

Read entire protocol before use.

Calcitonin (CT) - 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 Calcitonin (CT) in serum and plasma.

II. GENERAL INFORMATION

- A. Name: Bio-Line Calcitonin-ELISA Kit
- B. Catalogue number: BL-31-E: 96 tests
- C. Manufactured by: Bio-Line S.A.
Rue André Fauchille.17 - B-1150 Bruxelles - Belgium
- D. For technical assistance or ordering information contact:
Tel: +32-2-736.62.18. Fax: +32-2-742.13.15.

III. CLINICAL BACKGROUND

A Biological activities

Calcitonin(CT) is a 32 amino acid peptide hormone secreted by the para-follicular C-cells of the thyroid gland under serum calcium control. After acute administration, this peptide acts as a potent hypocalcemic and hypophosphatemic hormone by increasing renal calcium clearance and reducing bone resorption. However, its precise physiological role in bone metabolism is not yet fully understood.

Various forms of CT may be detected in blood samples, including a CT monomer, an oxidized monomer, a dimer, higher molecular weight forms, and possibly precursor of CT. The concentrations of these peptides vary with clinical status, renal function and tissular origin of CT (normal or ectopic production).

Medullary thyroid carcinoma (MTC) is a malignant tumor, developed from the C-cells, secreting calcitonin in large excess. This disease occurs either as a sporadic (80%) or a familial (20%) form, which is transmitted as an autosomal dominant gene or as a component of multiple endocrine neoplasia (IIb).

Moderate hypercalcitoninemia is also observed in pregnancy, pernicious anaemia, renal failure and during the neonatal period. Preferably, monomer form of CT is detected in this assay.

B Clinical Application


The measurement of CT is used for :

- Diagnosis of medullary thyroid carcinoma (MTC),
- Follow up of malignant tumors, to check the success of surgery and to monitor for recurrence,
- Diagnosis of the preclinical cases of the familial forms of MTC (MEN II or Sipple syndrome) by the use of stimulation tests (calcium or pentagastrin),
- Study of the pathophysiology of the calcium-phosphate and bone metabolism.

IV. PRINCIPLES OF THE METHOD

The Bio-Line Calcitonin Elisa a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. Calibrators and samples react with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAb 1 – human CT – MAb 2 – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB ready for use) is added and incubated. 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 proportional to the Calcitonin concentration. A calibration curve is plotted and Calcitonin concentration in samples is determined by interpolation from the calibration curve. The use of the EASIA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range.

V. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution			
 Microtiterplate with 96 anti CT (monoclonal antibodies) coated wells	96 wells	blue	Ready for use			
<table border="1" data-bbox="71 884 295 929"> <tr> <td>Ab</td> <td>HRP</td> <td>CONC</td> </tr> </table> Conjugate: HRP labelled anti-CT (monoclonal antibodies) in Stabilizing Buffer	Ab	HRP	CONC	1 vial 0.125 ml	red	Dilute 100 x with conjugate buffer
Ab	HRP	CONC				
<table border="1" data-bbox="71 1008 231 1052"> <tr> <td>CONJ</td> <td>BUF</td> </tr> </table> Conjugate buffer: TRIS-Maleate buffer with bovine serum albumin, EDTA and thymol	CONJ	BUF	1 vial 11 ml	red	Ready for use	
CONJ	BUF					
<table border="1" data-bbox="71 1131 215 1176"> <tr> <td>CAL</td> <td>N</td> </tr> </table> Calibrator N = 0 to 5 (see exact values on vial labels) in CT-Free human serum	CAL	N	6 vials lyophil.	yellow	Add 0.5 ml distilled water	
CAL	N					
<table border="1" data-bbox="71 1276 151 1321"> <tr> <td>SERUM</td> </tr> </table> CT free human serum (to be used for samples dilution) with thymol	SERUM	1 vial lyophil.	black	Add buffer (see reconstitution volume on the label)		
SERUM						
<table border="1" data-bbox="71 1377 151 1422"> <tr> <td>BUF</td> </tr> </table> Buffer (serum free): borate buffer	BUF	1 vial 8 ml	black	Ready for use		
BUF						
<table border="1" data-bbox="71 1467 271 1512"> <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="71 1556 239 1601"> <tr> <td>CONTROL</td> <td>N</td> </tr> </table> Controls - N = 1 or 2 in human serum with gentamycin	CONTROL	N	2 vials lyophil.	silver	Add 0.5 ml distilled water	
CONTROL	N					
<table border="1" data-bbox="71 1668 223 1713"> <tr> <td>CHROM</td> <td>TMB</td> </tr> </table> Chromogenic TMB solution (Tetramethylbenzidine)	CHROM	TMB	1 vial 25 ml	white	Ready for use	
CHROM	TMB					
<table border="1" data-bbox="71 1792 215 1836"> <tr> <td>STOP</td> <td>SOLN</td> </tr> </table> Stop Solution: HCl: 2N	STOP	SOLN	1 vial 25 ml	white	Ready for use	
STOP	SOLN					

Note: 1. CT free human serum is to be used for samples dilution.
2. 1 pg of our reference preparation is equivalent to 0.19 µIU NIBSC 89/620.

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 and 1 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 microtiterplate
- Microtiterplate reader capable of reading at 450 nm, 490 nm and 650 nm (in case of polychromatic reading) or capable of reading at 450 nm and 650 nm (monochromatic reading)
- Optional equipment: The ELISA-AID™ necessary to read the plate according to polychromatic reading (see paragraph XI.A.) can be purchased from Robert Maciels Associates, Inc. Mass. 02174 USA.

VII. REAGENT PREPARATION

- Calibrators** : Reconstitute the calibrators with 0.5 ml distilled water.
- Controls** : Reconstitute the controls with 0.5 ml distilled water.
- 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.
- CT Free Serum**: Reconstitute the CT Free Serum with the amount of Buffer as indicated on the vial label. Allow it to remain undisturbed until completely dissolved, and then mix well by gentle inversion.
- Working anti-CT-HRP conjugate**: Following the number of wells to be used, dilute the concentrated conjugate (100x) with the conjugate buffer in a clear glass vial according to the table below. Extemporaneous dilution is recommended.

Number of wells	Concentrated conjugate (100x) (ml)	Conjugate Buffer (ml)	Working Volume (ml)
8	0.01	1	1.01
16	0.02	2	2.02
24	0.03	3	3.03
32	0.04	4	4.04
48	0.05	5	5.05
96	0.10	10	10.1

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits 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, controls and CT free serum should be frozen immediately after use and kept at -20°C for 3 months. Only one freeze thawing cycle is allowed, discard the calibrators, controls and CT free serum after the second use.
- The concentrated Wash Solution is stable at room temperature until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, the concentrated conjugate (100x) is stable until expiry date, if kept in the original well-closed vial at 2 to 8°C.
- The Working anti-CT-HRP conjugate is stable for 1 week at 4°C.
- The chromogenic TMB solution and the Stop Solution are stable at 2°C to 8°C until the expiry date.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- Serum and plasma 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.
- Serum or EDTA plasma provides similar results.
 $Y(\text{serum}) = 0.84 \times (\text{EDTA plasma}) - 2.4 \text{ pg/ml}$ $r=0.90$ $n=15$
- Do not use haemolysed samples.
- Do not use lipemic 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.
 Prepare a calibration curve for each run, do not use data from previous runs.
 The chromogenic solution should be colourless. If a dark blue colour develops within a few minutes after preparation, this indicates that the preparation is unusable and must be discarded.
 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

- 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.
- Secure the strips into the holding frame.
- Pipette 50 µl of each Calibrator, Control and Sample into the appropriate wells.
- Pipette 100 µl of Working anti-CT-HRP conjugate into all the wells.
- Incubate for 18 ± 1 hour at 2-8 °C.
- Aspirate the liquid from each well.
- Wash the plate 4 times by:
 - Dispensing 0.4 ml of Wash Solution into each well
 - Aspirating the content of each well
- Pipette 100 µl of the Chromogenic solution into each well within 15 minutes following the washing step.
- Incubate the microtiterplate for 30 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
- Pipette 200 µl of Stop Solution into each well.
- Read the absorbances at 450 nm and 490 nm (reference filter 630 nm or 650 nm) within 3 hours and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

A. Polychromatic Reading:

- In this case, the ELISA-AID™ software will do the data processing.
- The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).
- A second reading is performed at 490 nm against the same reference filter.
- The ELISA-AID™ Software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.
- The principle of polychromatic data processing is as follows:
 - $X_i = OD$ at 450 nm
 - $Y_i = OD$ at 490 nm
 - Using a standard unweighted linear regression, the parameters A & B are calculated : $Y = A \cdot X - B$
 - If $X_i < 3$ OD units, then X calculated = X_i
 - If $X_i > 3$ OD units, then X calculated = $(Y_i - B)/A$
 - A 4-parameter logistic curve fitting is used to build up the calibration curve.
 - The CT concentration in samples is determined by interpolation on the calibration curve.

B. Bichromatic Reading

- Read the plate at 405 nm against a reference filter set at 650 nm (or 630 nm).
- Calculate the mean of duplicate determinations.
- On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of Calcitonin (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
- Read the concentration for each control and sample by interpolation on the calibration curve.

- Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.

XII. TYPICAL DATA

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

CT-U.S.-EASIA		Polychromatic model	Bichromatic model (OD)
Calibrator	0 pg/ml	0.100	0.039
	7 pg/ml	0.204	0.068
	43 pg/ml	0.472	0.146
	129 pg/ml	1.784	0.513
	385 pg/ml	5.827	1.675
	790 pg/ml	8.009	2.315

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 1.3 pg/ml.

B. Specificity

Some potentially interfering hormones have been tested in this assay. At concentrations up to 100 ng/ml, none of the following hormones showed significant interference :

- CGRP
- Salmon-calcitonin
- PDN 21
- Procalcitonin N terminal.

C. Precision

INTRA ASSAY				INTER ASSAY			
Serum	N	<X> ± SD (pg/ml)	CV (%)	Serum	N	<X> ± SD (pg/ml)	CV (%)
A	10	36.7 ± 1.0	2.8	A	20	41.3 ± 3.6	8.6
B	10	259.7 ± 6.3	2.4	B	20	167.3 ± 10.5	6.3

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Added CT (pg/ml)	Recovered CT (pg/ml)	Recovery (%)
475	414	87
135	124	92
65	63	97
20	20	100

DILUTION TEST

Sample	Dilution	Theoretical Concent. (pg/ml)	Measured Concent. (pg/ml)
Serum 1	1/1	-	624
	1/2	312	325
	1/4	156	158
	1/8	78	84
	1/16	39	42
	1/32	20	21
	1/64	9.8	9.1
	1/128	4.9	4.1

Samples were diluted with CT Free Serum.

E. Hook effect

A sample spiked with CT up to 830000 pg/ml gives higher OD's than the last calibrator point.

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.

Normal values

50 samples from normal subjects obtained values below 11 pg/ml.

Pentagastrin test:

Patient with MTC with elevated base levels of CT:

Time after Pentagastrin Stimulation (min)	CT measured with CALITININ ELISA(pg/ml)
0	4800
1	8200
2	34400
5	29300
10	21900
20	14900

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 chromogenic solution 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.

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5) Controls, Samples Working Anti-CT-HRP conjugate	50 - 100	- 50 100
Incubate for 18 ± 1 hours at 2 – 8 °C. Aspirate the contents of each well. Wash 4 times with 400 µl of Wash Solution and aspirate.		
Chromogenic TMB Solution	100	100
Incubate for 30 min at room temperature with continuous shaking at 700 rpm.		
Stop Solution	200	200
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm)		

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