DRG® Anti-Thyroglobulin (Anti-Tg) (EIA-4110)

CE

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INTENDED USE

The Quantitative Determination of Thyroglobulin (Tg) Autoantibodies in Human Serum or Plasma by a Microplate Enzyme Immunoassay. Measurements of Tg autoantibodies may aid in the diagnosis of certain thyroid diseases such as Hashimoto's and Grave's as well as nontoxic goiter.

SUMMARY AND EXPLANATION OF THE TEST

Antibodies to thyroglobulin have been shown to be characteristically present from patients with thyroiditis and primary thyrotoxicosis1, 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 EIA, permits the detectability of subclinical levels of antibodies to Tg. In addition, the results are quantitated by a spectrophotometer, which eliminates subjective interpretation.

DRG®'s microplate enzyme 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, and then the reactants are mixed. Reaction results between the autoantibodies 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 enzyme anti-human IgG 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 color.

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

PRINCIPLE

A Sequential ELISA Method (TYPE 1)

The reagents required for the sequential ELISA assay include immobilized antigen, circulating autoantibody 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 autoantibody, reaction results between the antigen and the antibody to form an immune-complex. The interaction is illustrated by the followed equation:

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

${}^{Bn}Ag_{(Tg)}$ = Biotinylated Antigen (Constant Quantity)

$h\text{-}Ab_{(X\text{-}Tg)}$ = Human Auto-Antibody (Variable Quantity)

$Ab_{(X\text{-}Tg)}\text{-}{}^{Bn}Ag_{(Tg)}$ = 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:

$$h\text{-}Ab_{(X\text{-}Tg)} - {}^{Bn}Ag_{(Tg)} + \underline{Streptavidin}_{c.w.} \Rightarrow \underline{immobilized\ complex}\ (IC)$$

$\underline{Streptavidin}_{c.w.}$ = Streptavidin immobolized on well

Immobilized complex (IC) = 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.

$$I.C._{(h\text{-}IgG)} + {}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)} \Rightarrow {}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)}\text{-}\ I.C._{(h\text{-}IgG)}$$

$I.C._{(h\text{-}IgG)}$ = Immobilized Immune complex (Variable Quantity)

${}^{ENZ}Ab_{(X\text{-}h\text{-}IgG}$ = Enzyme-antibody Conjugate (Constant Quantity)

${}^{ENZ}Ab_{(X\text{-}h\text{-}IgG)}\text{-}\ I.C._{(h\text{-}IgG)}$ = 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 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

REAGENTS

Materials Provided:

A. Anti-Thyroglobulin Calibrators - 1m/lvial

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 using a 1st International Reference Preparation, which was assayed against the Medical Research Council (MRC)
Research Standard A 65/93 for anti-thyroglobulin activity.

B. Thyroglobulin Biotin Reagent – 13ml/vial

One (1) vial of biotinylated thyroglobulin in a buffering matrix.
A preservative has been added. Store at 2-8°C.

C. x-Tg Enzyme-antigen Reagent – 13ml/vial

One (1) vial of anti-human IgG-horseradish peroxidase (HRP) conjugates in a buffered matrix. A preservative has been added. Store at 2-8°C.

D. Streptavidin Coated Microplate -- 96 wells

One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Serum Diluent - 20ml

One (1) vial of serum diluent containing buffer salts and a dye. Store at 2-8°C.

F. Wash Solution Concentrate -- 20ml

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

G. Substrate A –7ml/vial

One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

H. Substrate B -- 7ml/vial

One (1) bottle containing hydrogen peroxide (H2O2) in buffer. Store at 2-8°C.

I. Stop Solution -- 8ml/vial

One (1) bottle of stop solution containing a strong acid (1N HCl). Store at 2-8°C.

J. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette capable of delivering 10µl & 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. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Test tube for patient dilution
9. Timer.
10. Quality control materials.

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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 nonreactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. 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.

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 (for serum) or evacuated tube(s) containing EDTA or heparin.. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.
Samples may be refrigerated at 2-8°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°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml of the diluted specimen is required.

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. Store at room temperature 20-27°C for up to 60 days.
3. Working Substrate Solution
Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.
Note: Do not use the working substrate if it looks blue.
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

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20-27°C).

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 Biotin Reagent.
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 60 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 (blot and tap) or aspirate. Repeat two (2) additional times for a total of three (3) 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 two (2) additional times.
8. Add 0.100 ml (100µl) of x-Tg Enzyme 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. Swirl the microplate gently, cover and incubate for thirty- (30) minutes at room temperature.
10. Repeat steps (6 & 7) as explained above.
11. Add 0.100 ml (100µl) of Working Substrate Solution 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

12. Incubate at room temperature for fifteen (15) minutes.
13. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
14. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty- (30) minutes of adding the stop 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.

CALCULATION OF RESULTS

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

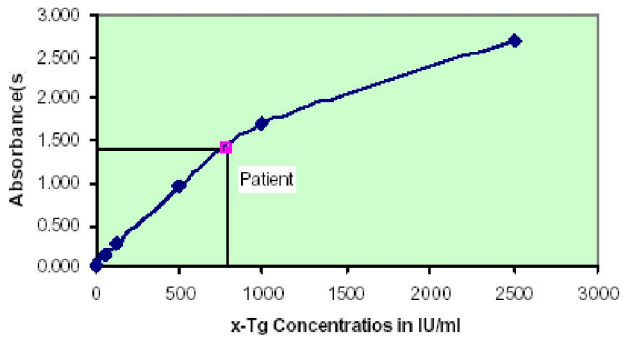
1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding anti-Tg activity in IU/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the level of anti-Tg activity for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in IU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance 1.387 (intersects the dose response curve) at 790 IU/ml anti-Tg concentration (See Figure 1). *

Example 1

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (IU/ml)
Cal A	A1	0.022	0.025	0
	B1	0.028		
Cal B	C1	0.135	0.133	50
	D1	0.131		
Cal C	E1	0.280	0.270	125
	F1	0.261		
Cal D	G1	0.962	0.949	500
	H1	0.936		
Cal E	A2	1.709	1.703	1000
	B2	1.698		
Cal F	C2	2.730	2.698	2500
	D2	2.667		
Patient	E2	1.390	1.387	**790**
	F2	1.383		

*The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a standard curve prepared with each assay.

Figure 1



Q.C. PARAMETERS

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

1. The absorbance (OD) of calibrator 'F' should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

Quality Control

Each laboratory should assay controls at levels in the normal, borderline and elevated 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. In addition, maximum absorbance 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.

RISK ANALYSIS

Assay Performance

1. It is important that the time of reaction in each well is held constant for 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. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be performed in the same sequence to eliminate any time deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Very high concentration of anti-Tg in patient specimens can contaminate samples immediately following these extreme levels. Bad duplicates are indicative of cross contamination. Repeat any sample, which follows any patient specimen with over 3.0 units of absorbance.
10. Samples, which are contaminated microbiologically, should not be used.
11. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from these instructions may yield inaccurate results.
12. 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.
13. 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.
14. Risk Analysis: as required by CE Mark IVD Directive 98/79/EC – for this and other devices, made by DRG, can be requested via e-mail: corp@drg-international.com

B. Interpretation

1. 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.
2. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
3. 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. DRG shall have no liability.
4. 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.
5. 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.
6. 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].

EXPECTED RANGES OF VALUES

A study of normal population was undertaken to determine expected values for the Anti-Tg Microplate 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 ELISA Test System
(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.

PERFORMANCE CHARACTERISTICS

A. Precision

The within and between assay precision of the Anti-Tg Microplate ELISA procedure 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	65.5	3.3	5.0%
Pool 2	20	385.5	15.5	4.0%
Pool 3	20	1554.4	55.4	3.6%

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

Sample	N	X	σ	C.V.
Pool 1	10	66.8	3.6	5.3%
Pool 2	10	374.2	18.5	4.9%
Pool 3	10	1625.5	65.2	4.0%

*As measured in ten experiments in duplicate.

B. Accuracy

The DRG® anti-Tg microplate ELISA was compared with a reference microplate anti-Tg ELISA. 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 82. The least square regression equation and the correlation coefficient were computed for the anti-Tg ELISA 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	415.6	y = 9.79 + 0.969 (x)	0.995
Reference	419.2		

Only slight amounts of bias between the anti-Tg ELISA 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.

C. Sensitivity

The anti-thyroglobulin (Tg) ELISA has a sensitivity of 5 IU/ml.

D. Specificity

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

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