat C-Peptide assay utilizes ^{125}I-labeled Rat C-Peptide and a Rat C-Peptide antiserum to determine the level of C-Peptide in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 250 tubes and contains the following reagents.

A. Assay Buffer
0.05M Phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, and 1% RIA Grade BSA
Quantity: 40 mL/vial
Preparation: Ready to use

B. Rat C-Peptide Antibody
Guinea Pig anti-Rat C-Peptide Antibody in Assay Buffer
Quantity: 26 mL/vial
Preparation: Ready to use

C. ^{125}I-Rat C-Peptide
^{125}I-Rat C-Peptide Label, HPLC purified (specific activity 630 µCi/µg) Lyophilized for stability. Freshly iodinated label contains <3 µCi (111 kBq), calibrated to the 1st Monday of each month.
Quantity: 27 mL/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to sit at room temperature for 30 minutes, with occasional gentle mixing.

D. Label Hydrating Buffer
Assay Buffer containing Normal Guinea Pig Serum as a carrier. Used to hydrate ^{125}I-Rat C-Peptide.
Quantity: 27 mL/vial
Preparation: Ready to use

E. Rat C-Peptide Standards
Purified Recombinant Rat C-Peptide in Assay Buffer at the following concentrations:
25, 50, 100, 200, 400, 800, 1600 pM
Quantity: 2 mL/vial
Preparation: Ready to use

F. Quality Controls 1 & 2
Purified Recombinant Rat C-Peptide in Assay Buffer
Quantity: 1 mL/vial
Preparation: Ready to use

G. Precipitating Reagent
Goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide
Quantity: 260 mL/vial
Preparation: Ready to use; chill to 4°C

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8°C for short term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer (RSO) is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.

2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.

3. Monitor hands, shoes, and clothing and immediate area surrounding the work station for contamination after each procedure and before leaving the area.

4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.

5. Never pipette radioactive material by mouth.

6. Dispose of radioactive waste in accordance with NRC rules and regulations.

7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.

8. Use absorbent pads for containing and easily disposing of small amounts of contamination.

9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

V. REAGENT PRECAUTIONS (continued)

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. 100 µL pipet with disposable tips
3. 100 µL & 1.0 mL repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets is not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A maximum of 100 µL per assay tube of serum or plasma can be used, although, 50 µL per assay tube is adequate for most applications. Tissue culture and other media may also be used.
2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.
3. Specimens can be stored at 4°C if they will be tested within 24 hours of collection. For longer storage, specimens should be stored at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles.
4. Avoid using samples with gross hemolysis or lipemia.
5. C-Peptide must be protected from proteolysis during assay procedures and sample storage. Collect blood samples in serum or plasma tubes containing 250 KIU Trasylol (Aprotinin) per mL of whole blood. This will result in a final concentration of approximately 500 KIU Trasylol per mL of serum or plasma. Aliquot and freeze at -20° to -70°C.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

Day One

1. Pipette 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 µL of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 100 µL of Assay Buffer to tubes seven through the end of the assay.
2. Pipette 100 µL of Standards and Quality Controls in duplicate (see assay flow chart).
3. Pipette 100 µL of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when C-Peptide concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 µL (e.g., when using 50 µL of sample, add 50 µL of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipette 100 µL of Rat C-Peptide Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
5. Vortex, cover, and incubate overnight (20-24 hours) at 4ºC.

Day Two

6. Pipet 100 µL of ^{125}I-Rat C-Peptide tracer to all tubes. Important: For preparation, see Section III, Part C.
7. Vortex, cover and incubate overnight (22-24 hours) at 4ºC.

VIII. ASSAY PROCEDURE (continued)

Day Three

8. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000 xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:

    $xg = (1.12 \times 10^{-5})(r)(rpm)^2$

    r = radial distance in cm (from axis of rotation to the bottom of the tube)

    rpm = revolutions per minute
11. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. Note: Invert tubes only one time. Pellets are fragile and slipping may occur.
12. Count all tubes in a gamma counter for 1 minute. Calculate the pM of Rat C-Peptide in unknown samples using automated data reduction procedures (see Section IX).

Assay Procedure Flow Chart

Day One					Day Two		Day Three	
Set-up	Step 1	Step 2&3	Step 4	Step 5	Step 6	Step 7	Step 8	Steps 9-12
Tube Number	Add Assay Buffer	Add Standard/QC Sample	Add Rat C-Peptide Antibody	**Vortex, Cover, and Incubate 20-24 hrs at 4°C**	Add I-125 Rat C-Peptide Tracer	**Vortex, Cover and Incubate 22-24 hrs at 4°C**	Add Precipitating Reagent	**Incubate 20 min. at 4°C, Centrifuge at 4°C for 20 min Decant and Count**
1,2	-	-	-		100 µl		-	
3,4	300 µl	-	-		100 µl		1.0 mL	
5,6	200 µl	-	100 µl		100 µl		1.0 mL	
7,8	100 µl	100 µl of 25 pM	100 µl		100 µl		1.0 mL	
9,10	100 µl	100 µl of 50 pM	100 µl		100 µl		1.0 mL	
11,12	100 µl	100 µl of 100 pM	100 µl		100 µl		1.0 mL	
13,14	100 µl	100 µl of 200 pM	100 µl		100 µl		1.0 mL	
15,16	100 µl	100 µl of 400 pM	100 µl		100 µl		1.0 mL	
17,18	100 µl	100 µl of 800 pM	100 µl		100 µl		1.0 mL	
19,20	100 µl	100 µl of 1600 pM	100 µl		100 µl		1.0 mL	
21,22	100 µl	100 µl of QC 1	100 µl		100 µl		1.0 mL	
23,24	100 µl	100 µl of QC 2	100 µl		100 µl		1.0 mL	
25-n	100 µl	100 µl of unknown	100 µl		100 µl		1.0 mL	

IX. CALCULATIONS

A. Explanation

The calculations for Rat C-Peptide can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data.

NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.

2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.

3. Calculate the percentage of tracer bound

   (Total Binding Counts/Total Counts) X 100

   This should be 35-50%.

4. Calculate the percentage of total binding (%B/Bo) for each standard and sample

   %B/Bo = (Sample or Standard/Total Binding) X 100

5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.

6. Construct the reference curve by joining the points with a smooth curve.

7. Determine the pM of Rat C-Peptide in the unknown samples and controls by interpolation of the reference curve.

NOTE: When sample volumes assayed differ from 100 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 µL of sample is used, then calculated data must be multiplied by 2).

X. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control Values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.

2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

3. The limit of sensitivity for the Rat C-Peptide assay is 25 pM (100 µL sample size).

4. The limit of linearity for the Rat C-Peptide assay is 1600 pM (100 µL sample size). Any result greater than 1600 pM should be repeated on dilution using Assay Buffer as a diluent.

XI. NORMAL FASTING RANGE

100-300 pM

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Rat C-Peptide that can be detected by this assay is 25 pM when using a 100 µL sample size.

B. Performance

The following parameters of assay performance are expressed as Mean $\pm$ Standard Deviation.

ED_{80} = 50 ± 5 pM
ED_{50} = 197 ± 13 pM
ED_{20} = 791 ± 51 pM

XII. ASSAY CHARACTERISTICS

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Rat C-Peptide	100 %
Mouse C-Peptide	100 %
Hamster C-Peptide	100 %
Human C-Peptide	< 0.1 %
Porcine C-Peptide	< 0.01 %
Human Proinsulin	< 0.01 %
Human Insulin	*
Rat Insulin	*
Glucagon	*
Somatostatin	*
Amylin	*
Human Pancreatic Polypeptide	*
Rat Pancreatic Polypeptide	*

*-not detectable

XII. ASSAY CHARACTERISTICS (continued)

D. Example of Assay Results

This data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.

Tube #	ID	CPM	Ave CPM	Ave Net CPM	% B/Bo	pM
1	Totals	20361				
2	″	21433	20897			
3	NSB	432				
4	″	481	457			
5	Bo	9124				
6	″	9231	9178	8721		
Standards						
7	25 pM	8156				
8		7932	8044	7587	87.0	
9	50 pM	7012				
10		7050	7031	6574	75.4	
11	100 pM	6096				
12		5957	6027	5570	63.9	
13	200 pM	4306				
14		4330	4318	3861	44.3	
15	400 pM	2964				
16		3038	3001	2544	29.2	
17	800 pM	1896				
18		1926	1911	1454	16.7	
19	1600 pM	1340				
20		1326	1333	876	10.1	
Controls/Unknown						
21	QC 1	5974				
22		6279	6127	5670	65.0	87.86
23	QC 2	2244				
24		2245	2245	1788	20.5	643.34
25-n	Unknown					

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

Recommended batch analysis decision using two controls (Westgard Rules) [4]:

1. When both controls are within ±2 SD.
   Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD.
   Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

   Technician check of systems:
   1. Check for calculation errors
   2. Repeat standards and controls
   3. Check reagent solutions
   4. Check instrument

XIV. REPLACEMENT REAGENTS

Reagent	**Cat #**
^{125}I-Rat C-Peptide (<3 µCi, 111 kBq)	9022
Label Hydrating Buffer (27mL)	LHB-P
Rat C-Peptide Standards (2 mL each)	8021-K
Rat C-Peptide Antibody (26 mL)	1021-K
Precipitating Reagent (260 mL)	PR-UV
Quality Control 1&2 (1 mL each)	6000-K
Assay Buffer (40 mL)	AB-P

XV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size
   NOTE: Appropriate license from NRC (or equivalent) must be on file at Millipore before radioactive orders can be shipped.

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.

XVI. REFERENCES

1. Morgan, C.R. and Lazarow, A. Immunoassay of Insulin: Two antibody system. Plasma insulin levels in normal, Subdiabetic, and diabetic rats. Diabetes 12:115-126, 1963.

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3. Feldman, H. and Rodbard, D. "Mathematical Theory of Radioimmunoassay," in: W.D. Odell and Doughaday, W.H. (Ed.), Principles of Competitive Protein-Binding Assays. Philadelphia: J.B. Leppincott Company; pp 158-203, 1971.

4. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.