Monobind Inc.
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AccuBind
ELISA Microwells

Calcitonin Test System
Product Code: 9325-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Calcitonin Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Calcitonin is a 32 amino acid alpha helix produced by the follicular cells of the thyroid gland. A cleavage product of procalcitonin (PCT), calcitonin is a product of the CALC1 gene in humans and provides support in regulating calcium homeostasis, lowering serum calcium concentrations and preventing hypercalcemia. Calcitonin is characterized by an N-terminal disulfide bridge, which contributes to its biological activity, and a C-terminal proline residue.

Calcitonin plays a role in calcium metabolism, with osteoclasts the most significant homeostatic targets. Calcitonin binds to CT receptors (CTRs) on osteoclasts, halting calcium resorption via prevention of cell differentiation and motility. CTR receptors are also found in the kidneys and hypothalamus, providing an excretion route for excess serum calcium. Calcitonin modulates calcium absorption via CTR receptors on renal tubules, preventing excess calcium uptake. CTR receptors belong to the family of G-protein coupled receptors, utilizing cAMP messengers to amplify and transduce signals initiated by calcitonin-CTR binding.

Calcitonin has emerged as a therapeutic avenue for hypercalcemia patients, utilized as a biomarker PCT for its rapid biomarker for medullary carcinoma of the thyroid (MCT), providing a facile and direct measurement of carcinogenic activity. Calcitonin levels are typically low in normal populations, and elevated levels suggest the presence of hypercalcemia or potential loss of thyroid function.

Medullary thyroid carcinoma is typically associated with elevated levels of calcitonin. Parafollicular C cells containing mutations in the RET gene will display elevated expression of calcitonin and the presence of nodules in the lymph nodes, potentially disrupting calcium homeostasis.

3.0 PRINCIPLE

Sandwich Equilibrium Method (Type 2):
The calcitonin immunoassay is an adapted two-site sandwich ELISA. In this assay, standards and patient samples are simultaneously incubated with the enzyme labeled detection antibody and a biotin coupled capture antibody on a coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stopping solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of calcitonin in the sample. Standards are used to generate a dose response curve of absorbance unit vs. concentration. Concentrations of calcitonin present in the controls and patient samples are determined directly from this curve.

The essential reagents required for a sandwich equilibrium assay include high affinity and specificity antibodies (signal and capture), with different and distinct epitope recognition, **in excess,** and native antigen. In this procedure, the calibrator, control or patient sample is added to the wells coated with anti-calcitonin antibody. Calcitonin from the sample binds to the anti-calcitonin (MoAb) on the wells. Subsequently an enzyme labeled anti-calcitonin is added to the wells. Calcitonin from the sample forms a sandwich between the two antibodies. Excess enzyme and sample is removed via a wash step. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{CT} + Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)} - Ag_{CT} - Ab_{(m)}$$

$Ab_{(m)}$ = Anti-calcitonin (MoAb) (On the Microwells in Excess Quantity)
Ag_{CT} = Native Antigen (Variable Quantity)
$^{Enz}Ab_{(CT)}$ = Enzyme labeled Mouse α CT (P) (Excess Quantity)
$^{Enz}Ab_{(CT)} - Ag_{CT} - Ab_{(m)}$ = Ag-Antibodies Sandwich complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Dissociation

The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

A suitable substrate is added to the wells to generate color in varying intensity depending upon the concentration of calcitonin in the wells. The intensity of the color in the sample can be visually compared to the known calibrators to obtain qualitative results or the color development can be read with the help of a microplate spectrophotometer to obtain semi-semi-quantitative results.

4.0 REAGENTS

Materials Provided:

A. Calcitonin Calibrators – 1.0 ml/vial (Dried) – Icons A-F
Six (6) vials of references for Calcitonin at levels of 0(**A**), 10(**B**), 40(**C**), 150(**D**), 400(**E**) and 1000(**F**) pg/ml. Store at 2-8°C. ***Reconstitute each vial with 1ml of distilled or deionized water***. The reconstituted calibrators are stable for 1 hour at 2 8°C. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

B. Calcitonin Control M – 1.0 ml/vial (Dried) – Icon M
One (1) vial of reference control for Calcitonin .Store at 2-8 °C. ***Reconstitute each vial with 1ml of distilled or deionized water***. The reconstituted control should be assayed immediately after reconstitution. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months .Freeze and thawed cycles should be minimized to one time only.

C. Calcitonin Enzyme Reagent – 6 ml/vial – Icon E
One (1) vial containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store at 2-8°C.

D. PCT Antibody Coated Plate – 96 wells – Icon
One 96-well microplate coated with procalcitonin antibody, packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate – 20 ml/vial – Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate Reagent – 12 ml/vial – Icon S^N
One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (0.5M H_2O_2) in buffer. Store at 2-8°C.

G. Stop Solution – 8 ml/vial – Icon STOP
One (1) vial containing a strong acid (0.5M H_2SO_4). Store at 2-8°C.

H. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not expose reagents to heat, sun, or strong light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C, unless otherwise specified. Kit and component stability are identified on label.**

Note 3: The above components are for a single 96-well microplate

4.1 Required But Not Provided:
1. Pipette capable of delivering 0.050ml (50µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl), and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent paper for blotting the microplate wells.
6. Plastic wrap or microplate covers for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8 °C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml (100µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high ranges of the dose response curve 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. 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.

8.0 REAGENT PREPARATION

1. Wash Buffer
Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.
3. Add 0.050 ml (50 µl) of the Enzyme Reagent to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds to mix (500 – 600 rpm) and cover.
5. Incubate 60 minutes (1 hour) at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100 µl) of Substrate Reagent to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**
   DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION
9. Incubate at room temperature for twenty (20) minutes.
10. Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
11. Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. **The results should be read within fifteen (15) minutes of adding the stop solution.**

Note 1: **For re-assaying specimens with concentrations greater than 1000 pg/ml, dilution should be performed.**
Note 2: **Do not use reagents that are contaminated or have bacterial growth.**
Note 3: **Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.**
Note 3: **It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the microwells at an angle while touching the side of the well.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Calcitonin in unknown specimens.
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate calibrator versus the corresponding calcitonin concentration in pg/ml on linear graph paper.
3. Connect the points with a best-fit curve.
4. To determine the concentration of calcitonin for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

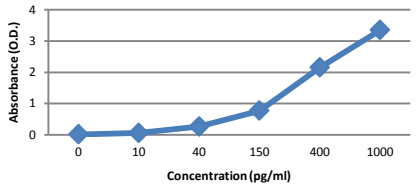
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Conc. (pg/ml)	Mean Abs
Cal A	0	0.016
Cal B	10	0.062
Cal C	40	0.268
Cal D	150	0.772
Cal E	400	2.150
Cal F	1000	3.347
Control M	80	0.365

*The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

Figure 1



*If the absorbance readout is off-scale or higher than the average absorbance of the highest calibrator, sample should be repeated with dilution.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator F (1000 pg/ml) should be ≥2.0
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system procedure have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problematic for all kinds of immunoassays. (*Boscato LM Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin.Chem. 1988:3427-33).* For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history, and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
7. The Calcitonin ELISA kit has exhibited no high dose hook effect with samples spiked with 1,000,000 pg/ml of calcitonin. Samples with calcitonin levels greater than the highest calibrator, however, should be diluted and re-assayed for correct values.

13.0 EXPECTED RANGES OF VALUES

Calcitonin levels were measured in thirty-one (31) apparently normal individuals. The values obtained ranged from 0.292 to 118.643 pg/ml. Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows the normal or Gaussian distribution as shown in histograms. The geometric mean ± 2 standard deviations of the mean were calculated to be 4.49 to 41.83 pg/ml.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precisions of the Calcitonin AccuBind® ELISA test system were determined by analysis of three different levels of pool control sera. The number (N), mean (X) value, standard deviation (σ) and coefficient of variation (C.V.) of each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V. %
Low	20	26.23	2.58	9.9
Normal	20	65.50	3.67	5.57
High	20	318.101	7.88	2.51

TABLE 3
Between Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V.%
Low	20	26.03	3.81	14.62
Normal	20	65.97	12.24	18.55
High	20	313.73	31.02	9.89

14.2 Sensitivity

The Calcitonin AccuBind® ELISA test system has a sensitivity of 4.4871 pg/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

15.0 REFERENCES

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3. Ganeshan, D., Paulson, E., Duran, C., Cabanillas, M.E., Busaidy, N.L., Charnsangavej, C. "Current Update on Medullary Thyroid Carcinoma". American Journal Roentgenology, 201, W867-W976, 2013.
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5. Masi, L.; Brandi, M.L. "Calcitonin and calcitonin receptors". Clin Cases Miner Bone Metab. 4(2): 117-122, 2007

Effective Date: 2019-Mar-05 Rev. 0 **DCO: N/A**
MP9325 **Product Code: 9325-300**

Size		96(A)	192(B)
Reagent (fill)	A)	1ml (Dried) set	1ml (Dried) set
	B)	1ml (Dried) set	1ml (Dried) set
	C)	1 (6ml)	2 (6ml)
	D)	1 plate	2 plates
	E)	1 (20ml)	1 (20ml)
	F)	1 (12ml)	2 (12ml)
	G)	1 (8ml)	2 (8ml)

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Please visit our website to learn more about our products and services.

Glossary of Symbols
(EN 980/ISO 15223)

- IVD — In Vitro - Diagnostic Medical Device
- 2°-8° — Temperature Limitation Storage Condition (2-8° C)
- Consult Instructions for Use
- REF — Catalogue Number
- Σ — Contains Sufficient Test for Σ
- LOT — Batch Code
- Used By (Expiration Day)
- Date of Manufacturer
- Manufacturer
- EC REP — Authorized Rep in European Country
- CE — European Conformity

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