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## Anti-Thyroid Peroxidase (Anti-TPO)



REF

E2-023

IVD

FOR INVITRO DIAGNOSTIC USE ONLY

**Intended Use:** The Quantitative Determination of Thyroid Peroxidase (TPO) Autoantibodies in Human Serum or Plasma by a Microplate Enzyme Immunoassay. Measurements of TPO 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 thyroid peroxidase have been shown to be characteristically present from patients with Hashimoto thyroiditis (95%), idiopathic myxedema (90%) and Graves Disease (80%)<sup>1</sup>.

In fact 72% of patients positive for anti-TPO exhibit some degree of thyroid dysfunction.<sup>2</sup> This has led to the clinical measurement becoming a valuable tool in the diagnosis of thyroid dysfunction.

Measurements of antibodies to TPO have been done, in the past, by Passive Hemagglutination (PHA). 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 TPO. In addition, the results are quantitated by a spectrophotometer, which eliminates subjective interpretation.

Immunospec 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 Thyroid Peroxidase Antigen (TPO) is added, and then the reactants are mixed. Reaction results between the autoantibodies to TPO and the biotinylated TPO 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.

### REAGENTS

#### A. x-TPO Calibrators – 1.0 ml/vial -

Six (6) vials of references for anti-TPO at levels of 0, 25, 50, 100, 250 and 500 IU/ml. Store at 2-8°C. A preservative has been added.

**Note:** The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the Medical Research Council (MRC) International Standard 66/387 for anti thyroid microsome.

#### B. TPO Biotin Reagent – 13ml/vial -

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

#### C. Anti-TPO Enzyme Reagent – 13ml/vial

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

#### D. 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 concentrate containing buffer salts and yellow 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 (H<sub>2</sub>O<sub>2</sub>) in buffer. Store at 2-8°C.

#### I. Stop Solution – 8ml/vial -

One (1) bottle 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. Pipettes capable of delivering 10, 25 & 50µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml 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(s) for patient dilution.
9. Timer.
10. Quality control materials.

### 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 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," 2<sup>nd</sup> 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 samples(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.05ml of the specimen is required.

**REAGENT PREPARATION:**

**1. Serum Diluent**

Dilute the serum diluent 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 0.025 ml (25µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.
3. Add 0.100 ml (100µl) of the TPO 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 300µl 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 the x-TPO 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. 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 500 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-TPO 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-TPO 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-TPO 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 (1.323) intersects the dose response curve at 200 IU/ml anti-TPO concentration (See Figure 1).

**Note:** Computer data reduction software designed for IEMA (ELISA) assays may also be used for the data reduction.

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (IU/ml)
Cal A	A1	0.022	0.026	0
	B1	0.030		
Cal B	C 1	0.240	0.244	25
	D 1	0.247		
Cal C	E 1	0.437	0.430	50
	F 1	0.422		
Cal D	G 1	0.795	0.788	100
	H 1	0.782		
Cal E	A 2	1.610	1.590	250
	B 2	1.572		
Cal F	C 2	2.659	2.600	500
	D 2	2.533		
Patient	E 2	1.294	1.323	200
	F 2	1.351		

\*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.

**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.

**LIMITATIONS OF PROCEDURE**

**A. Assay Performance**

1. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

2. 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 added in the same sequence to eliminate any time deviation during reaction.
3. Plate readers measure vertically. Do not touch the bottom of the wells.
4. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
5. Very high concentration of anti-TPO 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.
6. Samples, which are contaminated microbiologically, should not be used.

#### B. Interpretation

1. 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.
2. The presence of autoantibodies to TPO is confirmed when the serum level exceeds 40 IU/ml. The clinical significance of the result, coupled with anti-thyroglobulin 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.

#### EXPECTED RANGES OF VALUES

A study of normal population was undertaken to determine expected values for the anti-TPO Immunospec ELISA test system.

The number (n), mean (x) and standard deviation ( $\sigma$ ) are given in Table 1. Values in excess of 40IU/ml are considered positive for the presence of anti-TPO autoantibodies.

**TABLE 1**  
Expected Values for the Anti-TPO ELISA Test System (In IU/ml)

Number	100
Mean	17.6
Standard deviation	10.8
Upper 95% (+2 $\sigma$ ) level	39.2

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.

#### PERFORMANCE CHARACTERISTICS

##### A. Precision

The within and between assay precisions of the anti-TPO Immunospec ELISA test system were determined by analyses on three different levels of pool control sera. The number (N), mean value (X), standard deviation ( $\sigma$ ) and coefficient of variation (C.V) 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	$\sigma$	C.V.
Pool 1	20	25.5	1.5	5.7%
Pool 2	20	120.5	4.6	3.8%
Pool 3	20	352.4	14.8	4.2%

**TABLE 3\***

Sample	Between Assay Precision (Values in IU/ml)			
	N	X	$\sigma$	C.V.
Pool 1	10	26.5	1.8	6.8%
Pool 2	10	118.5	5.3	4.5%
Pool 3	10	365.4	22.5	6.2%

\*As measured in ten experiments in duplicate.

##### B. Accuracy

The anti-TPO Immunospec ELISA test system was compared with a reference anti-TPO ELISA microplate. Biological specimens from normal 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-TPO Immunospec ELISA method in comparison with the referencemethod. The data obtained is displayed in Table 4.

**TABLE 4**  
Least Square Regression

Method	Mean (x)	Analysis	Correlation Coefficient
This Method	122.9	$y = 1.02(x) - 5.1$	0.989
Reference	127.0		

##### C. Sensitivity

The anti-TPO Immunospec ELISA test system has a sensitivity of 1.5 IU/ml.

##### D. Specificity

Interferences from ANA, DNA, thyroglobulin (TPO) and rheumatoid antibodies were found to be insignificant

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