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*Please use only the valid version of the package insert provided with the kit.*

#### Name and Intended Use

The Calcitonin ELISA is intended for the quantitative determination of Calcitonin in human serum.

**The test is for in vitro diagnostic use only.**

#### Summary and Explanation

Calcitonin, a 32-amino-acid polypeptide, is secreted primarily by the thyroidal parafollicular C-cells. Its main biological effect is to inhibit osteoclastic bone resorption. This property has led to Calcitonin's use for disorders characterized by increased resorption such as Paget's disease, for some patients with osteoporosis.

#### Clinical Relevance

The most prominent clinical syndrome associated with a disordered hypersecretion of Calcitonin is medullary carcinoma of the thyroid (MTC). MTC is a tumor of the Calcitonin producing C-cells of the thyroid gland. Although MTC is rare, comprising 5 - 10% of all thyroid cancer, it is often fatal. It may occur sporadically or in a familial form that is transmitted as an autosomal dominant trait. MTC has great clinical importance because of its familial distribution. Further, it leans itself to be diagnosed early by serum Calcitonin and total cure for early sub-clinical disease is possible[1]. This is frequently associated with other clinical features and it has good potential for cure with surgery. Although a rare tumor, it can occur in a familial pattern[1,3,4] as a Type II multiple endocrine neoplasia. These tumors usually produce diagnostically elevated serum concentrations of Calcitonin. Therefore, the immunoassay for Calcitonin in serum can be used to diagnose the presence of MTC with an exceptional degree of accuracy and specificity. In the small but increasing percentage of patients, however, basal hormone levels are indistinguishable from normal[1]. Some of these subjects represent the early stages of C-cell neoplasia or hyperplasia that are most amenable to surgical cure. To identify these patients with early disease, provocative tests for Calcitonin secretion is necessary to preclude false negatives if only basal Calcitonin determination are performed. Most tumors respond with increased Calcitonin level to the administration of either calcium[5] or pentagastrin[6] or their combination[7], but either agent can still give misleading results. Therefore, in cases with clinical manifestations, both agents should be considered for diagnostic testing. Further, Calcitonin measurements can also be used to monitor the efficacy of therapy in patients with Calcitonin producing tumors. It has been reported[8] that multiple forms of immunoreactive calcitonin are found in either normal subjects or patients with MTC. These various forms of calcitonin have molecular weights varying from 3,400 (monomeric) up to 70,000 Dalton (polymeric).

Neoplastic disorders of other neuroendocrine cells can also elevate Calcitonin. The best example is small cell lung cancer. Other tumors such as carcinoids and islet cell tumors of the pancreas can also result in elevated serum Calcitonin.

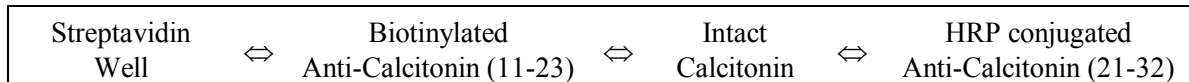
Increases in serum Calcitonin has also been noted in both acute and chronic renal failure, hypercalciuria and hypercalcemia.

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### Principle of the Test

The DRG Calcitonin Immunoassay is a two-site ELISA [Enzyme-Linked ImmunoSorbent Assay] for the measurement of the biologically intact 32 amino acid chain of Calcitonin. It utilizes two different mouse monoclonal antibodies to human calcitonin specific for well-defined regions on the calcitonin molecule. One antibody binds only to Calcitonin 11-23 and this antibody is biotinylated. The other antibody binds only to Calcitonin 21-32 and this antibody is labeled with horseradish peroxidase [HRP] for detection.



In this assay, calibrators, controls, or patient samples are simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. Thus the calcitonin in the sample is “sandwiched” between these two antibodies. 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. A dose response curve of absorbance unit vs. concentration is generated using results obtained from the calibrators. Concentrations of calcitonin present in the controls and patient samples are determined directly from this curve.

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#### Kit Components

Kit Components	Description	Quantity
<b>RGT 1</b> = Reagent 1	Biotinylated Calcitonin Antibody	1 x 7.0 mL
<b>RGT 2</b> = Reagent 2	Peroxidase (Enzyme) labeled Calcitonin Antibody	1 x 7.0 mL
<b>RGT 3</b> = Reagent 3	Reconstitution Solution containing EDTA	1 x 10 mL
<b>RGT A</b> = Reagent A	ELISA Wash Concentrate [Saline with surfactant]	1 x 30 mL
<b>RGT B</b> = Reagent B	TMB Substrate [tetramethylbenzidine]	1 x 20 mL
<b>SOLN</b> = Stopping Solution	ELISA Stop Solution [1 N sulfuric acid]	1 x 20 mL
<b>PLA</b> = Microplate	One holder with Streptavidin Coated Strips.	12 x 8-well strips
<b>CAL</b> = Calibrators A: 0 pg/mL B – F: Refer to vial labels for exact concentrations	Lyophilized synthetic h-Calcitonin. Lyophilized Zero calibrator [BSA solution]. All other calibrators consist of synthetic h-Calcitonin (1-32) in BSA solution, calibrated to <i>WHO 2<sup>nd</sup> IS 89/620</i>	1 x 2 mL for the zero calibrator  1 x 1 mL for all other calibrators
<b>CTRL</b> = Controls 1 & 2 Refer to vial labels for exact ranges	Lyophilized. 2 Levels. Synthetic h-Calcitonin (1-32) in BSA solution.	1 x 1 mL per level

#### Materials required but not supplied

- Microplate reader capable of measuring absorbance at wavelengths of 450 nm and 405 nm.
- Microplate washer [if washer is unavailable, manual washing may be acceptable].
- Precision Pipettors to deliver 50, 100 and 150 µL.
- (Optional): A multi-channel dispenser or a repeating dispenser for 50, 100 and 150 µL.

#### Warnings and Precautions for Users

Although the reagents provided in this kit have been specifically designed to contain no human blood components, the human patient samples, which might be positive for HBsAg, HBcAg or HIV antibodies, must be treated as potentially infectious biohazard. Common precautions in handling should be exercised, as applied to any untested patient sample.

Stopping Solution consists of 1 N Sulfuric Acid. This is a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves and eye protection, with appropriate protective

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clothing. Any spill should be wiped immediately with copious quantities of water. Do not breath vapor and avoid inhalation.

**Sample Collection and Storage**

The determination of Calcitonin should be performed with serum. To assay the specimen in duplicate, 200 µL of serum is required. Collect whole blood without anticoagulant. After allowing blood to clot, the serum should be promptly separated, preferably in a refrigerated centrifuge, and stored at –20°C or lower. Avoid grossly hemolyzed or grossly lipemic samples.

**Reagent Preparation and Storage**

Store all kit components at 2 °C - 8 °C except Wash Concentrate and Stop Solution upon receipt prior to use

1. All reagents except the calibrators, kit controls and the Wash Concentrate are ready-to-use. Store all reagents at 2 °C - 8 °C, except the Wash Concentrate, which should be kept at room temperature until dilution to avoid precipitation.
2. Reconstitute Calibrator A (Zero standard) with 2.0 mL distilled or deionized water and mix. For each of the non-zero calibrators (Calibrator B through F) and kit controls 1 and 2, reconstitute each vial with 1.0 mL of Reagent 3 (Reconstitution Solution) and mix. Allow the vials to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution. **Use the calibrators and controls as soon as possible upon reconstitution. Freeze (-20 °C) the remaining calibrators and controls as soon as possible after use in a non-self-defrosting freezer.** Standards and controls are stable at –20 °C for 6 weeks after reconstitution with up to 3 freeze thaw cycles when handled as recommended in “Procedural Notes” section.
3. **ELISA Reagent A:** Wash Concentrate:  
Mix contents of wash concentrate thoroughly. If precipitate is present in the Wash Concentrate due to storage at lower temperature such as 4 °C, dissolve by placing the vial in a 37 °C water bath or oven with swirling or stirring. Add wash concentrate (30 mL) to 570 mL of distilled or deionized water and mix.  
The diluted working wash solution is stable for 90 days when stored at room temperature.

**Assay Procedure**

1. Place sufficient Streptavidin Coated Strips in a holder to run all the six (6) calibrators, A - F of the Calcitonin CALIBRATORS [Exact concentration is stated on the vial label], Quality Control Sera and patient samples.
2. Pipet 100 µL of calibrators, controls, and samples into the designated or mapped well. **Freeze (-20°C) the remaining calibrators and controls as soon as possible after use, in a non-self-defrosting freezer.**
3. Add or dispense 50 µL of Reagent 1 (Biotinylated Antibody) into each of the wells which contain the calibrators, controls, and samples.
4. Add or dispense 50 µL of Reagent 2 (Enzyme Labeled Antibody) into each of the same wells.

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5. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light, and place it on an orbital shaker or rotator set at  $170 \pm 10$  rpm for 4 hours  $\pm$  30 minutes at room temperature ( $22\text{ }^{\circ}\text{C}$  -  $28\text{ }^{\circ}\text{C}$ ).
6. First aspirate the fluid completely and then wash/aspirate each well five (5) times with the Working Wash Solution (prepared from Reagent A), using an automatic microplate washer. The wash solution volume should be set to dispense 0.35 mL into each well.
7. Add or dispense 150  $\mu\text{L}$  of the ELISA Reagent B (TMB Substrate) into each of the wells.
8. With appropriate cover to avoid light exposure, place the microplate(s) on an orbital shaker or rotator set at  $170 \pm 10$  rpm for  $30 \pm 5$  minutes at room temperature ( $22\text{ }^{\circ}\text{C}$  -  $28\text{ }^{\circ}\text{C}$ ).
9. Add or dispense 100  $\mu\text{L}$  of the Stopping Solution into each of the wells. Mix gently.
10. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm against 250  $\mu\text{L}$  of distilled or deionized water. Read the plate again with the reader set to 405 nm against distilled or deionized water.  
Note: The second reading is designed to extend the analytical validity of the calibration curve to the value represented by the highest calibrator, which is approximately 1,000 pg/mL. Hence, patient samples with calcitonin  $> 300$  pg/mL can be quantified against a calibration curve consisting of the readings all the way up to the concentration equivalent to the highest calibrator using the 405 nm reading, away from the wavelength of maximum absorbance. In general, patient and control samples should be read using the 450 nm for calcitonin concentrations up to 300 pg/mL. Calcitonin concentrations above 300 pg/mL should be interpolated using the 405-nm reading.
11. By using the final absorbance values obtained in the previous step, construct a calibration curve via cubic spline, 4 parameter logistics, or point-to-point interpolation to quantify the concentration of the calcitonin.

**Procedural Notes**

- Calcitonin 1-32 is a very labile molecule. Set up the assay immediately upon the reconstitution or the thawing of all calibrators, controls, and patient samples.
- It is recommended that all calibrators, controls, and patient samples are assayed in duplicate. The average absorbance units of duplicate sets should then be used for reduction of data and the calculation of results.
- The samples should be pipetted into the well with minimum amount of air-bubble. To achieve this, “reverse pipet” described in the package insert of the manufacturers of Pipettors is recommended.
- Patient samples with values greater than the highest calibrator (Calibrator F), which is approximately 1,000 pg/mL (see exact concentration on vial label), may be diluted with Calibrator A (Zero Calibrator) and reassayed. Multiply the result by the dilution factor.
- Reagents from different lot numbers must not be interchanged.
- If preferred, mix in equal volumes, in sufficient quantities for the assay, Reagent 1 (Biotinylated Antibody) and Reagent 2 (Enzyme Labeled Antibody) in a clean amber bottle. The combined reagent is stable for seven (7) days when stored at  $4\text{ }^{\circ}\text{C}$ . Then use 100  $\mu\text{L}$  of the mixed antibody into each well. This alternative method should replace Step (3) and (4), to be followed with the incubation with orbital shaker.
- When mixing avoid splashing of reagents from wells. This will affect assay precision and accuracy.

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### Calculation of Results

#### Manuel Method

1. For the 450 nm readings, construct a dose response curve (calibration curve) using the first five calibrators provided, i.e. Calibrators A, B, C, D and E. For the 405 nm readings, construct a second dose response curve using the three calibrators with the highest concentrations, i.e. Calibrators D, E and F.
2. Assign the concentration for each calibrator stated on the vial in pg/mL. Plot the data from the calibration curve on linear graph paper with the concentration on the X-axis and the corresponding A.U. on the Y-axis.
3. Draw a straight line between 2 adjacent points. This mathematical algorithm is commonly known as the "point-to-point" calculation. Obtain the concentration of the sample by locating the absorbance unit on the Y-axis and finding the corresponding concentration value on the X-axis. Patient and control samples should be read using the 450 nm for Calcitonin concentrations up to 300 pg/mL. Calcitonin concentrations above 300 pg/mL should be interpolated using the 405 nm reading.

#### Automated Method

Computer programs using cubic spline or 4 PL [4 Parameter Logistics] or Point-to-Point can generally give a good fit.

**Sample Data at 450 nm** [raw A.U. readout against distilled or deionized water]

Microplate Well	1 <sup>st</sup> Reading Absorbance Unit	2 <sup>nd</sup> Reading Absorbance Unit	Average Absorbance Unit	Calcitonin pg/mL	Calcitonin pg/mL – Result to report
Calibrator A	0.008	0.009	0.0085		0
Calibrator B	0.059	0.064	0.0615		10
Calibrator C	0.186	0.194	0.190		30
Calibrator D	0.578	0.602	0.590		100
Calibrator E	1.900	1.882	1.891		300
Control 1	0.127	0.122	0.125	20.6	20.6
Control 2	2.554	2.565	2.560	> 300	*
Patient Sample 1	0.034	0.040	0.037	4.7	4.7
Patient Sample 2	0.104	0.098	0.101	16.3	16.3
Patient Sample 3	0.397	0.411	0.404	68.7	68.7
Patient Sample 4	2.195	2.173	2.184	> 300	*

\* Because the concentration readout is > 300 pg/mL, it is recommended to use the data obtained at 405 nm as shown in **Sample Data at 405 nm** in the table below.

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Sample Data *at 405 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	1 <sup>st</sup> Reading Absorbance Unit	2 <sup>nd</sup> Reading Absorbance Unit	Average Absorbance Unit	Calcitonin p g/mL	Calcitonin pg/mL – Result to report
Calibrator A	0.005	0.005	0.005		0
Calibrator D	0.187	0.198	0.193		100
Calibrator E	0.602	0.597	0.599		300
Calibrator F	1.898	1.910	1.904		1000
Control 1	0.045	0.044	0.045	< 300	¶
Control 2	0.814	0.816	.815	403	403
Patient Sample 1	0.016	0.020	0.018	< 300	¶
Patient Sample 2	0.039	0.035	0.037	< 300	¶
Patient Sample 3	0.128	0.134	0.131	< 300	¶
Patient Sample 4	0.697	0.689	0.693	345	345

¶ For samples with readout < 300 pg/mL, it is recommended to use the data obtained at 450 nm as shown in **Sample Data *at 450 nm*** in the table above. This practice should give the results with optimum sensitivity of the assay.

*NOTE: The data presented are for illustration purposes only and must not be used in place of data generated at the time of the assay.*

### Quality Control

Control serum or serum pools should be analyzed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the patient sample may not be valid.

### Limitation of the Procedure

- The DRG Calcitonin ELISA kit has exhibited no “high dose hook effect” with samples spiked with 1,000,000 pg/mL of pure intact calcitonin (1-32). The spiked sample gave a result greater than the highest standard, i.e. 1,000 pg/mL. Samples with calcitonin levels greater than the highest calibrator, however, should be diluted and reassayed for correct values.
- Like any analyte used as a diagnostic adjunct, calcitonin results must be interpreted carefully with the overall clinical presentations and other supportive diagnostic tests.

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### Expected Values

It is recommended that each laboratory establish its own reference range. The data provided should be used only as a guideline. Calcitonin levels were measured in fifty-nine (59) apparently normal female individuals and fifty-two (52) apparently normal male individuals with the DRG Calcitonin ELISA.

The values obtained on the normal females ranged from 0.1 to 10.9 pg/mL and the values obtained on the normal males ranged from 0.2 to 27.7 pg/mL.

Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows the normal or Gaussian distribution as shown in the histograms.

The geometric mean  $\pm$  2 standard deviations of the mean for the normal females were calculated to be 0.07 to 12.97 pg/mL and 0.68 to 30.26 pg/mL for the normal males.

Consistent with the literature [2,9], calcitonin levels were found to be generally lower in normal females than in normal males. Hence, the reference range should be less than 13 and 30 pg/mL, for females and males, respectively.

### Performance Characteristics

#### Accuracy

Seventy-seven patient samples, with calcitonin values ranging from 0.8 to 3,113 pg/mL were assayed by the DRG ELISA procedure and an ImmunoRadioMetric Assay Calcitonin (IRMA Kit). Linear regression analysis gives the following statistics:

$$\text{DRG ELISA} = 0.940 \text{ IRMA Kit} + 6.55 \text{ pg/mL} \quad r = 0.993, \quad N = 123$$

Further, fifty-one patient samples, with calcitonin values ranging from < 0.7 to 2,240 pg/mL were assayed by the DRG ELISA procedure and Chemiluminescence Immunoassay for Calcitonin Kit [or

ImmunoChemiluminescentMetricAssay (ICMA)]. Linear regression analysis gives the following statistics:

$$\text{DRG ELISA} = 1.094 \text{ ICMA Kit} - 6.13 \text{ pg/mL} \quad r = 0.995, \quad N = 123$$

#### Sensitivity

The sensitivity, or minimum detection limit, of this assay is defined as the smallest single value, which can be distinguished from zero at the 95% confidence limit.

The DRG Calcitonin ELISA has a calculated sensitivity of 1.0 pg/mL.

### Precision and Reproducibility

The precision (intra-assay variation) of the DRG Calcitonin ELISA Test was calculated from 20 replicate determinations on each of the three samples.



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**Intra-Assay Variation**

Sample	Mean Value (pg/mL)	N	Coefficient of Variation %
A	24.3	20	5.7
B	94.9	20	4.3
C	403	20	2.8

The total precision (inter-assay variation) of the DRG Calcitonin ELISA Test was calculated from data on three samples obtained in 15 different assays, by three technicians on two different lots of reagents, over a three-week period.

**Inter-Assay Variation**

Sample	Mean Value (pg/mL)	N	Coefficient of Variation %
A	16.5	15	7.4
B	64.5	15	7.4
C	340	15	6.1

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### Recovery

Various amounts of Calcitonin were added to four different patient sera to determine the recovery. The results are described in the following table:

Serum Sample	Endogenous Calcitonin (pg/mL)	Calcitonin added (pg/mL)	Expected Value (pg/mL)	Measured Value (pg/mL)	Recovery (%)
A	0	--	--	--	--
	0	100	100	110	110%
	0	200	200	217	109%
B	9.7	--	--	--	--
	8.7	100	109	106	97%
	7.8	200	208	207	100%
C	0	--	--	--	--
	0	100	100	104	104%
	0	200	200	205	103%
D	5.7	--	--	--	--
	5.1	126	131	119	91%
	4.6	220	225	203	90%

### Specificity and Cross-Reactivity

Cross reactant	Concentration of Cross reactant	Calcitonin without Cross reactant (pg/mL)	Calcitonin with Cross reactant (pg/mL)	Change in Calcitonin (pg/mL)	Cross reactivity (%)
PTH	100,000 pg/mL	186	194	8	0.00800
	30,000 pg/mL	186	200	14	0.04667
	10,000 pg/mL	186	194	8	0.08000
Calcitonin Gene Related Peptide	1,000,000 pg/mL	200	202	2	0.00020
	100,000 pg/mL	200	204	4	0.00400
Salmon Calcitonin	1,000,000 pg/mL	191	194	3	0.00030
	100,000 pg/mL	191	199	8	0.00800
TSH	5000 µIU/mL	198	203	5	0.00061
	500 µIU/mL	198	198	0	0.00000
	50 µIU/mL	198	199	1	0.01220

Each cross reactant is spiked into a sample containing Calcitonin. Calcitonin level is measured before and after the spike. None of the cross reactants interfere with this Calcitonin ELISA. The small changes in Calcitonin measured are well within the intra-assay precision statistics.

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### Kinetic Effect of the Assay

To determine whether there is any systematic kinetic effect between the beginning of the run and the end of the run, three spiked patient serum pools, selected to represent a good cross section of the calcitonin concentration, were placed in sequence throughout the run of one microplate or 96 wells [with twelve 8-well strips].

Linearity of Patient Sample Dilutions: Parallelism

Six patient serum samples were diluted with Calibrator A (Zero Calibrator). Results in pg/mL are shown below:

Sample	Dilution	Expected (E)	Observed (O)	% O/E
A	Undiluted	-	343	-
	1:2	172	168	98%
	1:4	85.8	81.3	95%
	1:8	42.9	40.3	94%
B	Undiluted	-	271	-
	1:2	136	131	97%
	1:4	67.8	70	103%
	1:8	33.9	34.3	101%
C	Undiluted	-	265	-
	1:2	133	134	101%
	1:4	66	70.4	106%
	1:8	33.1	32.5	98%
D	Undiluted	-	>1000	-
	1:2	-	1060	-
	1:4	530	504	95%
	1:8	265	271	102%
E	Undiluted	-	231	-
	1:2	116	116	100%
	1:4	57.8	58.8	102%
	1:8	28.9	27.1	94%
	1:16	14.4	12.1	84%
F	Undiluted	-	>1000	-
	1:2	-	997	-
	1:4	499	429	86%
	1:8	249	223	89%
	1:16	125	119	95%

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