Monobind Inc.
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AccuLite
CLIA Microwells

Total Thyroxine *(T4)*
Test System
Product Code: *275-300*

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Total Thyroxine Concentration in Human Serum or Plasma by a Microplate Chemiluminescence Immunoassay (CLIA)

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum thyroxine concentration is generally regarded as an important *in-vitro* diagnostic test for assessing thyroid function. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the last three decades. This procedural evolution can be traced from the empirical protein bound iodine (PBI) test (1) to the theoretically sophisticated radioimmunoassay (2).

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T4 conjugate is added, and then the reactants are mixed. A competition reaction results between the enzyme conjugate and the native thyroxine for a limited number of antibody combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound enzyme-thyroxine conjugate is separated from the unbound enzyme-thyroxine conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

The employment of several serum references of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyroxine concentration.

3.0 PRINCIPLE

Competitive Enzyme Immunoassay (Type 5)
The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen. Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubulized binding sites. The interaction is illustrated by the following equation:

$$Enz_{Ag} + Ag + Ab_{C.W.} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{C.W.} + Enz_{AgAb_{C.W.}}$$

$Ab_{C.W.}$ = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
Enz_{Ag} = Enzyme-antigen Conjugate (Constant Quantity)
$AgAb_{C.W.}$ = Antigen-Antibody Complex
$Enz_{Ag\,Ab_{C.W.}}$ = Enzyme-antigen Conjugate -Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity, determined by reaction with a substrate that generates light, in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. T4 Calibrators – 1.0 ml/vial - Icons A-F
Six (6) vials of serum reference for thyroxine at concentrations of 0 (**A**), 2.0 (**B**), 5.0 (**C**), 10.0 (**D**), 15.0 (**E**) and 25.0 (**F**) µg/dl. Store at 2-8°C. A preservative has been added. For SI units: µg/dl x 12.9 = nmol/L.

B. Total T4-Tracer – 1.5 ml/vial - Icon Ⓔ
One (1) vial of thyroxine-horseradish peroxidase (HRP) conjugate in a stabilized protein matrix. A preservative has been added. Store at 2-8°C.

C. Total T3/T4 Tracer Buffer – 13 ml - Icon Ⓑ
One (1) bottle reagent containing buffer, red dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. T4 Light Reaction Wells – 96 wells - Icon
One 96-well white microplate coated with sheep anti-thyroxine serum and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

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

F. Signal Reagent A – 7.0ml/vial - Icon C[A]
One (1) bottle containing luminol in a buffer. Store at 2-8°C.

G. Signal Reagent B -- 7.0ml/vial - Icon C[B]
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Product Insert.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8ºC. Kit and component stability are indentified on the label.**
Note 3: Above reagents are for a 96-well microplate. For other kit configurations, see table at the end of the IFU.

4.1 Materials Required [But Not Provided]:

1. Pipette capable of delivering 25µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.350ml volumes with a precision of better than 1.5%.
3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and signal preparation.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Luminometer
6. Test tubes for preparation of enzyme conjugate and signal reagent A and B.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. 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 required tests. 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 or plasma 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. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8^{0}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^{0}C for up to 30 days. Avoid use of contaminated devices. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range 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 standard curve for run-to-run reproducibility. In addition, maximum light intensity 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.

8.0 REAGENT PREPARATION:

1. **Working T4 Tracer Reagent**
   Dilute the T4-Tracer 1:11 with Total T3/T4 tracer buffer in a suitable container. For example, dilute 160µl of conjugate with 1.6ml of buffer for 16 wells (A slight excess of solution is made). This reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.
   General Formula:
   Amount of Buffer required = Number of wells * 0.1
   Quantity of T4 Enzyme necessary = # of wells * 0.01
   i.e. = 16 x 0.1 = 1.6ml for Total T3/T4 Conjugate Buffer
   16 x 0.01 = 0.16ml (160µl) for T4 enzyme conjugate
2. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.
3. **Working Signal Reagent Solution -** Store at 2 - 8°C.
   Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references 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, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8ºC.**
2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of Working T4 Tracer Reagent to all wells (see Reagent Preparation Section).
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 45 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) 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 four (4) additional times.**
8. Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**
9. Incubate at room temperature for five (5) minutes in the dark.
   DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
10. Read the relative light units in each well with a Chemiluminescence microplate reader for 0.5-1.0 seconds. **The results should be read within 30 minutes after adding signal reagent.**

Note: For reassaying specimens with concentrations greater than 25 µg/dl, pipet 12.5µl of the specimen and 12.5µl of the 0 serum reference into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the thyroxine concentration.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

1. Record the RLU's obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU's for each duplicate serum reference versus the corresponding T4 concentration in µg/dl on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of T4 for an unknown, locate the average RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/dl) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (49979) of the unknown intersects the calibration curve at (6.6µg/dl) T4 concentration (See Figure 1).

Note 1: Computer data reduction software designed for chemiluminescence assays may also be used for the data

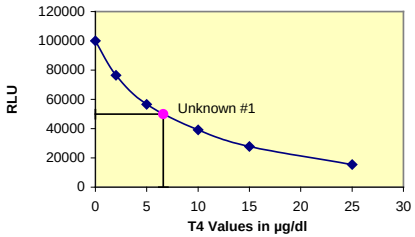
reduction. If such software is utilized the software should be ascertained.

Example 1

Sample I.D.	Well Number	RLU's (A)	Mean RLU's (B)	Value (µg/dl)
Cal A	A1	99846	100000	0
	B1	100154		
Cal B	C1	75846	76437	2
	D1	77028		
Cal C	E1	57556	56647	5
	F1	55738		
Cal D	G1	39680	39196	10
	H1	38712		
Cal E	A2	27634	27833	15
	B2	28033		
Cal F	C2	15381	15417	25
	D2	15454		
Ctrl 1	E2	53675	53593	***5.8***
	F2	53512		
Ctrl 2	G2	29566	29355	***14.0***
	H2	29144		
Patient 1	A3	50095	49979	***6.6***
	B3	49864		

* 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. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1



11.0 Q.C. PARAMETERS

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

1. The Dose Response Curve should be within established parameters.
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 signal reagent initiates a kinetic reaction, therefore the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. 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.
9. Patient Specimens with T4 concentrations greater than 25 µg/dl can be diluted by pipeting12.5µl of the specimen and 12.5µl of the 0 serum reference into the sample well; this maintains a uniform protein concentration. Multiply the readout value by 2 to obtain the thyroxine concentration.
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 AccuLite® CLIA procedure have been formulated to eliminate maximal interference; however, potential interactions between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems 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. Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of thyroxine to TBG (3, 4). Thus, total thyroxine concentration alone is not sufficient to assess clinical status.
8. Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.
9. A decrease in total thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates. A table of interfering drugs and conditions which affect total thyroxine values has been compiled by the Journal of the American Association of Clinical Chemists.

"NOT INTENDED FOR NEWBORN SCREENING"

13.0 EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values for the T4 AccuLite™ CLIA method. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1.

TABLE I

Expected Values for the T4 AccuLite™ CLIA (in µg/dl)

	Male	Female *
Number of Specimens	46	61
Mean (X)	7.5	8.3
Std.Dev (σ)	1.6	1.8
Expected Ranges (±2 σ)	4.3 – 10.7	4.7 – 11.9

*Normal patients with high TBG levels were **not** excluded except if pregnant.

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 precision of the T4 AccuLite™ CLIA were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2

Within Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	16	3.7	0.25	6.8%
Normal	16	8.6	0.31	3.6%
High	16	14.5	0.82	5.7%

TABLE 3

Between Assay Precision (Values in µg/dl)

Sample	N	X	σ	C.V.
Low	10	3.4	0.22	6.4%
Normal	10	8.8	0.37	4.3%
High	10	14.2	0.77	5.5%

*As measured in ten experiments in duplicate over a ten day period.

14.2 Sensitivity

The thyroxine procedure has a sensitivity of 80 pg/well. This is equivalent to a sample containing a concentration of 0.318µg/dl. The sensitivity was ascertained by determining the variability of the 0 µg/dl serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The T4 AccuLite™ CLIA method was compared with a microplate Elisa method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.5µg/dl – 28µg/dl). The total number of such specimens was 120. The least square regression equation and the correlation coefficient were computed for this method in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method	8.37	y = 0.18+0.965(x)	0.965
Reference	8.25		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

14.4 Specificity

The cross-reactivity (Specificity) of the thyroxine antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of thyroxine needed to displace the same amount of tracer.

Substance	Cross Reactivity	Concentration
l–Thyroxine	1.0000	-
d-Thyroxine	0.9800	10µg/dl
d-Triiodothyronine	0.0150	100µg/dl
l–Triiodothyronine	0.0300	100µg/dl
Iodothyrosine	0.0001	100µg/ml
Diiodothyrosine	0.0001	100µg/ml
Diiodothyronine	0.0001	100µg/ml

15.0 REFERENCES

1. Barker, S.B., "Determination of Protein Bound Iodine." *Journal Biological Chemistry* **173-175.** (1948)
2. Chopra, I.J., Solomon, D.H., an Ho, R.S., "A Radioimmunoassay of Thyroxine," *J. Clinical EndocrinoL* **33,** 865. (1971)
3. Young, D.S., Pestaner, L.C., and Gilberman, U., "Effects of Drugs on Clinical Laboratory Tests." *Clinical Chemistry* **21,** 3660. (1975)
4. Sterling, L., Diagnosis and Treatment of Thyroid Disease, Cleveland *CRC Press* **P. 19-51.** (1975)
5. Rae P, Farrar J, Beckett G, Toft A, 'Assessment of thyroid status in elderly people.' *British Med. Jour* **307,** 177-180. (1993)
6. Charkes ND, "The many causes of subclinical hyperthyroidism." *Thyroid* **6,** 391-396. (1996)
7. Chou FF, Wang PW, Huang SC, 'results of Subtotal Thyroidectomy for Graves' disease.' *Thyroid* **9,** 253-256.
8. Muzzaffari EL, Gharib H, "Thyroxine suppressive therapy in patients with nodular thyroid disease." *Ann Intern Med* **128,** 386-394. (1998)
9. Attwood EC, Seddon RM, Probert DE: "The T4/TBG ratio and the investigation of thyroid function." *Clin Biochem* **11,** 218. (1978)
10. Jain R, Isaac RM, Gottschalk ME et al: I'Transient central hypothyroidism as a cause of failure to thrive in newborns and infants.' *J. Endocrinology Invest* **17,** 631-637. (1994)

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Cat #: *275-300*

Size		96(A)	192(B)	480(D)	960(E)
Reagent (fill)	A)	1ml set	1ml set	2ml set	2ml set x2
	B)	1 (1.5ml)	2 (1.5ml)	1 (8ml)	2 (8ml)
	C)	1 (13ml)	2 (13ml)	1(60ml)	2 (60ml)
	D)	1 plate	2 plates	5 plates	10 plates
	E)	1 (20ml)	1 (20ml)	1 (60ml)	2 (60ml)
	F)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	G)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)

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