CANINE C-PEPTIDE RIA KIT
125 TUBES (Cat. # CCP-24HK)

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I. INTENDED USE

Millipore's Canine C-Peptide Radioimmunoassay kit is for the quantitative determination of Canine C-Peptide in serum, plasma, and other biological media. It is a completely homologous assay since the antibody was raised against purified Canine C-Peptide and both the tracer and the standard are prepared with Canine C-Peptide. ***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 calibration or 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 Milliore Canine C-Peptide assay utilizes ^{125}I-labeled Canine C-Peptide and a Canine 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 125 tubes and contains the following reagents:

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

B. Canine C-Peptide Antibody
Guinea Pig anti-Canine C-Peptide Antibody in Assay Buffer
Quantity: 13 mL/vial
Preparation: Ready to use

C. ^{125}I-Canine C-Peptide
^{125}I-Canine C-Peptide Label, HPLC purified (specific activity 628 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains 1.5µCi (56 kBq), calibrated to the 1st Monday of each month.
Quantity: 13.5 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.

III. REAGENTS SUPPLIED (continued)

D. Label Hydrating Buffer
Assay Buffer containing Normal Guinea Pig Serum as a carrier. Used to hydrate ^{125}I-Canine C-Peptide.
Quantity: 13.5 mL/vial
Preparation: Ready to use

E. Canine C-Peptide Calibrator
Purified Canine C-Peptide in Assay Buffer
Lyophilized for stability
Quantity: 2 mL/vial upon hydration
Preparation: Hydrate with 2 mL deionized/distilled water.
Note: The actual calibrator concentration of Canine C-Peptide present in the vial will be lot dependent.
Please refer to the Canine C-Peptide analysis sheet for exact Canine C-Peptide calibrator concentration present in a specific lot.

F. Quality Controls 1 & 2
Purified Canine C-Peptide in Assay Buffer
Quantity: Lyophilized, 1 mL/vial upon hydration
Preparation: Hydrate with 1 mL deionized/distilled water.

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: 130 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. Reconstituted Canine C-Peptide Calibrator should be stored 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 there from, 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 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.

V. REAGENT PRECAUTIONS (continued)

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.

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 & 1.0 mL 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 are 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[2]. Use no more than 10 IU heparin per mL of blood collected.
3. Canine C-Peptide must be protected from proteolysis during assay procedures and sample storage. Trasylol (Aprotinin) at a concentration of 500 KIU per mL of serum or plasma should be added to samples to protect from proteolysis.
4. 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 $\leq$ -20°C. Avoid multiple (>5) freeze/thaw cycles.
5. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

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

A. Canine C-Peptide Calibrator Preparation

Use care in opening the lyophilized calibrator vial. Using a pipette, reconstitute the Canine C-Peptide Calibrator with 2 mL distilled or deionized water to give a concentration described in the analysis sheet. Invert and mix gently, let sit for five minutes then mix well.

Label seven glass tubes 1, 2, 3, 4, 5, 6, and 7. Add 0.5 mL Assay Buffer to each of the seven tubes. Prepare serial dilutions by adding 0.5 mL of the reconstituted calibrator to tube 1, mix well and transfer 0.5 mL from tube 1 to tube 2, mix well and transfer 0.5 mL from tube 2 to tube 3, mix well and transfer 0.5 mL from tube 3 to tube 4, mix well and transfer 0.5 mL from tube 4 to tube 5, mix well and transfer 0.5 mL from tube 5 to tube 6, mix well and transfer 0.5 mL from tube 6 to tube 7 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤-20°C. Avoid multiple freeze/thaw cycles.

	Volume of Deionized Water to Add	Volume of Calibrator to Add	Calibrator Concentration ng/mL
	2 mL	0	X (Refer to calibrator insert sheet for exact concentration)

Tube #	Volume of Assay Buffer to Add	Volume of Calibrator to Add	Calibrator Concentration ng/mL
1	0.5 mL	0.5 mL of reconstituted calibrator	X/2
2	0.5 mL	0.5 mL of tube 1	X/4
3	0.5 mL	0.5 mL of tube 2	X/8
4	0.5 mL	0.5 mL of tube 3	X/16
5	0.5 mL	0.5 mL of tube 4	X/32
6	0.5 mL	0.5 mL of tube 5	X/64
7	0.5 mL	0.5 mL of tube 6	X/128

B. Canine C-Peptide Quality Control 1 and 2 Preparation

Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute Quality Control 1 and Quality Control 2 with **1 mL** distilled or deionized water. Invert and mix gently, let sit for five minutes or until completely dissolved then mix well.

III. ASSAY PROCEDURE (continued)

C. Assay Set-Up, Day One

1. Pipet 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µL to Reference (Bo) tubes (5-6), and 100 µL in tubes 7 through the end of the assay.
2. Pipet 100 µL of Calibrators and Quality Controls in duplicate (see flow chart).
3. Pipet 100 µL of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Canine 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. Pipet 100 µL of hydrated ^{125}I- Canine C-Peptide to all tubes. Important: For preparation, see Section III, Part C.
5. Pipet 100 µL of Canine C-Peptide Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

D. Day Two

7. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes (except Total Count Tubes).
8. Vortex and incubate 20 minutes at 4°C.
9. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, 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

10. Immediately decant the supernatant of all tubes except Total Count tubes (1-2), drain tubes for at least 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.
11. Count all tubes in a gamma counter for 1 minute each. Calculate the ng/mL of Canine C-Peptide in unknown samples using automated data reduction procedures.

Assay Procedure Flow Chart

Day One						Day Two	
Set-up	Step 2	Steps 3-4	Step 5	Step 6	Step 7	Step 8	Steps 9-12
Tube #	Add Assay Buffer	Add Calibrator / QC / Sample	Add I-125 Canine C-Peptide Tracer	Add Canine C-Peptide Antibody	**Vortex, Cover, and Incubate 20-24 hrs at 4°C**	Add Precipitating Reagent	**Vortex and 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 tube 7	100 µl	100 µl		1.0 mL	
9,10	100 µl	100 µl of tube 6	100 µl	100 µl		1.0 mL	
11,12	100 µl	100 µl of tube 5	100 µl	100 µl		1.0 mL	
13,14	100 µl	100 µl of tube 4	100 µl	100 µl		1.0 mL	
15,16	100 µl	100 µl of tube 3	100 µl	100 µl		1.0 mL	
17,18	100 µl	100 µl of tube 2	100 µl	100 µl		1.0 mL	
19,20	100 µl	100 µl of tube 1	100 µl	100 µl		1.0 mL	
21,22	100 µl	100 µl of reconstituted calibrator	100 µl	100 µl		1.0 mL	
23,24	100 µl	100 µl of QC 1	100 µl	100 µl		1.0 mL	
25,26	100 µl	100 µl of QC 2	100 µl	100 µl		1.0 mL	
27, n	100 µl	100 µl of unknown	100 µl	100 µl			

IX. CALCULATIONS

A. Explanation

The calculations for Canine 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 30-50%.

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 ng/mL of Canine 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.

ASSAY CHARACTERISTICS

A. Sensitivity

The limit of sensitivity for the Canine C-Peptide assay is 0.15 ng/mL (100 µL sample size).

B. Performance

The following parameters of assay performance are expressed as Mean ± 3 Standard deviations.

ED_{80}=0.56 ± .15 ng/mL

ED_{50}=2.03 ± .54 ng/mL

ED_{20}=7.21 ± 2.76 ng/mL

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.

Canine C-Peptide	100%
Human C-Peptide	15%
Rat C-Peptide	ND
Human Proinsulin	ND
Porcine Proinsulin	ND
Bovine Proinsulin	ND
Human Insulin	ND
Glucagon	ND

ND- Not Detectable

D. Precision

Within and Between Assay Variation

Sample number	Mean Canine C-Peptide level (ng/mL)	Assay Variation (%CV)
		Intra-assay
1	1.07	3.06
2	4.68	3.33
		Inter-assay
3	1.26	6.91
4	4.84	5.61

XI. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike and Recovery of Canine C-Peptide in Canine serum

Serum Sample #	Canine C-Peptide Added (ng/mL)	Observed (ng/mL)	% of Recovery
1	0	0.16	100
	1.2	1.32	97.06
	5	5.24	101.55
	10	10.12	99.61
2	0	0.20	100
	1.2	1.40	100
	5	5.24	100.77
	10	10.92	107.06
3	0	0.08	100
	1.2	1.28	100
	5	5.2	102.36
	10	11.2	111.11

Varying concentrations of Canine C-Peptide were added to three different canine serum samples and the Canine C-Peptide content was determined by RIA. Mean of the observed levels from duplicate determinations in one assay are shown. Percent recovery was calculated as the observed over expected multiplied by 100.

F. Linearity

Effect of Serum Dilution

Sample No.	Volume sampled	Observed ng/mL	Expected ng/mL	% Expected
1	100 µl	9.8	9.8	100
	50 µl	4.96		101
	25 µl	2.32		95
2	100 µl	11.04	11.04	100
	50 µl	5.56		101
	25 µl	2.68		97
3	100 µl	11.28	11.28	100
	50 µl	5.68		101
	25 µl	2.68		95

Canine Serum containing varying concentrations of Canine C-Peptide were analyzed in the volumes indicated. Dilution factors of 1, 2, and 4 representing 100 µL, 50 µL, and 25 µL, respectively, were applied in calculating observed concentrations.

XI. ASSAY CHARACTERISTICS (continued)

G. 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	ng/mL
1	Totals	16031	16131			
2	"	16231				
3	NSB	426	413			
4	"	399				
5	Bo	6854	6892	6479		
6	"	6930				
		Standards				
7	0.156 ng/mL	6394	6499	6086	93.9	
8		6603				
9	0.313 ng/mL	6279	6264	5851	90.3	
10		6248				
11	0.625 ng/mL	5438	5442	5029	77.6	
12		5445				
13	1.25 ng/mL	4740	4678	4265	65.8	
14		4616				
15	2.5 ng/mL	3494	3464	3051	47.1	
16		3433				
17	5.0 ng/mL	2344	2251	1838	28.4	
18		2157				
19	10.0 ng/mL	1505	1440	1027	15.8	
20		1374				
21	20.0 ng/mL	899	896	483	7.5	
22		893				
		Controls/Unknowns				
23		4543	4472	4059	62.7	1.38
24		4401				
25		2237	2283	1870	28.9	4.95
26		2329				

XII. NORMAL FASTING RANGE

Normal fasting range:

To Be Established

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control 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[3]):

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

Reagents	**Cat. #**
^{125}I-Canine C-Peptide (<1.5 µCi, 56 kBq)	9024-HK
Label Hydrating Buffer (13.5mL)	LHB-PHK
Canine C-Peptide Calibrator (Lyophilized)	8024-K
Canine C-Peptide Antibody (13 mL)	1024-HK
Precipitating Reagent (130 mL)	PR-UVHK
QC 1&2 (Lyophilized)	6000L-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. 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.

2. Thorell, J.I. *Scand J. Clin. Lab. Invest.* 31:187, 1973

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