



Read entire protocol before use.

C-PEP-Elisa

Bio-Line S.A. - Rue André Fauchille.17 - B-1150 Bruxelles - Belgium

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of human C-Peptide (C-PEP) in serum.

II. GENERAL INFORMATION

- A. Name: Bio-Line **C-Peptide-Elisa** Kit
- B. Catalogue number: **BL-30-E**: 96 tests
- C. Manufactured by: Bio-Line S.A.
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III. CLINICAL BACKGROUND

A. Biological Activity

Insulin is synthesized in the beta-cells of the islets of Langerhans as a precursor molecule, proinsulin. In the secretory granules of the beta-cells, proinsulin is cleaved into insulin and into a 31-amino-acid peptide, called the Connecting Peptide or C-Peptide. Insulin and C-Peptide are secreted in equimolar amounts. However, because of its longer half-life, the plasma concentration of C-peptide is higher than that of insulin. The determination of plasma C-Peptide allows an assessment of the endogenous insulin production, even in the presence of exogenous insulin administration or in the presence of circulating anti-insulin antibodies. Moreover, the determination of C-Peptide in urine provides a reliable index of the insulin production when blood sampling is difficult or when an integrated estimation of C-Peptide secretion over a period of several hours is requested.

B. Clinical applications

- . Assessment of residual beta-cell function in diabetics under insulin therapy
- . Detection and monitoring of the remission phase of type I diabetes
- . Adjunct in the differential diagnosis between type I (insulin-dependent) and type II (non insulin-dependent) diabetes
- . Diagnosis of insulin-induced factitious hypoglycaemia
- . Contribution to the diagnosis of insulinoma (insulin suppression test)
- . Prognostic index of foetal outcome in pregnant diabetic women
- . Evaluation of insulin secretion in liver disease
- . Monitoring of pancreatectomy

IV. PRINCIPLES OF THE METHOD


The Bio-Line C-PEP-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on a microtiterplate. A fixed amount of C-PEPTIDE labelled with horseradish peroxidase (HRP), compete with unlabelled C-PEPTIDE present in the calibrators, controls and samples for a limited number of binding sites on a specific antibody.

After 2 hours incubation at room temperature, the microtiterplate is washed to stop the competition reaction.

The chromogenic solution (TMB – H₂O₂) is added and incubated for 30 min. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is inversely proportional to the C-PEPTIDE concentration.

A calibration curve is plotted and C-PEPTIDE concentration in samples is determined by interpolation from the calibration curve.

V. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution			
 Microtiterplate with 96 anti C-PEPTIDE (monoclonal antibodies) coated	96 wells	blue	Ready for use			
<table border="1" data-bbox="65 846 296 891"> <tr> <td>Ag</td> <td>HRP</td> <td>CONC</td> </tr> </table> Conjugate: HRP labelled C-PEPTIDE (HPLC grade) in TRIS-HCl buffer with bovine serum albumin and thymol	Ag	HRP	CONC	1 vial 0.2 ml	red	Dilute 100 x with conjugate buffer
Ag	HRP	CONC				
<table border="1" data-bbox="65 1025 260 1070"> <tr> <td>CONJ</td> <td>BUF</td> </tr> </table> Conjugate buffer : in TRIS-MALEATE buffer with bovine casein and thymol	CONJ	BUF	1 vial 5.5 ml	red	Ready for use	
CONJ	BUF					
<table border="1" data-bbox="65 1193 196 1238"> <tr> <td>CAL</td> <td>0</td> </tr> </table> Zero calibrator in human serum and thymol	CAL	0	1 vial lyophilized	yellow	Add 2.0 ml distilled water	
CAL	0					
<table border="1" data-bbox="65 1317 204 1361"> <tr> <td>CAL</td> <td>N</td> </tr> </table> Calibrator N = 1 to 5 (see exact values on vial labels) in human serum and thymol	CAL	N	5 vials lyophilized	yellow	Add 1.0 ml distilled water	
CAL	N					
<table border="1" data-bbox="65 1485 260 1529"> <tr> <td>WASH</td> <td>SOLN</td> <td>CONC</td> </tr> </table> Wash Solution (Tris-HCl)	WASH	SOLN	CONC	1 vial 10 ml	brown	Dilute 200 x with distilled water (use a magnetic stirrer).
WASH	SOLN	CONC				
<table border="1" data-bbox="65 1608 233 1653"> <tr> <td>CONTROL</td> <td>N</td> </tr> </table> Controls - N = 1 or 2 in human serum with thymol	CONTROL	N	2 vials lyophilized	silver	Add 0.5 ml distilled water	
CONTROL	N					
<table border="1" data-bbox="65 1731 260 1776"> <tr> <td>CHROM</td> <td>TMB</td> </tr> </table> Chromogen TMB (Tetramethylbenzidine)	CHROM	TMB	1 vial 25 ml	white	Ready for use	
CHROM	TMB					
<table border="1" data-bbox="65 1865 260 1910"> <tr> <td>STOP</td> <td>SOLN</td> </tr> </table> Stop solution: HCl 1.0N	STOP	SOLN	1 vial 25 ml	white	Ready for use	
STOP	SOLN					

- Note:** 1. Use the zero calibrator for sample dilutions.
2. 1 ng of the calibrator is equivalent to 1 ng of the IRR 84/510.

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

- High quality distilled water
- Pipettes for delivery of: 50 µl, 100 µl, 200 µl, 500 µl, 1 ml and 2 ml (the use of accurate pipettes with disposable plastic tips is recommended)
- Vortex mixer
- Magnetic stirrer
- Horizontal microtiterplate shaker capable of 700 rpm ± 100 rpm
- Washer for microtiterplates
- Microtiterplate reader capable of reading at 450 nm and 650 nm (or 630 nm)

VII. REAGENT PREPARATION

- Calibrators** : Reconstitute the zero calibrator with 2.0 ml distilled water and the other calibrators with 1.0 ml distilled water.
- Controls** : Reconstitute the controls with 0.5 ml distilled water.
- Working C-Peptide-HRP Conjugate** : pipette 50 µl of the conjugate (concentrated C-Peptide-HRP solution) into 5 ml of conjugate buffer for 96 wells used. Extemporaneous preparation is recommended
- Working Wash solution** : Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kit components are stable until the expiry date, indicated on the vial label, if kept at 2 to 8°C.
- Unused strips must be stored, at 2-8°C, in a sealed bag containing a desiccant until expiration date.
- After reconstitution, calibrators and controls are stable for 1 week at 2 to 8°C. For longer storage periods, aliquots should be made and kept at -20°C. Avoid successive freeze thaw cycles.
- The concentrated Wash Solution is stable at room temperature until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- The Working C-PEPTIDE-HRP conjugate is stable for 18 hours at 2 to 8°C.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- Serum must be kept at 2 - 8°C.
- If the test is not run within 24 hours, storage in aliquots at -20°C is recommended. Avoid subsequent freeze thaw cycles.
- Prior to use, all samples should be at room temperature. It is recommended to vortex the samples before use.
- Do not use haemolysed samples.

X. PROCEDURE

A. Handling notes

- Do not use the kit or components beyond expiry date.
- Do not mix materials from different kit lots.
- Bring all the reagents to room temperature prior to use.
- Thoroughly mix all reagents and samples by gentle agitation or swirling.
- Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
- Use a clean plastic container to prepare the Wash Solution.
- In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
- For the dispensing of the Chromogenic Solution and the Stop Solution avoid pipettes with metal parts.
- High precision pipettes or automated pipetting equipment will improve the precision.
- Respect the incubation times.
- To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section XIII paragraph E (Time delay).
- Prepare a calibration curve for each run, do not use data from previous runs.
- Dispense the Chromogenic Solution within 15 minutes following the washing of the microtiterplate.
- During incubation with Chromogenic Solution, avoid direct sunlight on the microtiterplate.

B. Procedure

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8 °C.
2. Secure the strips into the holding frame.
3. Pipette 100 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 50 µl of diluted anti-C-PEPTIDE-HRP conjugate into all the wells.
5. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
 - dispensing 0.4 ml of Wash Solution into each well
 - aspirating the content of each well
8. Pipette 100 µl of the chromogenic solution into each well within 15 minutes following the washing step.
9. Incubate the microtiterplate for 30 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
10. Pipette 100 µl of Stop Solution into each well.
11. Read the absorbencies at 450 nm (reference filter 630 nm or 650 nm) within 3 hours and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. Calculate for each calibrator, control and sample:

$$B/B_0 (\%) = \frac{OD (\text{Calibrator, Control or Sample})}{OD (\text{Zero Calibrator})} \times 100$$

4. Using either linear-linear or semi-logarithmic graph paper, plot the (B/B₀(%)) values for each calibrator point as a function of the C-PEPTIDE concentration of each calibrator point. Reject obvious outliers.
5. Computer assisted methods can also be used to construct the calibration curve. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.
6. By interpolation of the sample (B/B₀ (%)) values, determine the C-PEPTIDE concentrations of the samples from the calibration curve.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

C-PEP-ELISA		OD units
Calibrator	0 pmol/ml	1.796
	0.05 pmol/ml	1.513
	0.13 pmol/ml	1.216
	0.48 pmol/ml	0.786
	1.6 pmol/ml	0.404
	4.9 pmol/ml	0.196

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.01 pmol/ml.

B. Specificity

The specificity was estimated by spiking a pool of C-Peptide serum with concentration lower than 0.03 pmol/ml with the following peptides :

Compound	Quantity added (ng/ml)	Cross-reaction (%)
- Biosynthetic human proinsulin	6.25	12.50
- Purified porcine proinsulin	50.00	ND
- Monkey C-Peptide	6.00	30.00
- Pork C-Peptide	6000.00	0.03
- Pork Insulin	18000.00	0.01
- Pork Glucagon	1000.00	ND
- Beef Glucagon	1000.00	ND

ND : No interference Detected

C. Precision

INTRA ASSAY				INTER ASSAY			
Serum	N	<X> ± SD (pmol/ml)	CV (%)	Serum	N	<X> ± SD (pmol/ml)	CV (%)
A	20	0.19 ± 0.01	5.8	A	20	0.28 ± 0.03	9.2
B	20	0.59 ± 0.05	8.4	B	20	0.74 ± 0.05	7.2

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Sample	Added C-PEPTIDE (pmol/ml)	Recovered C-PEPTIDE (pmol/ml)	Recovery (%)
Serum	0.60	0.49	82
	1.20	1.16	97
	2.41	2.32	96
	4.12	4.02	98

DILUTION TEST

Sample	Dilution	Theoretical Concent. (pmol/ml)	Measured Concent. (pmol/ml)
Serum	1/1	-	4.13
	1/2	2.07	1.99
	1/4	1.03	1.07
	1/8	0.52	0.54
	1/16	0.26	0.24
	1/32	0.13	0.11

Samples were diluted with zero calibrator.

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls that contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values.

Identification	Number of subjects	Range pmol/ml)
Diabetes type I	32	0.01 – 0.28
Diabetes type II	41	0.43 – 2.60
Normal	41	0.28 - 2.00

The ranges are based on 2.5% to 97.5% percentiles.

XVI. PRECAUTIONS AND WARNINGS

Safety

For *in vitro* diagnostic use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl, the chromogen contains TMB and H₂O₂. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVII. BIBLIOGRAPHY

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (μ l)	SAMPLE(S) CONTROLS (μ l)
Calibrators (0-5) Controls, Samples Working Anti-C-PEPTIDE- HRP conjugate	100 - 50	- 100 50
Incubate for 2 hours at room temperature with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 3 times with 400 μ l of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 30 min at room temperature with continuous shaking at 700 rpm.		
Stop Solution	100	100
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm)		

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