Monobind Inc.
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AccuLite
CLIA Microwells

Thyroglobulin Ab (Anti-Tg) Test System

Product code: 1075-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Thyroglobulin (Tg) Autoantibodies in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Antibodies to thyroglobulin have been shown to be characteristically present from patients with thyroiditis and primary thyrotoxicosis[1, 2]. This has lead to the clinical measurement becoming a valuable tool in the diagnosis of thyroid dysfunction. Passive Hemaglutination (PHA) methods have been employed in the past for measurements of antibodies to Tg. PHA tests do not have the sensitivity of enzyme immunoassay and are limited by subjective interpretation. This procedure, with the enhanced sensitivity of chemiluminescence, permits the detectability of subclinical levels of antibodies to Tg. In addition, the results are quantitated by a luminometer, which eliminates subjective interpretation.

Autoantibodies to Tg are often present in patients with autoimmune thyroid disease. Approximately 10 percent of healthy individuals have autoantibodies to Tg at low levels; higher concentrations are found in 30% and 85% of patients with Graves' disease and Hashimoto's Thyroiditis respectively. Antibodies to thyroid peroxidase (TPO) occur more frequently than autoantibodies to Tg in these conditions thus rendering anti-Tg assays without any practical use. However, anti-Tg assays are useful while determining Tg levels in patients with thyroid conditions. The presense of Tg autoantibodies produces false results in determination of Tg levels both by competitive assays and by sandwitch immunoassays.

Monobind's microplate chemiluminescence immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, diluted patient specimen, or control is first added to a microplate well. Biotinylated thyroglobulin (Tg) is added, then the reactants are mixed. Reaction results between the auto-antibodies to Tg and the biotinylated Tg to form an immune complex, which is deposited to the surface of streptavidin coated wells through the high affinity reaction of biotin and streptavidin.

After the completion of the required incubation period, aspiration or decantation separates the reactants that are not attached to the wells. An anti-human IgG- enzyme conjugate is then added to permit quantitation of reaction through interacting with human IgG of the immune complex. After washing, the enzyme activity is determined by reaction with substrate to produce light (*luminescence)*.

The employment of several serum references of known antibody activity permits construction of a dose response curve (graph) of enzyme and antibody activities. From comparison to the dose response curve, an unknown specimen's enzyme activity can be correlated with auto-immune antibody level. The intensity of light is directly proportional to the concentration of anti-thyroglobulin antibody in the specimen.

3.0 PRINCIPLE

A Sandwich Sequential CLIA Method (Type 1)
The reagents required for the sequential CLIA assay include immobilized antigen, circulating auto-antibody and enzyme-linked species specific antibody. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated thyroglobulin antigen.

Upon mixing biotinylated antigen and a serum containing the auto-antibody, reaction results between the antigen and the antibody to form an immune-complex. The interaction is illustrated by the followed equation:

$$h\text{-}Ab_{(X\text{-}Tg)} + {}^{Btn}Ag_{(Tg)} \underset{k_a}{\overset{k_a}{\rightleftharpoons}} h\text{-}Ab_{(X\text{-}Tg)} - {}^{Btn}Ag_{(Tg)}$$

${}^{Btn}Ag_{(Tg)}$ = Biotinylated Antigen (Constant Quantity)
$h\text{-}Ab_{(X\text{-}Tg)}$= Human Auto-Antibody (Variable Quantity)
$Ab_{(X\text{-}Tg)} - {}^{Btn}Ag_{(Tg)}$ = Immune Complex (Variable Quantity)
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antigen. This interaction is illustrated below:
$h\text{-}Ab_{(X\text{-}Tg)} - {}^{Btn}Ag_{(Tg)} + \underline{Streptavidin}_{C.W.} \Rightarrow$ <u>immobilized complex</u> (IC)
<u>Streptavidin</u>$_{C.W.}$ = Streptavidin immobolized on well
<u>Immobilized complex (IC)</u> = sandwich complex bound to the solid surface

After the incubation time, the well is washed to separate the unbound components by aspiration and/or decantation. The enzyme linked species specific antibody (anti-h-IgG) is then added to the microwells. This conjugates binds to the immune complex that formed.
$I.C._{(h\text{-}IgG)} + {}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)} \Rightarrow {}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)} - I.C._{(h\text{-}IgG)}$
$I.C._{(h\text{-}IgG)}$ = Immobilized Immune complex (Variable Quantity)
${}^{ENZ}Ab_{(X\text{-}h\text{-}IgG}$ = Enzyme-antibody Conjugate (Constant Quantity)
${}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)} - I.C._{(h\text{-}IgG)}$ = Ag-Ab Complex (Variable Quantity)

The anti-h-IgG enzyme conjugate that binds to the immune complex in a second incubation is separated from unreacted material by a wash step. The enzyme activity, determined by reaction with a substrate that generates light, in this fraction is directly proportional to the antibody concentration in the specimen. By utilizing several different serum references of known antibody activity, a reference curve can be generated from which the antibody activity of an unknown can be ascertained

4.0 REAGENTS

Materials Provided:

A. Anti-Tg Calibrators – 1.0 ml/vial Icons A-F
Six (6) vials of references for anti-Tg at levels of 0(**A**), 50(**B**), 125(**C**), 500(**D**), 1000(**E**), and 2000(**F**) IU/ml. Store at 2-8°C. A preservative has been added.
Note: The calibrators, human serum based, were calibrated against the Medical Research Council (MRC) Research Standard A 65/93 for anti-thyroglobulin activity.

B. Tg Biotin Conjugate – 13 ml/vial – Icon ∇
One (1) vial of biotinylated thyroglobulin in a buffered matrix. A preservative has been added. Store at 2-8°C**.**

C. Anti-Tg Tracer Reagent – 13 ml/vial - Icon Ⓔ
One (1) vial of anti-human IgG-horseradish peroxidase (HRP) conjugate in a bufferred matrix. A preservative has been added. Store at 2-8°C**.**

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

E. Serum Diluent Concentrate – 20ml/vial
One (1) vial of serum diluent containing buffer salts and a dye. Store at 2-8°C (see Reagent Preparation Section).

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

G. Signal Reagent A – 7.0ml/vial - Icon C[A]
One (1) vial containing luminol in buffer. Store at 2-8°C (see Reagent Preparation Section).

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

I. 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 2-8°C. Kit and component stability are identified on the label.**
Note 3: Above reagents are for a single 96-well microplate.

4.1 Materials Required But Not Provided:
1. Pipet(s) capable of delivering 0.010ml (10µl) and 0.050ml (50µl) volume with a precision of <1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Microplate washer (optional) or squeeze bottle to wash plates.
4. Microplate luminometer.
5. Test tubes for dilution of patient samples and signal A /B.
6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.

5.0 PRECAUTIONS

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

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

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

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

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, medium and high 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. **Serum Diluent**
Dilute the serum diluent concentrate to 200ml in a suitable container with distilled or deionized water. Store at 2-8°C.
2. **Wash Buffer**
Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
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.
4. **Patient Sample Dilution (1/100)**
Dispense 0.010ml (10µl) of each patient specimen into 1ml of serum diluent. Cover and vortex or mix thoroughly by inversion. Store at 2-8°C for up to forty-eight (48) hours

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 2-8°C**
2. Pipette 0.050 ml (50µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.
3. Add 0.100 ml (100µl) of Tg Biotinylated Conjugate Solution.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 30 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 0.350ml (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 anti-Tg Tracer Reagent to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**
DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION
9. Incubate for thirty (30) minutes at room temperature.
10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
11. Add 0.350ml (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.**.
12. 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 SIGNAL ADDITION
13. Incubate at room temperature for five (5) minutes in the dark.
14. Read the RLUs (Relative Light Units) in each well in a microplate luminometer for at least 0.2 seconds/ well. **The results can be read within 30 minutes of adding the signal solution.**

Note: For re-assaying specimens with concentrations greater than 2000 IU/ml, dilute the sample an additional 1:5 or 1:10 using the original diluted material. Multiply by the dilution factor to obtain the concentration of the specimen.

10.0 CALCULATION OF RESULTS

A reference curve is used to ascertain the concentration of anti-Tg (x-Tg) in unknown specimens.

1. Record the RLUs obtained from the printout of the microplate luminometer as outlined in Example 1.
2. 2 Plot the mean RLUs for each duplicate serum reference versus the corresponding x-Tg activity in IU/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of x-Tg for an unknown, locate the average RLUs for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLUs (77757) of the unknown intersects the calibration curve at 1403 IU/ml x-Tg concentration (See Figure 1)*.

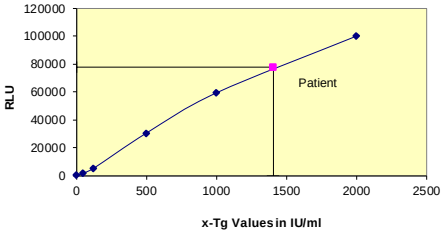
Note: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	RLUs (A)	Mean RLU (B)	Value (IU/ml)
Cal A	A1	197	209	0
	B1	220		
Cal B	C1	1851	1739	50
	D1	1626		
Cal C	E1	5513	5464	125
	F1	5416		
Cal D	G1	30663	30553	500
	H1	30443		
Cal E	A2	59172	58361	1000
	B2	57569		
Cal F	C2	99297	100000	2000
	D2	100703		
Ctrl 1	E2	806	843	***24.2***
	F2	880		
Ctrl 2	G2	82221	82403	***1512***
	H2	82586		
Patient 1	A3	77809	77757	***1403***
	B3	77705		

* 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 RLUs of the calibrators have been normalized to 100,000 RLUs for the F 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. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be 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 in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.
7. The presence of autoantibodies to Tg is confirmed when the serum level exceeds 125 IU/ml. The clinical significance of the result, coupled with anti-thyroid peroxidase activity, should be used in evaluating the thyroid condition. However, clinical inferences should not be solely based on this test but rather as an adjunct to the clinical manifestations of the patient and other relevant tests.
8. The cost benefits should be considered in the use of thyroglobulin antibodies testing when performed in concert with anti- thyroid perxidase (TPO). The widespread practice of performing both tests has been questioned[4].

13.0 EXPECTED RANGES OF VALUES

A study of normal population was undertaken to determine expected values for the Anti-Tg AccuLite® CLIA test system. The number (n), mean (x) and standard deviation (σ) are given in Table 1. Values in excess of 125IU/ml are considered positive for the presence of anti-Tg autoantibodies.

TABLE I
Expected Values for the Anti-Tg AccuLite® CLIA (in IU/ml)

Number (n)	100
Mean (X)	74.3
Standard deviation (σ)	25.2
Upper 95% (+2σ) level	124.7

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 Anti-Tg AccuLite® CLIA test system were determined by analyses on three different levels of pool control sera. The number, mean value, 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 IU/ml)

Sample	N	X	σ	C.V.
Pool 1	20	63.3	3.3	5.2%
Pool 2	20	224.3	14.5	6.5%
Pool 3	20	1498.1	67.4	4.5%

TABLE 3*
Between Assay Precision (Values in IU/ml)

Sample	N	X	σ	C.V.
Pool 1	10	67.0	5.7	8.5%
Pool 2	10	237.6	18.5	7.8%
Pool 3	10	1518.3	78.4	5.2%

*As measured in ten experiments in duplicate.

14.2 Sensitivity

This procedure has a sensitivity of 1.95 IU/ml. The sensitivity was ascertained by determining the variability of the '0 IU/ml' calibrator and using the 2σ (95% certainty) statistics to calculate the minimum dose.

14.3 Accuracy

The Anti-Tg AccuLite® CLIA test system was compared with a reference method. Biological specimens from normals, and disease states populations were used. The disease states included; Hashimoto's thyroiditis, Graves Disease, thyroid nodules as well as thyroid carcinoma. The total number of such specimens was 122. The least square regression equation and the correlation coefficient were computed this assay 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
Monobind	387.6	y = -11.2 + 0.989 (x)	0.992
Reference	404.2		

Only slight amounts of bias between the Anti-Tg AccuLite® CLIA test system 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

Interferences from ANA, DNA, thyroid perodidase (TPO) and rhematoid antibodies were found to be insignificant in the assay system.

15.0 REFERENCES

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

Glossary of Symbols
(EN 980/ISO 15223)

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