



Human C-Peptide

250 Tubes

Cat. # HCP-20K

**HUMAN C-PEPTIDE RIA KIT
250 TUBES (Cat. # HCP-20K)**

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HUMAN C-PEPTIDE RIA KIT 250 TUBES (Cat. # HCP-20K)

I. INTENDED USE

EMD Millipore's Human C-Peptide radioimmunoassay kit is for the quantitative determination of Human C-Peptide in serum, plasma, and other tissue culture media. It is a completely homologous assay since the antibody was raised against purified Human C-Peptide and both the tracer and the standard are prepared with Human C-Peptide. This assay has a very low crossreactivity (<4.0%) to Human Proinsulin. ***For Research Use Only. Not for Use in Diagnostic Procedures.***

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 EMD Millipore Human C-Peptide assay utilizes ¹²⁵I-labeled Human C-Peptide and a Human 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, 1% RIA Grade BSA
Quantity: 40 mL/vial
Preparation: Ready to use

B. Human C-Peptide Antibody

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

C. ¹²⁵I-Human C-Peptide

¹²⁵I-Human C-Peptide Label, HPLC purified (specific activity 658 $\mu\text{Ci}/\mu\text{g}$)
Lyophilized for stability. Freshly iodinated label contains $<3 \mu\text{Ci}$ (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 set 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 ¹²⁵I-Human C-Peptide.
Quantity: 27 mL/vial
Preparation: Ready to use

E. Human C-Peptide Standards

Purified Human C-Peptide in Assay Buffer at the following concentration: 5 ng/mL
Quantity: 3 mL/vial
Preparation: Ready to use

F. Quality Controls 1 & 2

Purified Human 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

Upon receipt, unused kit may be stored between 2 and 8°C for short term storage. For prolonged storage (>2 weeks), freeze unused kit at $\leq -20^\circ\text{C}$. Lyophilized components upon hydration should be stored at $\leq -20^\circ\text{C}$ immediately after use, or discarded. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at $\leq -20^\circ\text{C}$. Do not mix reagents from different kits unless they have the same lot number and are unopened.

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 (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.

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. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full labels of hazardous components in this kit:

Ingredient, Cat #		Full Label	
Guinea Pig anti Human C-Peptide Antibody	1020-K		Warning. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Human C-Peptide Standard	8020-K		Warning. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
125I-Human C-Peptide	9020	 	Danger. Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention.
Precipitating Reagent	PR-UV		Warning. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

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 μ L pipet with disposable tips
3. 100 μ L & 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 μ L per assay tube of serum or plasma can be used, although, 50 μ l 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. Human 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.

For unprotected samples there is a loss of approximately 25% HCP after 3 months storage at $\leq -20^{\circ}\text{C}$. No loss was observed when unprotected sample were stored at $\leq -70^{\circ}\text{C}$ for up to 12 months.
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 $\leq -20^{\circ}\text{C}$. Avoid multiple (>5) freeze/thaw cycles.
5. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

Standard Preparation

Use care in opening the Standard vial.

Label five glass tubes 1, 2, 3, 4, and 5. Add 1.0 mL Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 1.0 mL of the 5.0 ng/mL standard to tube 1, mix well and transfer 1.0 mL of tube 1 to tube 2, mix well and transfer 1.0 mL of tube 2 to tube 3, mix well and transfer 1.0 mL of tube 3 to tube 4, mix well and transfer 1.0 mL of tube 4 to tube 5, 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 $\leq -20^{\circ}\text{C}$. Avoid multiple freeze/thaw cycles.

Tube #	Standard Concentration	Volume of Assay Buffer to Add	Volume of Standard to Add
1	2.5 ng/mL	1.0 mL	1.0 mL of 5.0 ng/mL
2	1.25 ng/mL	1.0 mL	1.0 mL of 2.5 ng/mL
3	0.625 ng/mL	1.0 mL	1.0 mL of 1.25 ng/mL
4	0.313 ng/mL	1.0 mL	1.0 mL of 0.625 ng/mL
5	0.156 ng/mL	1.0 mL	1.0 mL of 0.313 ng/mL

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

A. Day One

1. Pipet 200 μL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4) and, 100 μL to Reference (Bo) tubes (5-6).
2. Pipet 100 μL of Standards 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 Human 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 ^{125}I -Human C-Peptide to all tubes. Important: For preparation, see Section III, Part C.
5. Pipet 100 μL of Human 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 .

VIII. ASSAY PROCEDURE (continued)

B. 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 supernate 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. Calculate the ng/mL of Human C-Peptide in unknown samples using automated data reduction procedures.

Assay Procedure Flow Chart

Day One						Day Two		
Set-up	Step 1	Steps 2 & 3	Step 4	Step 5	Step 6	Step 7	Step 8	Steps 9-11
Tube Number	Add Assay Buffer	Add Standard / QC Sample	Add I-125 Human C-Peptide Tracer	Add Human C-Peptide Antibody	Vortex, Cover, and Incubate 20-24 hrs at 4°C	Add Precipitating Reagent	Vortex, cover, and Incubate 20 min. at 4°C	Centrifuge at 4°C for 20 min., Decant, and Count
1,2	-	-	100 µL	-		-		
3,4	200	-	100 µL	-		1.0 mL		
5,6	100	-	100 µL	100 µL		1.0 mL		
7,8	-	100 µL of 0.156	100 µL	100 µL		1.0 mL		
9,10	-	100 µL of 0.313	100 µL	100 µL		1.0 mL		
11,1	-	100 µL of 0.625	100 µL	100 µL		1.0 mL		
13,1	-	100 µL of 1.25	100 µL	100 µL		1.0 mL		
15,1	-	100 µL of 2.5	100 µL	100 µL		1.0 mL		
17,1	-	100 µL of 5.0	100 µL	100 µL		1.0 mL		
19,2	-	100 µL of QC 1	100 µL	100 µL		1.0 mL		
21,2	-	100 µL of QC 2	100 µL	100 µL		1.0 mL		
23-	-	100 µL of	100 µL	100 µL		1.0 mL		
25,n	-	100 µL of	100 µL	100 µL	1.0 mL			

IX. CALCULATIONS

A. Explanation

The calculations for Human 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: $(\text{Total Binding Counts}/\text{Total Counts}) \times 100$. This should be 35-50%.
4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
 $\%B/Bo = (\text{Sample or Standard}/\text{Total Binding}) \times 100$
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 Human C-Peptide in the unknown samples (unknowns 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 Human C-Peptide assay is 0.065 ng/mL + 2SD (100 μL sample size).
4. The limit of linearity for the Human C-Peptide assay is 5.0 ng/mL (100 μL sample size). Any result greater than 5.0 ng/mL should be repeated on dilution using Assay Buffer as a diluent.

XI. NORMAL FASTING RANGE

Normal fasting range:

0.5-1.5 ng/mL

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of C-Peptide that can be detected by this assay is 0.065 ng/mL + 2 SD when using a 100 µL sample size.

B. Performance

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

ED₈₀ = 0.3 ± 0.1 ng/mL

ED₅₀ = 1.2 ± 0.2 ng/mL

ED₂₀ = 4.9 ± 0.5 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.

Human C-Peptide	100%
Monkey C-Peptide	90%
Human Proinsulin	<4%
Human Insulin	ND
Glucagon	ND
Somatostatin	ND
Pancreatic Polypeptide	ND
Rat C-Peptide	ND
Porcine C-Peptide	ND
ND - not detectable	

D. Precision

Within and Between Assay Variation

Sample No.	Mean ng/mL	Within % CV	Between % CV
1	0.4	3.4	9.3
2	0.9	4.4	4.4
3	1.8	6.4	4.2
4	2.1	4.2	3.2
5	2.8	4.5	2.4

Within and between assay variations were performed on five human serum samples containing varying concentrations of Human C-Peptide. Data (mean and % CV) shown are from five duplicate determinations of each serum sample in five separate assays.

XII. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike and Recovery of C-Peptide in Human Serum

Sample No.	Human C-Peptide Added ng/mL	Observed ng/mL	Expected ng/mL	% Recovery
1	0	0.39	-	-
2	0.25	0.64	0.64	100
3	0.50	0.90	0.89	101
4	1.00	1.37	1.39	99
5	2.50	2.76	2.89	96

Varying concentrations of Human C-Peptide were added to five human serum samples and the Human C-Peptide content was determined by RIA. Mean of the observed levels from five duplicate determinations in five separate assays are shown. Percent recovery was calculated on the observed vs. expected.

F. Linearity

Effect of Serum Dilution

Sample #	Volume Sampled	Observed ng/mL	Expected ng/mL	% Of Expected
1	100 μ L	2.8	2.8	100
	75 μ L	2.8		100
	50 μ L	2.7		99
	25 μ L	2.5		92
2	100 μ L	2.1	2.1	100
	75 μ L	2.0		98
	50 μ L	2.0		97
	25 μ L	1.9		89
3	100 μ L	1.8	1.8	100
	75 μ L	1.7		96
	50 μ L	1.7		94
	25 μ L	1.5		83
4	100 μ L	1.8	1.8	100
	75 μ L	1.8		99
	50 μ L	1.8		99
	25 μ L	1.6		90

Aliquots of pooled human serum containing varying concentrations of Human C-Peptide were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2 and 4 representing 100 μ L, 75 μ L, 50 μ L and 25 μ L, respectively, were applied in calculating observed concentrations. Mean Human C-Peptide levels and percent of expected for five separate assays are shown.

XII. 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	17717	17737			
2	"	17756				-
3	NSB	415	402			
4	"	389				-
5	Bo	8592	8391	7989		
6	"	8190				-
<u>Standards</u>						
7	0.156 ng/mL	7094	7093	6691	0.838	
8		7092				-
9	0.313 ng/mL	6081	6090	5688	0.712	
10		6099				-
11	0.625 ng/mL	4817	4923	4521	0.566	
12		5029				-
13	1.25 ng/mL	3444	3493	3091	0.387	
14		3542				-
15	2.5 ng/mL	2409	2379	1977	0.247	
16		2349				-
17	5 ng/mL	1586	1571	1169	0.146	
18		1555				-
<u>Controls/Unknown</u>						
19	QC 1	6483	6325	5923	0.741	0.28
20		6167				
21	QC 2	3367	3433	3031	0.379	1.3
22		3499				
23-n	unknown					

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 EMD Millipore website emdmillipore.com using the catalog number as the keyword.

Recommended batch analysis decision using two controls (Westgard Rule)⁴:

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-Human C-Peptide (<3 uCi, 111 kBq)	9020
Label Hydrating Buffer (27mL)	LHB-P
Human C-Peptide Standards (3 mL each)	8020-K
Human C-Peptide Antibody (26 mL)	1020-K
Precipitating Reagent (260 mL)	PR-UV
QC 1&2 (1 mL each)	6000-K
Assay Buffer (40 mL)	AB-P

XV. ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at emdmillipore.com/msds.

XVI. REFERENCES

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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.