User Guide

Human C-Peptide ELISA

96-Well Plate

EZHCP-20K
EZHCP-20BK

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 Use Only. Not for use in Diagnostic Procedures.

Principles of Assay

This assay is a based sequentially on:

- Capture of Human C-Peptide from samples by a monoclonal antibody immobilized to the wells of a microtiter plate
- Binding of the biotinylated monoclonal HCP antibody to capture Human C-Peptide molecules
- Wash away of unbound materials including free materials from samples and free detection antibody
- Conjugation of SA-HRP (Poly-HRP-labeled streptavidin) enzyme to the biotinylated antibodies
- 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.

Reagents Supplied

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

Note: Store all reagents at 2-8 °C

Reagents Supplied	Volume	Quantity	Cat. No.
Human C-Peptide ELISA Plate with 2 plate sealers **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2–8 °C	-	1 plate 2 sealers	EP20
Human C-Peptide Standards Human C-Peptide in Assay Buffer: 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL **Note:** The standard(s) in this kit have been calibrated to an International Reference standard, NIBSC cod #84/510, Version 03.	0.5 mL/vial	7 vials	E8020-K
Human C-Peptide Quality Controls 1 and 2	0.5 mL/vial	1 vial each	E6020-K
Matrix Solution	1 mL	1 vial	EMTX-CP
Assay Buffer	8 mL	1 vial	EABIR-2
10X Wash Buffer	50 mL	2 bottles	EWB-HRP
Human C-Peptide Detection Antibody	3 mL	1 bottle	E1020
Enzyme Solution	12 mL	1 bottle	EHRP
Substrate Solution	12 mL	1 bottle	ESS-TMB
ELISA Stop Solution (**Caution:** Corrosive Solution)	12 mL	1 bottle	ET-TMB

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8°C. Once opened, liquid standards and controls can be stored up to 30 days 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.

Reagent Precautions

Sodium Azide

Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, 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.

Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. Harmful if swallowed. Causes respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

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

Symbol Definitions

Ingredient	Cat. No.	Full Label	
Human C-Peptide Detection Antibody	E1020		**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 Standards	E8020-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 Quality Control 1 and 2	E6020-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.
Stop Solution	ET-TMB		**Warning:** May be corrosive to metals.
10X HRP Wash Buffer Concentrate	EWB-HRP		**Warning:** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Materials Required

(Not Provided)

- Multi-channel Pipettes and pipette tips: 50-300 µL
- Pipettes and pipette tips: 10 µL-200 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- Refrigerator
- De-ionized water
- Microtiter Plate Reader capable of reading absorbency at 450 nm
- Microtiter Plate Shaker
- Absorbent Paper or Cloth

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.

Human C-Peptide ELISA Assay Procedure

Warm all reagents to room temperature 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. 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 Microtiter Plate Arrangement 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, for example 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.
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. Wipe the bottom of the microtiter plate to remove any residue prior to reading on plate reader. Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there are 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

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 8-10	Step 11	Step 11-13	Step 14		
Well #	Dilute both bottles of 10X Wash Buffer with 900 mL 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/ QCs/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 mins 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								
E1, F1			40 µL	10 µL	10 µL of 0.5 ng/mL Standard								
G1, H1			40 µL	10 µL	10 µL of 1 ng/mL Standard								
A2, B2			40 µL	10 µL	10 µL of 2 ng/mL Standard								
C2, D2			40 µL	10 µL	10 µL of 5 ng/mL Standard								
E2, F2			40 µL	10 µL	10 µL of 10 ng/mL Standard								
G2, H2			40 µL	10 µL	10 µL of 20 ng/mL Standard								
A3, B3			40 µL	10 µL	10 µL of QC 1								
C3, D3			40 µL	10 µL	10 µL of QC 2								
E3, F3			50 µL	-	10 µL of Sample								

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									

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.

Assay Characteristics

Sensitivity

The lowest level of Human C-Peptide that can be detected by this assay is 0.05 ng/mL.

Cross Reactivity

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	-
Porcine C-Peptide	n.d.*
Rat C-Peptide	n.d.*
Canine C-Peptide	n.d.*
Human C-Peptide	n.d.*

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

Precision

Intra- and Inter- Assay Variations

Sample No.	Mean HCP Levels (ng/mL)	Intra-Assay Variations % CV	Inter-Assay Variations % CV
1	1	4.75	8.72
2	3	2.95	5.00
3	7	1.60	-

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

Recovery

Spike and Recovery of Human C-Peptide in Human Serum

Sample No.	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%.

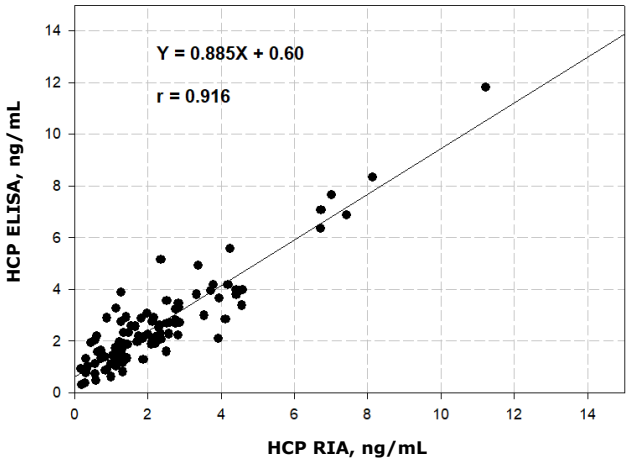
Linearity

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

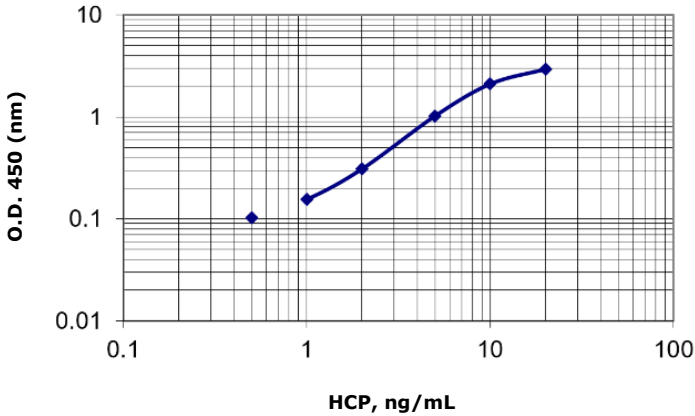
Dilutions of human sera containing varying concentrations of Human C-Peptide were analyzed. The mean Human C-Peptide level and percent of expected from four duplicates determinations are shown.

Correlation Graph of HCP ELISA vs. RIA



Note: One hundred human serum samples were analyzed using this HCP ELISA kit and HCP RIA kit (Cat No. HCP-20K).

Human C-Peptide Standard Curve



Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

Low or No Signal with Standards

- Standards were left at room temperature. Standards should be stored at −20 °C.
- 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

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Cat. No.
ELISA Plate	EP20
10X HRP Wash Buffer Concentrate	EWB-HRP
Human C-Peptide ELISA Standard	E8020-K
Human C-Peptide Quality Control 1 and 2	E6020-K
Matrix Solution	EMTX-CP
Assay Buffer	EABIR-2
Human C-Peptide Detection Antibody	E1020
Enzyme Solution	EHRP
Substrate Solution	ESS-TMB
Stop Solution	ET-TMB
10-pack of Human C-Peptide (HCP) ELISA Kits	EZHCP-20BK

References

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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.

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20142583 Rev 09/25