HUMAN C-PEPTIDE ELISA KIT
96-Well Plate (Cat. #EZHCP-20K)

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HUMAN C-PEPTIDE ELISA KIT
96-Well Plate (Cat. #EZHCP-20K)

I. INTENDED USE

This kit is for non-radioactive quantification of Human C-Peptide (HCP) in serum, plasma and other biological media. One kit is sufficient to measure 38 unknown samples in duplicate. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

This assay is based, sequentially, on: 1) capture of Human C-Peptide from samples by a monoclonal antibody immobilized to the wells of a microtiter plate, 2) binding of the biotinylated monoclonal HCP antibody to capture Human C-Peptide molecules, 3) wash away of unbound materials including free materials from samples and free detection antibody, 4) conjugation of SA-HRP (Poly-HRP-labeled streptavidin) enzyme to the biotinylated antibodies, and 5) quantification of bound detection conjugate by monitoring SA-HRP enzyme activity in the presence of TMB (tetramethylbenzidine) substrates. The enzyme activity is measured spectrophotometrically by the absorbency at 450 nm due to production of the photometric product. Since the amount of photometric product is directly proportional to the concentration of Human C-Peptide in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of HCP.

III. REAGENTS SUPPLIED

Each kit is sufficient to run one 96-well microtiter plate and contains the following reagents:

A. Human C-Peptide ELISA Plate
Coated with anti-Human C-Peptide Monoclonal Antibody
Quantity: 1 plate
Preparation: Ready to use
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C.

B. Adhesive Plate Sealer
Quantity: 1 Sheet
Preparation: Ready to use

C. 10X HRP Wash Buffer Concentrate
10X concentrate of 50 mM TBS Buffer containing 0.05% Tween 20
Quantity: 2 bottles containing 50 mL each
Preparation: Dilute 1:10 with deionized water

III. REAGENTS SUPPLIED (continued)

D. Human C-Peptide Standards
Human C-Peptide in Assay Buffer: 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL
Quantity: 0.5 mL/vial
Preparation: Ready to use

E. ELISA Human C-Peptide Quality Controls 1 and 2
Human C-Peptide in QC Buffer
Quantity: 0.5 mL/vial
Preparation: Ready to use

F. Matrix Solution
Quantity: 1 mL
Preparation: Ready to use

G. Assay Buffer
0.05M PBS containing 0.025 M EDTA, 1% BSA, with
0.08% Sodium Azide, with proprietary reagent, pH 7.4
Quantity: 8 mL
Preparation: Ready to use

H. Human C-Peptide Detection Antibody
Biotinylated anti-Human C-Peptide Monoclonal Antibody
Quantity: 3.0 mL
Preparation: Ready to use

I. Enzyme Solution
Pre-titered Streptavidin-Horseradish Peroxidase Conjugate (SA-HRP)
Quantity: 12 mL
Preparation: Ready to use

J. Substrate (TMB)
3, 3', 5, 5'-tetramethylbenzidine (TMB)
Quantity: 12 mL
Preparation: Ready to use

K. ELISA Stop Solution
0.3M HCl
Quantity: 12 mL
Preparation: Ready to use

IV. STORAGE AND STABILITY

All components of the kit should be stored at 2-8°C upon receipt. For prolonged storage (> 2 weeks), store the Wash Buffer, Standards and Matrix Solution at ≤ -20°C. Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8°C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

V. REAGENT PRECAUTIONS

A. Sodium Azide

Sodium Azide has been added to 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.

B. Hydrochloric Acid

Hydrochloric Acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eyes.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipets with tips, 10 µl-200 µl
2. Multi-channel pipette, 50 µl-300 µl
3. Buffer and Reagent Reservoirs
4. Vortex Mixer
5. Refrigerator
6. Deionized Water
7. Microtiter plate reader capable of reading absorbency at 450 nm
8. Microtiter Plate Shaker
9. Absorbent Paper or Cloth

VII. SAMPLE COLLECTION AND STORAGE

1. 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.

2. To prepare serum samples, whole blood is directly drawn into a Vacutainer® serum tube that contains no anticoagulant. Let blood clot at room temperature for 30 minutes. Promptly centrifuge the clotted blood at 2,000 to 3,000 x g for 15 minutes at 4 ± 2°C. Transfer and store serum samples in separate tubes.

3. Samples can be stored at 4°C if they will be tested within 3 hours of collection. For longer storage, specimens should be stored at ≤ -20°C. Avoid multiple (>3) freeze/thaw cycles. Aliquot samples before freezing if necessary.

4. To prepare plasma samples, whole blood should be collected into Vacutainer® EDTA-plasma tubes and centrifuged immediately after collection. Observe the same precautions in the preparation of serum samples.

5. If heparin is to be used as an anticoagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.

6. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

Pre-warm all reagents to room temperature (20-25°C) immediately before setting up the assay.

1. Dilute the concentrated 10X Wash Buffers 10 fold. Mix the entire contents of both bottles of 10X Wash Buffer with 900 mL distilled or deionized water.

2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8°C. Assemble the strips in an empty plate holder and fill each well with 300 µl of 1X Wash Buffer. Incubate at room temperature for 5 minutes. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step.

3. Wash the wells two additional times with 1X Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

4. Add 40 µl of Assay Buffer into each Blank, Standard, and QC well. Refer to Section IX for suggested well orientations.

5. Add 50 µl of the Assay Buffer into each Sample well.

6. Add 10 µl Assay Buffer to assay background well, i.e. the blank wells #A1 and #B1.

7. Add 10 µl of Matrix Solution into each Blank, Standard, and QC well.

8. Add 10 µl of Standards in duplicate into appropriate wells.

9. Add 10 µl of QC1 and QC2 in duplicate into appropriate wells.

10. Add 10 µl of serum or plasma samples in duplicate into appropriate wells.

11. Add 20 µl of the Detection Antibody into each well. **For best results all additions should be completed within one hour.**

12. Cover the plate with plate sealer.

13. Incubate at room temperature (20-25°C) for 2 hours *while shaking on a microtiter plate shaker.*

14. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the well.

15. Wash the wells 5 times with 1X Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

VIII. ASSAY PROCEDURE (continued)

16. Add 80 µl of the Enzyme Solution into each well.

17. Cover the plate with plate sealer. Incubate 30 minutes at room temperature *while shaking on a microtiter plate shaker.*

18. Wash the wells 5 times with 1X Wash Buffer, 300 µl per well per wash. Decant and tap after each wash to remove residual buffer.

19. Add 80 µl of the Substrate Solution to each well, cover plate with sealer and shake on the plate shaker for **approximately** 12 to 19 minutes. Blue color should be formed in wells of Human C-Peptide standards with intensity proportional to increasing concentrations of Human C-Peptide.

NOTE: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time.

20. Remove the plate sealer, and stop the reaction by adding 80 µl of Stop Solution into each well of the plate. Shake the plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.

21. Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. The absorbance of highest Human C-Peptide standard should be approximately 2.4 ~ 2.8

Assay Procedure for Human C-Peptide ELISA Kit (Cat. # EZHCP-20K)

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14	Step 14	Step 14	Step 14
Well #	**Dilute both bottles of 10X Wash Buffer with 900mL Deionized Water.**	**Add 300 µl Wash Buffer to plate and incubate at room temperature for 5 minutes. Remove residual buffer by tapping smartly on absorbent towels. Wash 2X with 300 µl Wash Buffer**	Assay Buffer	Matrix Solution	Standards/ Controls/ Samples	Detection Antibody	**Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 5X with 300 µl Wash Buffer**	Enzyme Solution	**Seal, Agitate, Incubate 30 minutes at Room Temperature . Wash 5X with 300 µl Wash Buffer**	Substrate	**Seal, Agitate, Incubate 12-19 minutes at Room Temperature.**	Stop Solution	**Read Absorbance at 450 nm.**
A1, B1			50 µl	10 µl	----------	20 µl		80 µl		80 µl		80 µl	
C1, D1			40 µl	10 µl	10 µl of 0.2 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
E1, F1			40 µl	10 µl	10 µl of 0.5 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
G1, H1			40 µl	10 µl	10 µl of 1 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
A2, B2			40 µl	10 µl	10 µl of 2 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
C2, D2			40 µl	10 µl	10 µl of 5 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
E2, F2			40 µl	10 µl	10 µl of 10 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
G2, H2			40 µl	10 µl	10 µl of 20 ng/mL Standard	20 µl		80 µl		80 µl		80 µl	
A3, B3			40 µl`	10 µl	10 µl of QC I	20 µl		80 µl		80 µl		80 µl	
C3, D3			40 µl	10 µl	10 µl of QC II	20 µl		80 µl		80 µl		80 µl	
E3, F3 ↓			50 µl	------	10 µl of Sample	20 µl		80 µl		80 µl		80 µl	

IX. MICROTITER PLATE ARRANGEMENT

Standard Human C-Peptide ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	2 ng/mL	EQC 1	etc…								
B	Blank	2 ng/mL	EQC 1	etc…								
C	0.2 ng/mL	5 ng/mL	EQC 2									
D	0.2 ng/mL	5 ng/mL	EQC 2									
E	0.5 ng/mL	10 ng/mL	Sample 1									
F	0.5 ng/mL	10 ng/mL	Sample 1									
G	1 ng/mL	20 ng/mL	Sample 2									
H	1 ng/mL	20 ng/mL	Sample 2									

X. CALCULATIONS

The dose-response curve of this assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function.

XI. ASSAY CHARACTERISTICS

Sensitivity

The lowest level of Human C-Peptide that can be detected by this assay is 0.2 ng/mL.

Crossreactivity

The specificity of the Human C-Peptide ELISA is the ability to selectively measure the analytes in the presence of other like components in the sample matrix.

Human C-Peptide	100%
Intact Human Proinsulin	63%
Human Insulin	n.d.*
Porcine C-Peptide	n.d.*
Rat C-Peptide	n.d.*
Canine C-Peptide	n.d.*

n.d.: Not detectable at concentrations up to 200 ng/mL.

Precision

Within and Between Assay Variation

Sample No.	Mean HCP Levels ng/mL	Within % CV	Between % CV
1	1	4.75	8.72
2	3	2.95	5.00
3	7	1.60	----

The between assay variation of Millipore Human C-Peptide ELISA kits were studied using two serum samples with varying concentrations of Human C-Peptide. The mean variation of each sample was calculated using results from eight separate assays with duplicate samples in each assay.

XI. ASSAY CHARACTERISTICS (continued)

Recovery

Spike & Recovery of Human C-Peptide in Human Serum

Sample #	HCP added ng/mL	Expected ng/mL	Observed ng/mL	% of Recovery
1	0	2.31	2.31	100
	0.5	2.81	2.83	104
	2.0	4.31	4.21	95
	5.0	7.31	7.70	108
2	0	1.68	1.68	100
	0.5	2.18	2.19	101
	2.0	3.68	3.57	93
	5.0	6.68	6.77	102
3	0	2.05	2.05	100
	0.5	2.55	2.50	98
	2.0	4.05	3.92	91
	5.0	7.05	6.93	97
4	0	3.84	3.84	100
	0.5	4.34	4.34	100
	2.0	5.84	5.87	102
	5.0	8.84	9.34	111

Varying concentrations of Human C-Peptide were added to four human serum samples and the Human C-Peptide content was determined by ELISA. Mean of the observed levels from four duplicate determinations are shown.
Percent recovery = observed ÷ expected x 100%.

XI. ASSAY CHARACTERISTICS (continued)

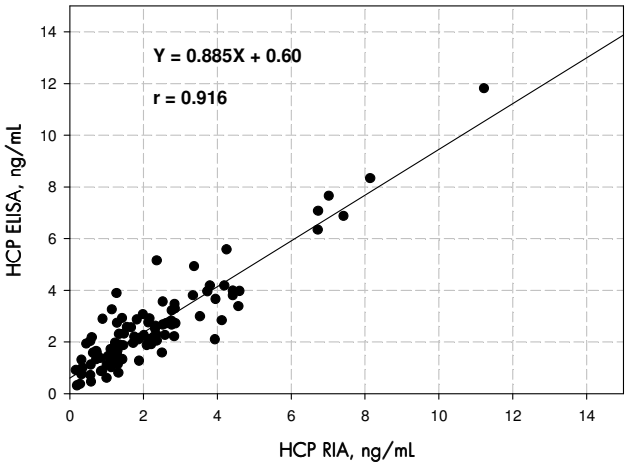
Linearity

Effect of Serum Dilution

Sample No.	Sample Dilution	Expected ng/mL	Observed ng/mL	% of Expected
1	0	3.38	3.38	100
	2	1.69	1.83	108
	4	0.85	0.88	104
	8	0.42	0.43	102
2	0	7.24	7.24	100
	2	3.62	3.17	88
	4	1.81	1.65	92
	8	0.91	0.88	98
3	0	8.42	8.42	100
	2	4.21	3.60	86
	4	2.11	2.10	100
	8	1.05	1.01	96

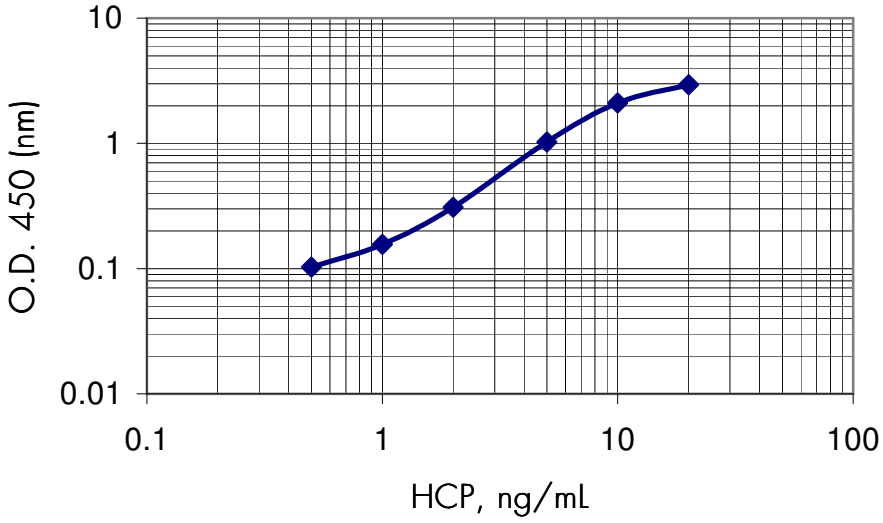
Dilutions of human sera containing varying concentrations of Human C-Peptide were analyzed. The mean Human C-Peptide level and percent of expected from four duplicates determinations are shown.

XII. CORRELATION GRAPH OF HCP ELISA vs RIA



Note: One hundard human serum samples were analized using this HCP ELISA kit and Millipore's HCP RIA kit (Cat #: HCP-20K).

XIII. Human C-Peptide Standard Curve



XIV. QUALITY CONTROLS

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.

XV. TROUBLESHOOTING GUIDE

Low or No Signal with Standards

* Standards were left at room temperature. Standards should be stored at -20ºC.
* Insufficient time for reaction with substrate. Allow substrate to react longer.
* Kit reagents have expired.
* Inadequate plate washing after sample incubation.
* Too much washing after conjugate incubation can reduce signal.

High Background

* Inadequate plate washing. After conjugate incubation, tap out plate on absorbent towels after decanting.
* Cross contamination between neighboring wells.

Samples too High

* Dilute sample with assay buffer to bring HCP concentration within standard range.

Signal too High on Highest Standard

* Plate incubated too long with substrate. Discard substrate, wash plate once and add freshly prepared substrate. Check RFU in less time.

High Variance in RFU of Duplicates

* Cross contamination in wells
* Bubbles in substrate at time of reading
* Loss of reagent or faulty pipetting in duplicates

XVI. REPLACEMENT REAGENTS

Reagents	**Catalogue #**
Human C-Peptide ELISA Plate with Plate Sealers	EP20
10X HRP Wash Buffer Concentrate	EWB-HRP
Human C-Peptide Standards (0.5 mL/vial)	E8020-K
ELISA HCP Quality Controls 1 & 2 (0.5 mL/vial)	E6020-K
Assay Buffer (8 mL/vial)	EABIR-2
Human C-Peptide Detection Antibody (3 mL/vial)	E1020
Enzyme Solution (12 mL/vial)	EHRP
Substrate (12 mL/vial)	ESS-TMB
Matrix Solution (1 mL/vial)	EMTX-CP
Stop Solution (12 mL/vial)	ET-TMB

XVII. 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

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 human 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.

XVIII. REFERENCES

1. Tijsen P. "Practice and Theory of Enzyme Immunoassays" in Burdon RH and Knippenberg PH (Ed.), Laboratory Techniques in Biochemisrty and Molecular Biology. Amsterdam/NY: Elsevier, 1985

2. O'Rahilly S & Moller DE *1992 Clin Endocrinol 36:121.*

3. Reaven GM *1991 Diab Care 14:195.*

4. Fontbonne AM & Eschwege EM *1991 Diab Care 14:461.*

5. Horwitz DL et al., *1976 New Engl J Med 295:207.*

6. Bommen M et al., *1984 Arch Dis Child 59:1096..*

7. Field JB *1989 Endocrinol Metab Clin North Am 18:27.*