Calcitonin
Immunoenzymatic determination of Calcitonin concentration in human serum

INTENDED USE
Immunoenzymatic colorimetric method for quantitative determination of Calcitonin concentration in human serum. Calcitonin kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
Calcitonin is a hormone secreted by the parafollicular C cells of the thyroid gland, that acts on calcium levels by modulating the ability to "clearance" of renal calcium and reducing bone resorption. It is unclear, however, the physiological result of this modulation. An excess of Calcitonin in the blood tissue may be indicative of the onset of malignant tumor "medullary thyroid cancer" (MTC), that mainly (80% of cases) appears sporadically, but that also has a familial component (20% of cases).
A moderate increase of Calcitonin in the blood can be found in the states of pregnancy, pernicious anemia, renal dysfunction, and during the neonatal period. Therefore, the quantification of Calcitonin in the blood tissue can be useful mainly in the diagnosis and follow-up of MTC, but also in the study of the pathophysiology of calcium and bone metabolism.

2. PRINCIPLE OF THE METHOD
Meridian Calcitonin assay is based on the simultaneous capture of the antigen by two monoclonal antibodies (one coated on the microplate, the other linked to horseradish peroxidase) and subsequent quantification via a chromogenic substrate.
In the first phase an incubation of calibrators, controls and patient samples in the microtiter plate with the conjugat reagent is carried out; antibodies to Calcitonin form a sandwich with the antigen, blocking Calcitonin in the wells of the microplate.
After incubation, the microplate is washed with a washing solution for the removal of components that have not reacted.
Finally, a chromogenic substrate solution containing TMB is added. After the incubation, the reaction is stopped by adding the Stop Solution. The color of the solution turns yellow.
The amount of color developed is directly proportional to the concentration of Calcitonin in the sample.

The concentration of Calcitonin in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit
1. Calibrators (6 vials, lyophilized)
   - CAL0
   - CAL1
   - CAL2
   - CAL3
   - CAL4
   - CAL5
   See Control concentration on the Certificate of Analysis
2. Controls (1 vial, lyophilized)
   - Control 1
   - Control 2
   See Control concentration on the Certificate of Analysis
3. 50X Conjugate (1 vial, 0.125 mL)
   Monoclonal anti Calcitonin antibodies conjugated to horseradish peroxidase (HRP)
4. Conjugate buffer (1 vial, 6 mL)
   Tris buffer, BSA
5. Coated Microplate (1 breakable microplate)
6. TMB Substrate (1 vial, 12 mL)
   H2O2-TMB (0.26 g/L) (avoid any skin contact)
7. Stop Solution (1 vial, 12 mL)
   Chloridric acid 0.1N (avoid any skin contact)
8. 200X Conc. Wash Solution (1 vial, 10 mL)
   Tris-HCl buffer
9. Serum (1 vial, lyophilized)
   Human serum
10. Serum buffer (1 vial, 8 mL)
   Borate buffer
3.2. Reagents necessary not supplied
Distilled water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm, 620-630 nm)

Notes
Store all reagents at 2-8°C in the dark.
Open the bag of reagent 5 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, it is stable up to expiry date of the kit.

4. WARNINGS
• This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
• Use appropriate personal protective equipment while working with the reagents provided.
• Follow Good Laboratory Practice (GLP) for handling blood products.
• Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
• All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the reagents should be handled in the same manner as potentially infectious material.
• Some reagents contain small amounts of Thymol as preservative. Avoid the contact with skin or mucosa.
• The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
• The Stop Solution consists of a diluted chloridric acid solution. Chloridric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
• Avoid the exposure of reagent TMB/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS
• Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
• All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
• Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
• Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
• If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
• The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
• It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
• Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
• Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled samples.
• Maximum precision is required for reconstitution and dispensation of the reagents.
• Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
• Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibration (C₀…C₅)
Calibrators should be reconstituted immediately before use with 0.5 mL of distilled or deionized water. Shake gently until solubilization of material. The exact concentrations of the Calibrators are stated on the labels and Certificate of Analysis for each specific lot.
Reconstituted calibrators must be stored at -20°C for a maximum of 3 months; carry out only 1 cycle of freezing and thawing (if necessary aliquot the reconstituted Calibrators).
The calibrators are calibrated against the International Standard NIBSC 89/620: 1 pg of calibrator corresponds to 0.19 μIU of International Standard.

6.2. Preparation of the Controls (1 and 2)
Proceed as the Calibrators.
Acceptability range of Controls is stated on the Certificate of Analysis.

6.3. Preparation of the Conjugate
Immediately before using, prepare the required amount of Conjugate by diluting 1:50 the Conjugate (Reagent 3) with Conjugate buffer (Reagent 4); diluted conjugate is stable 1 week at 2-8°C.
After opened, the Conjugate (reagent 3) not diluted is stable until expiry date stated on the label at 2-8°C, tightly closed.

6.4. Preparation of Wash Solution
Dilute 1:200 the "200X Conc. Wash Solution" with deionized or distilled water in the needed quantity; prepare the diluted washing solution immediately before use and discard the diluted solution not used.

Concentrated wash solution is stable at room temperature (22-28°C) until the expiry date stated on the label.

6.5. Preparation of the Sample
The determination of the Calcitonin with this kit can be carried out on human serum.
If the determination is not made within 24 hours of collection sample, storage the sample in aliquots at -20°C.
Avoid cycles of freezing and thawing.
Do not use hemolyzed or lipemic samples.

6.6. Preparation of the Serum (reagent 9)
Serum reagent is used to dilute any samples whose quantification is outside the calibration curve.
Reconstitute the lyophilized Serum (reagent 9) with Serum buffer (reagent 10) according to the quantity in mL indicated on the label for each specific lot.
Mix well before use.
Reconstituted Serum must be stored at -20°C for a maximum of 3 months; carry out only 1 cycle of freezing and thawing (if necessary aliquot the reconstituted Serum).

6.7. Procedure
• Allow all reagents to reach room temperature (22-28°C).
• Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
• To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
• As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀-C₅), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator C₀-C₅</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Sample/Control</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Diluted Conjugate</td>
<td>50 µL</td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>

Incubate 18 (±1) hours at 2-8°C.
Remove the contents from each well. Wash the wells 3 times with 400 µL of diluted wash solution.

Important note: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.

<table>
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<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB Substrate</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Incubate 30 minutes in the dark at room temperature (22-28°C).

Stop Solution
<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Sample/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
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Shake the microplate gently.
Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of Calcitonin for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the calibration curve (C₀-C₅) and of each sample.

8.2. Calibration Curve
Plot the mean value of absorbance (Em) of the calibrators (C₀-C₅) against concentration. Draw the best-fit curve through the plotted points. (es: Four Parameter Logistic).
8.3. Calculation of Results
Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in pg/mL.
If the sample is outside the calibration curve, dilute with reconstituted Serum (reagent 9).

9. REFERENCE VALUES
80 samples from healthy subjects were tested to establish the following normal range:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>No</td>
</tr>
<tr>
<td>Salmon Calcitonin</td>
<td>No</td>
</tr>
<tr>
<td>PDN 21</td>
<td>No</td>
</tr>
<tr>
<td>N-terminal procalcitonin</td>
<td>No</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision
10.1.1. Intra Assay
Within run variation was determined by replicate the measurement of two different samples in one assay. The within assay variability is ≤ 3.9%.
10.1.2. Inter Assay
Between run variation was determined by replicate the measurement of two different samples in different lots. The between assay variability is ≤ 6.0%.

10.2. Specificity
Specificity of Calcitonin kit has been checked against the following potentially interfering substances tested in concentrations of 100 ng/mL:

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10.3. Sensitivity
The lowest detectable concentration of Calcitonin that can be distinguished from the Calibrator zero is 0.7 pg/mL at the 95% confidence limit.

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
| DE  | In vitro Diagnostikum       | DE  | Hergestellt von     |
| ES  | Producto sanitario para diagnóstico In vitro | ES  | Elaborado por     |
| FR  | Dispositif medical de diagnostic in vitro | FR  | Fabriqué par     |
| GB  | In vitro Diagnostic Medical Device | GB  | Manufacturer     |
| IT  | Dispositivo medico-diagnostico in vitro | IT  | Produttore     |
| PT  | Dispositivos medicos de diagnostico in vitro | PT  | Produzido por     |

| DE  | Achtung, Begleitdokumente  | DE  | Herstellungs datum |
| ES  | Precaución, consulte los documentos adjuntos | ES  | Fecha de fabricacion |
| FR  | Attention, veuillez consulter les documents d’accompagnement | FR  | Date de fabrication |
| GB  | Caution, consult accompanying documents  | GB  | Date of manufacture |
| IT  | Attenzione, consultare la documentazione allegata | IT  | Data di produzione |
| PT  | Atenção, consultar os documentos de acompanhamento | PT  | Data de produção |

| DE  | Verwendbar bis  | DE  | Biogefährdung     |
| ES  | Establa hasta (usar antes de último día del mes) | ES  | Riesgo biológico |
| FR  | Utiliser avant (dernier jour du mois indiqué) | FR  | Risque biologique |
| GB  | Use by (last day of the month) | GB  | Biological risk |
| IT  | Utilizzare prima del (ultimo giorno del mese) | IT  | Rischio biologico |
| PT  | Utilizar (antes ultimo dia do mês) | PT  | Risco biológico |

| DE  | Gebrauchsanweisung beachten  | DE  | Chargenbezeichnung |
| ES  | Consultar las instrucciones | ES  | Codigo de lote |
| FR  | Consulter le mode d’emploi | FR  | Numero de lot |
| GB  | Consult instructions for use | GB  | Batch code |
| IT  | Consultare le istruzioni per l’uso | IT  | Codice del lotto |
| PT  | Consultar instruções para uso | PT  | Codigo do lote |

| DE  | Ausreichend für “n” Tests | DE  | Inhalt     |
| ES  | Contenido suficiente para "n" tests | ES  | Contenido del estuche |
| FR  | Contenu suffisant pour “n” tests | FR  | Contenu du coffret |
| GB  | Contains sufficient for “n” tests | GB  | Contents of kit |
| IT  | Contenuto sufficiente per “n” saggi | IT  | Contenido del kit |
| PT  | Contém o suficiente para “n” testes | PT  | Conteúdo do kit |

| DE  | Temperaturbereich       | DE  | Bestellnummer     |
| ES  | Limitaciôn de temperatura | ES  | Número de catálogo |
| FR  | Limites de température de conservation | FR  | Références du catalogue |
| GB  | Temperature limitation | GB  | Catalogue number |
| IT  | Limiti di temperatura | IT  | Numero di Catalogo |
| PT  | Temperaturas limites de conservação | PT  | Número do catálogo |

| DE  | Vor direkter sonneneinstrahlung schützen | DE  |     |
| ES  | Mantener alejado de la luz solar | ES  |     |
| FR  | Tenir à l’écart de la lumière du soleil | FR  |     |
| GB  | Keep away from sunlight | GB  |     |
| IT  | Tenere lontano dalla luce solare | IT  |     |
| PT  | Mantenha longe da luz solar | PT  |     |
SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORE CAUSE POSSIBILI/ SUGGERIMENTI

Nessuna reazione colorimetrica del saggio
- mancata dispensazione del coniugato
- contaminazione del coniugato e/o del Substrato
- errori nell’esecuzione del saggio (es. Dispensazione accidentale dei reagenti in sequenza errata o provenienti da flaconi sbagliati, etc.)

Reazione troppo blanda (OD troppo basse)
- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo breve, temperatura di incubazione troppa bassa

Reazione troppo intensa (OD troppo alte)
- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo lungo, temperatura di incubazione troppa alta
- qualità scadente dell’acqua usata per la soluzione di lavaggio (basso grado di deionizzazione,)
- lavaggi insufficienti (coniugato non completamente rimosso)

Valori inspiegabilmente fuori scala
- contaminazione di pipette, puntali o contenitori- lavaggi insufficienti (coniugato non completamente rimosso)
  CV% intrasaggio elevato
- reagenti e/o strip non portate a temperatura ambiente prima dell’uso
- il lavatore per micropiastre non lava correttamente (suggerimento: pulire la testa del lavatore)
  CV% intersaggio elevato
- condizioni di incubazione non costanti (tempo o temperatura)
- controlli e campioni non dispensati allo stesso tempo (con gli stessi intervalli) (controllare la sequenza di dispensazione)
- variabilità intrinseca degli operatori

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation