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Calcitonin ELISA

Catalog Number M046010

For the quantitative determination of calcitonin in human serum samples.

For research use only.
This product insert must be read in its entirety before use.

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 SIGNIFICANCE

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 leant 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 Calci- tonin. 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 pentagastrin6 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.

PRINCIPLE OF THE ASSAY

The 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.

Streptavidin Well--Biotinylated Anti-Calcitonin (11-23)--Intact Calcitonin--HRP conjugated Anti-Calcitonin (21-32)

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 re- sults obtained from the calibrators. Concentrations of calcitonin present in the controls and patient samples are determined directly from this curve.

KIT COMPONENTS

Microplate Plate - The plate contains 12 x 8-well strips coated with streptavidin. Ready for use.

Calibrator A - 1 vial (0 pg/mL) solution containing BSA serum. Lyophilized.

Calibrators B through F - Synthetic human Calcitonin (1-32) in a BSA serum solution. Lyophilized. See vial label for concentrations.

Controls - Synthetic human Calcitonin (1-32) in a BSA serum solution. Lyophilized. See vial label for concentration ranges.

Biotinylated Calcitonin Antibody - 1 vial of Biotinylated Calcitonin Antibody. Ready to use.

Enzyme Conjugate - 1 vial of Peroxidase labeled Calcitonin Antibody. Ready to use.

Reconstitution Solution - 1 vial of reconstitution solution containing EDTA.

Wash Buffer Concentrate - 1 vial of 20-fold concentrated saline solution with surfactant.

Substrate - 1 vial of TMB Substrate. Ready to use.

Stop Solution - 1 vial of 1 N Sulfuric Acid. Ready to use.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8º C. Do not use past the kit expiration date.</td></tr>
<tr><td rowspan="9">Opened Reagents</td><td>Calibrators</td><td rowspan="2">Store unused calibrators and controls at -20 ºC for up to 6 weeks.</td></tr>
<tr><td>Controls</td></tr>
<tr><td>Biotinylated Calcitonin Antibody</td><td rowspan="4">Store at 2 - 8 ºC.</td></tr>
<tr><td>Enzyme Conjugate</td></tr>
<tr><td>Substrate</td></tr>
<tr><td>Reconstitution Solution</td></tr>
<tr><td>Stop</td><td rowspan="2">Store at room temperature.</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>Microplate wells</td><td>Return unused wells to the foil pouch containing the desiccant and seal. Store at 2 - 8 ºC.</td></tr>
</table>

SUPPLIES REQUIRED BUT NOT PROVIDED

- Microplate Reader
- Microplate Washer
- Pipettes or pipetting equipment with disposable polypropylene tips
- Multi-channel pipette
- Disposable polypropylene test tubes
- Glass measuring cylinders
- Distilled or deionised water

PRECAUTIONS

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.

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

CRITICAL PARAMETERS

- Allow samples and all reagents to equilibrate to room temperature (22–25 °C) prior to performing the assay. This is especially important for the TMB Substrate.
- Adhere to recommended incubation temperatures in the protocol as variations may cause inconsistent or poor assay results.
- It is essential that all wells are washed thoroughly and uniformly. When washing is done by hand, use a squeeze bottle and ensure that all wells are completely filled and emptied at each step.
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
- A separate standard curve must be run on each plate.
- Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.
- Mix all reagents thoroughly prior to use, but avoid foaming!
- Keep the wells sealed with the plate sealer except when adding reagents and during reading.
- Any variation in the protocol can cause variation in binding!
- The kit should not be used beyond the expiration date on the kit label.
- The values obtained by the samples should be within the standard range. If this is not the case, dilute the sample and repeat the assay.
- We take great care to ensure that this product is suitable for all validated sample types, as designated in this manual. Other sample types may be tested and validated by the user.

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, theserum should be promptly separated, preferably in a refrigerated centrifuge, and stored at -20° C or lower.

REAGENT PREPARATION

Note: All reagents should be stored at the recommended temperatures. Bring all reagents to room temperature (22 - 25 °C) before use. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.

Wash buffer Concentrate - Bring to room temperature before use. Wash Buffer may exhibit precipitation when stored at cold temperatures. Mix thoroughly before use. Add 30 mL of Wash Buffer Concentrate to 570 mL deionized or distilled water and mix.

Calibrators - Reconstitute Calibrator A with 2 mL of distilled or deionized water and mix. For Calibrators B through F, reconstitute each vial with 1 mL of Reconstitution Solution and mix. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to ensure complete reconstitution. **use immediately following reconstitution.** Store unused calibrators at -20°C for up to 6 weeks.

Controls - Reconstitute each vial with 1 mL of Reconstitution Solution and mix. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to ensure complete reconstitution. **use immediately following reconstitution.** Store unused controls at -20°C for up to 6 weeks.

ASSAY PROTOCOL

Read the entire protocol before beginning the assay. It is recommended that all standards and samples be assayed in dulplicate. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommend- ed to briefly centrifuge these reagents before use. *Note: Reagents and samples may require specific handling temperatures and need preparation prior to the assay. See the Reagent and Sample Preparation sections before proceeding*.

1. Prepare all reagents and samples as described in the previous sections.
2. Remove any excess microplate strips from the plate fram and return them to the foil pouch containing the desiccant pack.

Calibrator/Sample/Antibody Incubation

3. Pipet 100 µL of calibrator, control or sample into duplicate wells. Freeze (-20 °C) the remaining calibrators and controls as soon as possible after use.
4. Pipette 50 µL of Biotinylated Antibodyinto each of the wells which already contain the sample.
5. Pipette 50 µL of Enzyme Conjugate into each of the same wells.
6. Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light. Incubate on an orbital shaker or rotator set at 170 ± 10 rpm for 4 hours ± 30 minutes at room temperature (22 - 25°C).

Wash

7. Aspirate and wash each well five (5) times with the Working Wash Solution, using an automatic microplate washer. Blot dry by inverting the plate on an absorbent material. The wash solution volume should be set to dispense 0.35 mL into each well.

Substrate Incubation

8. Add or dispense 150 µL of the Substrate Solution into each of the wells.
9. With appropriate cover to avoid light exposure, place the microplate(s) on an orbital shaker or rotator set at 170 ± 10 rpm for 30 ±5 minutes at room temperature (22 - 28°C).

Stop reaction

10. Add or dispense 100 µL of the Stop Solution into each of the wells. Mix gently.
11. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm against 250 µL 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.

SUMMARY

Prepare reagents and samples as previously described.

↓

Pipette 100 µL calibrator, control or sample in duplicate into the wells.

↓

Pipette 50 µL Biotinylated Antibody into the wells.

↓

Pipette 50 µL Enzyme Conjugate into the wells.

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Cover and Incubate 4 hours at RT (22 - 25 °C) on a shaker.

↓

Aspirate and wash 5 times.

↓

Add 150 µL of Substrate to each well. Cover and Incubate 30 minutes at RT on a shaker.

↓

Add 100 µL of Stop Solution to each well. Read at 450 nm

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, Biotinylated Antibody and Enzyme Labeled Antibody in a clean amber bottle. The combined reagent is stable for seven (7) days when stored at 4°C. Then use 100 µL of the mixed antibody into each well. This alternative method should replace Step (4) and (5), to be followed with the incubation with orbital shaker.

CALCULATION OF RESULTS

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.

Manual 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] can generally give a good curve fit.

SAMPLE DATA

Data obtained at 450 nm (raw A.U. readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	Calcitonin pg/mL	Calcitonin result to report (pg/mL)
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	*
Sample 1	0.034	0.040	0.037	4.7	4.7
Sample 2	0.104	0.098	0.101	16.3	16.3
Sample 3	0.397	0.411	0.404	68.7	68.7
Sample 4	2.195	2.173	2.184	>300	*

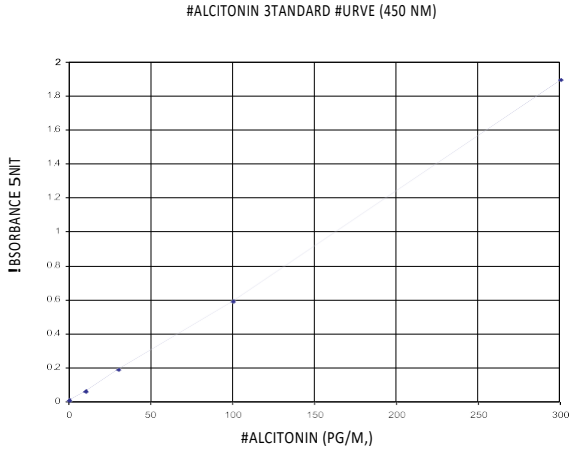
**Because the concentration is >300 pg/mL, it is recommended to use the data obtained at 405 nm.*

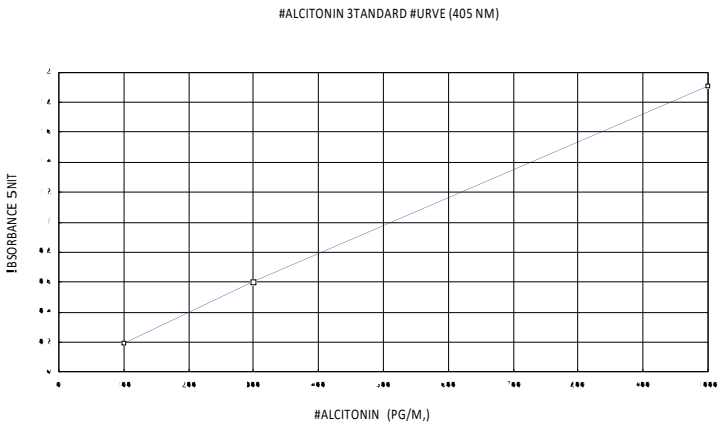
Data obtained at 405 nm (raw A.U. readout against distilled or deionized water):

Well	1st Reading (A.U.)	2nd Reading (A.U.)	Average A.U.	Calcitonin pg/mL	Calcitonin result to report (pg/mL)
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	0.815	403	403
Sample 1	0.016	0.020	0.018	<300	*
Sample 2	0.039	0.035	0.037	<300	*
Sample 3	0.128	0.134	0.131	<300	*
Sample 4	0.697	0.689	0.693	345	345

For samples with a readout <300 pg/mL, it is recommended to use the data obtaind at 450 nm.This practice should give the results with optimum sensitivity of the assay.

NOTE: The data presented is 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.

LIMITATIONS OF THE PROCEDURE

The 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. Samples from patients routinely exposed to animal or animal serum products may contain heterophilic antibodies causing atypical results. This assay has been formulated to mitigate the risk of this type of interference. However, potential interactions between rare sera and test components an occur.

EXPECTED VALUES

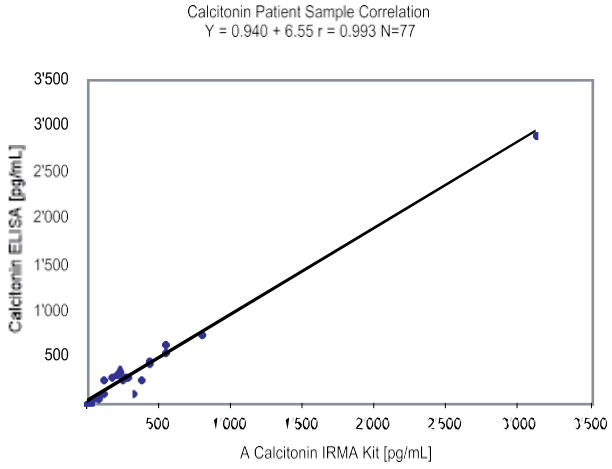
It is recommended that each laboratory establish its own reference range. The data provided should be used only as a guideline. Calcitonin levels were measured in fifty-nine (59) apparently normal female individuals and fifty-two (52) apparently normal male individuals with the Calcitonin ELISA. The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

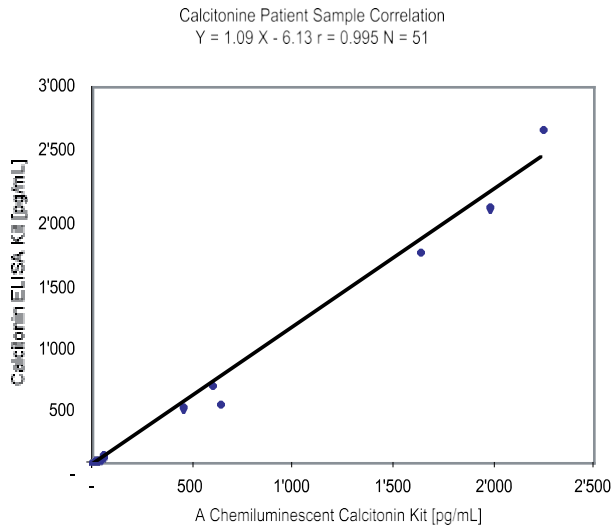
Seventy-seven (77) patient samples, with calcitonin values ranging from 0.8 to 3,113 pg/mL were assayed by the ELISA procedure and an ImmunoRadioMetricAssay Calcitonin (IRMA Kit). Linear regression analysis gives the following statistics:

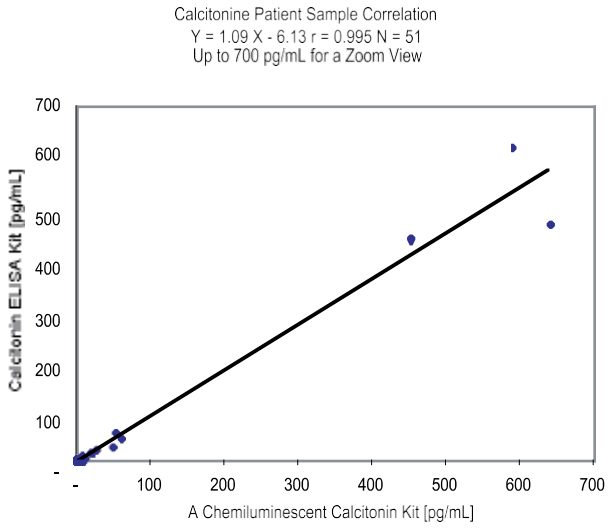
Calcitonin ELISA = 0.940 IRMA kit + 6.55 pg/mL
r = 0.993, N = 123



Further, fifty-one (51) patient samples, with calcitonin values ranging from < 0.7 to 2,240 pg/mL were assayed by the ELISA procedure and a Chemiluminescence Immunoassay for Calcitonin Kit [or ImmunoChemiluminescentMetricAssay (ICMA)]. Linear regression analysis gives the following statistics:

Calcitonin ELISA = 1.094 ICMA kit - 6.13 pg/mL
r = 0.995, 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 Calcitonin ELISA has a calculated sensitivity of 1.0 pg/mL.

Reproducibility

Intra-assay precision (Precision within an assay) - The intra-assay precision was measured by assaying three control samples 20 times on one plate.
Inter-assay precision (Precision between assays) - The inter-assay precison was assessed by repeated measurements of three control samples in 15 different assays by 3 technicians on 2 different lots of reagents, over a 3 week period.

	Intra-assay precision			Inter-assay precision		
Control	A	B	C	A	B	C
mean (pg/mL)	24.3	94.9	403	16.5	64.5	340
n	20	20	20	15	15	15
Cv (%)	5.7	4.3	2.8	7.4	7.4	6.1

SPECIFICITY AND CROSS-REACTIVITY

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

Cross-reactant	Concentration of Cross-reactant (pg/mL)	Calcitonin without cross-reactant (pg/mL)	Calcitonin with cross-reactant (pg/mL)	Change in Calcitonin (pg/mL)	% Cross-reactivity
PTH (1-84)	100,000	186	194	8	0.008%
	30,000	186	200	14	0.047%
	10,000	186	194	8	0.08%
Calcitonin Gene Related Peptide	1,000,000	200	202	2	0.0002%
	100,000	200	204	4	0.004%
Salmon Calcitonin	1,000,000	191	194	3	0.0003%
	100,000	191	199	8	0.008%
TSH	5000 μIU/mL	198	203	5	0.00061%
	500 μIU/mL	198	193	0	0%
	50 μIU/mL	198	199	1	0.0122%

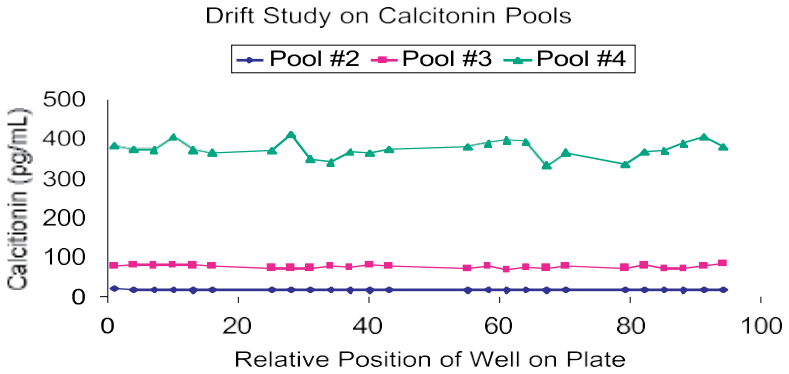
RECOVERY

Various amounts of Calcitonin were added to four different patient sera to determine the recovery.

Serum Sample	Endogenous	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%

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). The results, displayed in the following graphs, show no significant assay drift.



LINEARITY OF PATIENT SAMPLE DILUTIONS: PARALLELISM

Six patient serum samples were diluted with Calibrator A (Zero Calibrator).

Sample	dilution	Expected (pg/mL)	observed (pg/mL)	%observed/Expected
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	88%
	1:8	249	223	89%
	1:16	125	119	95%

TROUBLESHOOTING

problem	recommendation
Low Absorbance	• Check reagents for proper storage • Control expiration date. • Check preparation of reagents. • Control incubation times and temperature. • Check reader wavelength.
High Absorbance/high zero standard value	• Check preparation of reagents. • Control incubation times and temperature. • Equilibrate ELISA reagents to room temperature (22 - 25 °C). • Ensure that every well of the ELISA plate is completely filled and emptied at every wash step. • Check that plates are blotted on tissue paper after washing.
Flat cure/poor reproduciblity	• Check reagents for proper storage. • Control expiration date. • Check preparation of working standards. • Check incubation times and temperatures. • Use separate reservoirs for pipetting different solutions with multichanned pipettes. Always use new pipette tips. • Check pipette calibration. • Ensure efficient washing procedure.

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