Monobind Inc.
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AccuLite
CLIA Microwells

Free Triiodothyronine (Free T3) Test System

Product Code: 1375-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Free Triiodothyronine Concentration in Human Serum by a Microplate Chemiluminescence Immunoassay (CLIA).

Levels of Free T3 are thought to reflect the amount of T3 available to the cells and may therefore determine the clinical metabolic status of T3.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Triiodothyronine, a thyroid hormone, circulates in blood almost completely bound (>99.5%) to carrier proteins (1, 2). The main transport protein is thyroxine-binding globulin (TBG). However, only the free (unbound) portion of triiodothyronine is believed to be responsible for the biological action. Further, the concentrations of the carrier proteins are altered in many clinical conditions, including pregnancy. In normal thyroid function as the concentrations of the carrier proteins alters, the total triiodothyronine level changes so that the free triiodothyronine concentration remains constant. Thus, measurements of free triiodothyronine concentrations correlate more reliably with clinical status than total triiodothyronine levels.

For example, the increase in total triiodothyronine levels associated with pregnancy, oral contraceptives and estrogen therapy result in higher total T3 levels while the free T3 concentration remains basically unchanged.

This microplate chemiluminescence immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations in a direct determination of free T3. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-T3 conjugate (analog method) is added, and then the reactants are mixed. A competition reaction results between the enzyme conjugate and the free triiodothyronine for a limited number of antibody combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound enzyme-triiodothyronine conjugate is separated from the unbound enzyme-triiodothyronine 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 free triiodothyronine 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 free triiodothyronine concentration.

3.0 PRINCIPLE

Competitive Chemiluminescence Immunoassay – Analog Method for Free T3 (Type 5)

The essential reagents required for a solid phase enzyme immunoassay include immobilized T3 antibody, enzyme-T3 conjugate and native free T3 antigen. The enzyme-T3 conjugate should have no measurable binding to serum proteins especially TBG and albumin. The method achieves this goal.

Upon mixing immobilized antibody, enzyme-T3 conjugate and a serum containing the native free T3 antigen, a competition reaction results between the native free T3 and the enzyme-T3 conjugate for a limited number of insolubulized binding sites. The interaction is illustrated by the followed equation:

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

$Ab_{C.W.}$ = Specific 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 free 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. Free T3 Calibrators – 1ml/vial - Icons A-F
Six (6) vials of serum reference for free triiodothyronine at approximate* concentrations of 0 (**A**), 1.0 (**B**), 3.0 (**C**), 5.0 (**D**), 8.0 (**E**) and 16.0 (**F**) pg/ml. Store at 2-8°C. A preservative has been added. For SI units: 1pg/ml x 1.536 = pmol/L .
* Exact levels are given on the labels on a lot specific basis.

B. Free T3 Tracer Reagent – 13ml/vial - Icon Ⓔ
One (1) vial of triiodothyronine -horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

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

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

E. Signal Reagent A – 7ml/vial - Icon C[A]
One (1) bottle containing luminol in buffer. Store at 2-8°C (see Reagent Preparation Section).

F. Signal Reagent B – 7ml/vial - Icon C[B]
One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C (see Reagent Preparation Section).

G. Product Insert.

4.1 Required but not provided:

1. Pipette capable of delivering 50µl 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 and (200-1000µl) dispenser(s) for substrate dilutions.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate luminometer.
6. Test tubes for dilution of signal A & 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.

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 2-8°C.Kit and component stability are identified on the label.**
Note 3: Above reagents are for a 96-well microplate. For other kit configurations, see table at the end of this IFU.

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 comonenets must be according to local regulatory and statutory requirements.

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. 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 repetitive freezing and thawing. When assayed in duplicate, 0.100ml 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. 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 Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at room 2-30°C for up to 60 days.
2. **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 microplate 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 2-8°C**
2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of fT3-Tracer Reagent to all wells.
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 to all wells (see Reagent Preparation Section).). **Always add reagents in the same order to minimize reaction time differences between wells.**
   DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
9. Incubate for five (5) minutes in the dark.
10. Read the relative light units in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the signal solution.**

10.0 CALCULATION OF RESULTS

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

1. Record the RLU's (*Relative Light Units*) 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 Free T3 concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration Free T3 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 pg/ml) 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 (79179) of the unknown intersects the calibration curve at (2.45pg/ml) Free T3 concentration (See Figure 1)*.

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

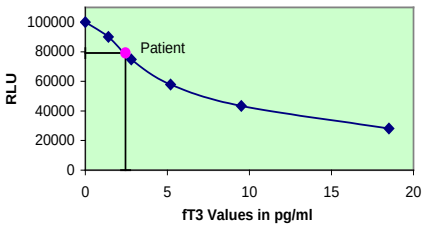
reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU (B)	Value (pg/ml)
Cal A	A1	98765	100000	0.00
	B1	101235		
Cal B	C1	90366	90042	1.0
	D1	89719		
Cal C	E1	74696	74761	3.0
	F1	74825		
Cal D	G1	58669	57943	5.0
	H1	57217		
Cal E	A2	41951	43463	8.0
	B2	44976		
Cal F	C2	28573	28138	16.0
	D2	27703		
Pat 1	E2	79942	79179	***2.45***
	F2	78416		

* 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 is 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. 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.
10. 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.
11. 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. ***Measurement 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. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. 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.
5. 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.
6. If a patient, for some reason, reads higher that the highest calibrator report as such (e.g. > 20pg/ml). ***Do not try to dilute the sample. TBG variations in different matrices will not allow Free T3 hormone to dilute serially***
7. Several drugs are known to affect the binding of Triiodothyronine to the thyroid hormone carrier proteins or its metabolism to T3 and complicate the interpretation of free T3 results (3).
8. Circulating autoantibodies to T3 and hormone-binding inhibitors may interfere (4).
9. Heparin has been reported to have *in vivo* and *in vitro* effects on free T3 concentration (5). Therefore, do not obtain samples in which this anti-coagulant has been used.
10. In severe nonthyroidal illness (NTI), the assessment of thyroid status becomes very difficult. TSH measurements are recommended to identify thyroid dysfunction (6).
11. Familial dysalbuminemic conditions may yield erroneous results on direct free T3 assays (7).

"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 Free T3 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 Free T3 AccuLite™ CLIA (in pg/ml)

	Adult	Pregnancy
Number of Specimens	110	75
Mean (X)	2.8	3.0
Standard Deviation (σ)	0.7	0.6
Expected Ranges (±2σ)	1.4 – 4.2	1.8 – 4.2

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 Free T3 AccuLite™ CLIA method were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation (σ) 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 pg/ml)

Sample	N	X	S.D.	C.V.
Low	20	2.00	0.18	9.0%
Normal	20	4.75	0.28	5.9%
High	20	8.24	0.54	6.6%

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

Sample	N	X	S.D.	C.V.
Low	10	2.11	0.22	10.4%
Normal	10	4.99	0.41	8.2%
High	10	8.06	0.70	8.7%

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

14.2 Sensitivity

This procedure has a sensitivity of 0.742 pg/ml. The sensitivity was ascertained by determining the variability of the 0 pg/ml serum calibrator and using the 2 σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The Free T3 AccuLite™ CLIA method was compared with a microplate enzyme immunoassay (**EIA**) method. Biological specimens from hypothyroid, euthyroid and hyperthyroid populations were used (The values ranged from 0.1pg/ml – 17pg/ml). The total number of such specimens was 181. The least square regression equation and the correlation coefficient were computed for this Free T3 CIA™ 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	3.1	y = 0.11+0.976(x)	0.985
Reference	3.2		

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 of the triiodothyronine 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 triiodothyronine needed to displace the same amount of tracer.

Substance	Cross Reactivity	Concentration
l-Triiodothyronine	1.0000	-
l-Thyroxine	< 0.0002	10µg/ml
Iodothyrosine	< 0.0001	10µg/ml
Diiodothyrosine	< 0.0001	10µg/ml
Diiodothyronine	< 0.0001	10µg/ml
Phenylbutazone	< 0.0001	10µg/ml
Sodium Salicylate	< 0.0001	10µg/ml

15.0 REFERENCES

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Cat #: 1375-300

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

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