



TPO Ab Elisa



IMMUNOENZYMOMETRIC ASSAY FOR THE QUANTITATIVE DETERMINATION OF ANTI-THYROPEROXIDASE (TPO) AUTOANTIBODIES IN HUMAN SERUM AND PLASMA

BL-07-E- 96 Assays

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CLINICAL APPLICATIONS AND PRINCIPLE OF THE ASSAY

Autoimmune thyroid diseases encompass a wide spectrum of different clinical symptoms varying from hypo- to hyperthyroidism (Hashimoto's disease and Graves' disease). The link between these extremes is the presence of serum auto-antibodies directed against the microsomal antigen (TMAb) and/or thyroglobulin (TgAb). Thyroid autoimmunity is more frequently registered in women. Antibody prevalence in women increases with age, rising from approximately 10% at the age of 18-24 up to 30% at the age of 55-65 for TgAb and from 15% at the age of 18-24 up to 24% at the age of 55-65 for TMAb. These significant TgAb and TMAb titers can lead to the development of chronic thyroiditis, often resulting in hypothyroidism. The main microsomal agent responsible for autoimmunity is an enzyme: thyroperoxidase (TPO). The detection of auto-antibodies against thyroperoxidase (anti-TPO) is known to be a highly sensitive as well as specific tool in diagnosing autoimmune diseases. Asymptomatic thyroid diseases with transient hyperthyroidism have been frequently noted in post-partum conditions. Subjects with moderate-sized goiters exhibit high anti-TPO levels. A rise in TgAb and anti-TPO has recently been proposed as an independent marker for "at-risk" pregnancies. In cases of primary myxoedema, significant TgAb and anti-TPO levels indicate the end-stage of autoimmune atrophic chronic thyroiditis. In younger patients, a firm goiter found in combination with high TgAb and anti-TPO levels is generally a sign of Hashimoto's disease, characterized by a progressive decrease in thyroid function leading to hypothyroidism. In Graves' disease, the toxic goiter is associated to chronic thyroiditis, as confirmed by high TgAb and anti-TPO levels. Monitoring of TgAb, TMAb and anti-TPO levels is frequently used for treatment follow-up. TgAb can also be used as a marker for identification of family members exposed to the risk of transmission of autoimmune conditions.

This test is based on an immunoenzymometric assay (IEMA). The solid phase (polystyrene microwells) is coated with immunopurified thyroperoxidase (>95% purity). During the first incubation, the autoantibodies possibly present in calibrators and samples are bound to the antigen coated on the solid phase. After washing to remove all unbound material, the tracer is added (anti-hlgG antibody conjugated to horseradish peroxidase) which binds to the antibodies captured during the first step, if any. Following this incubation, the unbound material is removed by aspiration and washing.

The residual enzyme activity found in the wells will thus be directly proportional to anti-TPO concentration in calibrators and samples and evidenced by incubating the solid phase with a Chromogen solution (Tetramethylbenzidine, TMB) in a Substrate-Buffer. Colorimetric reading will be performed by using a spectrophotometer at 450 nm.

REAGENTS PROVIDED WITH THE KIT

- Reagents are sufficient for 96 wells.
- Store the kit at 2-8 °C.
- The expiry date of each reagent is shown on the vial label.
- 1 Coated Microtiterplate:** 96 breakable wells coated with human thyroperoxidase. Keep unused wells at 2-8 °C, protected from moisture, in the provided aluminum bag and carefully sealed.
- 2 Calibrators:** 6 vials (2 ml) of anti-TPO in prediluted human serum, at the following concentrations: 0, 50, 150, 300, 1000 and 3000 U/ml. Ready for use. Preservative: NaN₃ (<0.1%).
- 3 Enzyme Tracer:** 1 vial (20 ml) of anti-hlgG conjugated to horseradish peroxidase (HRPO), in Tris buffer containing BSA and stabilizers. Preservative: Neomycin. Ready for use. This calibrator has been calibrated against the MRC 66/387 International Reference.

- 4 Control Serum:** 1 vial (2 ml) of prediluted human serum. Ready for use. Preservative: NaN₃ (<0.1%).
- 5 Diluent (concentrated):** 1 vial (30 ml) of phosphate buffer with BSA. Preservative: NaN₃ (<0.1%). Bring vial content to 300 ml with distilled water. The diluted sample diluent is stable for 3 months at 2-8 °C.
- 6 Washing Solution (concentrated):** 1 vial (100 ml) of Phosphate buffer with detergent. Preservative: Thimerosal (<0.1%). Bring vial content to 1,000 ml with distilled water. The diluted washing solution is stable for 1 month at 2-8 °C. If undissolved crystals are detected, put them back into solution by placing the vial at 37 °C for a few minutes.
- 7 Chromogen:** 1 vial (15 ml) of Tetramethylbenzidine in citrate-phosphate buffer and DMSO.
- 8 Substrate Buffer:** 1 vial (15 ml) of citrate-phosphate buffer containing H₂O₂.
Note: Before use, make a 1+1 dilution with equal volumes of Chromogen and Substrate Buffer in glassware. Avoid direct light exposure and use within 1 hour from preparation.
- 9 Blocking Reagent:** 1 vial (15 ml) of 0.5 M H₂SO₄. Ready for use.

KIT REAGENTS

Reagents	Quantity	Physical state
Wells	96	Ready for use
Calibrator	6 x 2 ml	Ready for use
Tracer	1 x 20 ml	Ready for use
Control Serum	1 x 2 ml	Ready for use
Diluent	1 x 30 ml	Concen. 10 x
Washing Solution	1 x 100 ml	Concen. 10 x
Chromogen	1 x 15 ml	Ready for use
Substrate Buffer	1 x 15 ml	Ready for use
Blocking Reagent	1 x 15 ml	Ready for use

MATERIAL REQUIRED BUT NOT SUPPLIED

- Adjustable, automatic micropipettes with disposable tips.
- Graduated cylinder.
- Dry Heater, adjustable at 37 °C ± 1 °C.
- Aspiration pump or automated well washing device.
- Microtiterplate spectrophotometer capable of measuring absorbances within a 0-3.0 A interval at 450 nm and 405 nm.
- Millimetric graph paper.
- Distilled H₂O.

WARNINGS AND PRECAUTIONS

In order to obtain reproducible results, the following rules must be observed:

- Do not mix reagents of different lots.
- Do not use reagents beyond their expiry date.
- Use thoroughly clean glassware, free from contamination of metal ions or oxidating substances.
- Use distilled water, stored in clean containers.
- Avoid any contamination among samples; for this purpose, disposable tips should be used for each sample and reagent.
- Follow exact incubation times. Dispense Chromogen and Blocking Reagent in not more than 3-4 minutes; dispense the two reagents in the same sequence.

In order to avoid personal and environmental contamination, the following precautions must be observed:

- Use disposable gloves while handling potentially infectious material and performing the assay.
- Do not pipette reagents by mouth.
- Do not smoke, eat, drink or apply cosmetics during the assay.
- Chromogen and Blocking Reagent should be handled with care. Avoid contact with skin, eyes and mucous membranes. In case of accident rinse thoroughly with running water.
- All material of human origin used for the preparation of this kit tested negative for HBsAg, anti-HIV and anti-HCV. Since no test at present can guarantee complete absence of these viruses, all samples and reagents used for the assay must be considered potentially infectious; therefore, the assay waste must be decontaminated and disposed of, in accordance with established safety procedures. Disposable ignitable material must be incinerated; disposable non-ignitable material must be sterilized in autoclave for at least 1 hour at 121 °C.
- Liquid wastes must be added with sodium hypochlorite at a final concentration of 3%. Let the hypochlorite act for at least 30 minutes. Liquid wastes containing acid must be neutralized with appropriate amounts of base before treating with sodium hypochlorite.
- Avoid splashing and aerosol formation; in case of spilling, wash carefully with a 3% sodium hypochlorite solution and dispose of this cleaning liquid as potentially infectious waste.
- Some reagents contain sodium azide as preservative; to prevent build-up of explosive metal azides in lead and copper plumbing, reagents should be discarded by flushing the drain with large amounts of water.

SPECIMEN COLLECTION AND PREPARATION

The assay can be performed in serum or plasma samples. Highly lipemic or hemolyzed samples must be discarded. Keep samples at 2-8°C for 1 day; for longer periods it is advisable to freeze samples in aliquots at -20°C. Plasma samples may present fibrin filaments which could interfere with the assay; make sure that samples are always perfectly clear before testing. Repeated freezing and thawing of samples should be avoided.

Sample pre-dilution

Before testing, samples must be diluted 1:301 as follows:
10 µl Sample + 3,000 µl Diluent.

Calibrators and Control Serum are already prediluted and ready for use.

ASSAY PROCEDURE*

- Allow reagents and samples to warm up at room temperature.
 - Mix samples by inversion before use.
- 1- Prepare the wells for: Blank, calibrators, Control Serum and samples.
 - 2- Pipette **100 µl** of each Calibrator, Control Serum and prediluted sample into the corresponding wells. Pipette directly in the bottom of the wells.
 - 3- Incubate for **60 minutes at 37°C**.
 - 4- Wash the wells **4 times** with **350 µl** of diluted washing solution. Aspirate all liquid from the wells.
 - 5- Add **100 µl** of Enzyme Tracer into all wells, except for the Blank wells.
 - 6- Incubate for **60 minutes at 37°C**.
 - 7- Wash the wells **4 times** with **350 µl** of diluted washing solution. Aspirate all liquid from the wells.
 - 8- Pipette **200 µl** of Substrate-Chromogen solution (see reagent paragraph) into all wells.
 - 9- Incubate for **15 minutes at 37°C**, avoid direct light exposure.

10 - Pipette **100 µl** of Blocking Reagent into all wells.

11 - Read the absorbance of the wells with a possibly bichromatic spectrophotometer at 450 and 405 nm, with reference wavelength at 620nm (setting the instrument at zero with the Blank well). Reading must be completed within 20 minutes from the end of the assay.

* Please refer to the instrument instruction sheet if using.

ASSAY SCHEME

Sample pre-dilution: 1/301

Wells	Blank	Calibrator (0-5)	Control Serum	Samples
Reagent				
Calibrator (0 - 5)	----	100 µl	----	----
Control	----	----	100 µl	----
Samples	----	----	----	100 µl
<ul style="list-style-type: none"> - Incubate: 37°C 60'. - Aspirate and wash: 4 x 350 µl. 				
Tracer	----	100 µl	100 µl	100 µl
<ul style="list-style-type: none"> - Incubate: 37°C 60'. - Aspirate and wash: 4 x 350 µl. 				
Chromogen Substrate	200 µl	200 µl	200 µl	200 µl
<ul style="list-style-type: none"> - Incubate: 37°C 15'. 				
Blocking Reagent	100 µl	100 µl	100 µl	100 µl
<ul style="list-style-type: none"> - Read : 450 nm – 405 nm and 620 nm as reference. 				

CALCULATION OF RESULTS

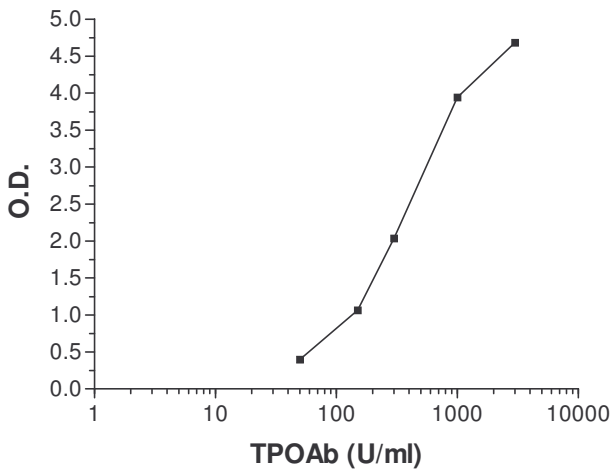
In order to obtain a better sensitivity, the present method employs spectrophotometric reading at two wavelengths (450 and 405 nm). For samples with Anti-TPO concentrations ranging from 0 to 300 U/ml, read at 450 nm; for samples with Anti-TPO levels higher than 300 U/ml, read at 405 nm. Draw a calibration curve on millimetric graph paper, by plotting the calibration concentration (x-axis) against the absorbance obtained for each calibrator (y-axis). Corresponding Anti-TPO concentrations in U/ml are obtained by interpolating the absorbances of each sample on the calibration curve.

EXAMPLE OF CALCULATION

The values shown below must be considered as an example and must not be used in place of experimental data.

Description			D.O.1	D.O.2	D.O.3	D.O. med.	Anti-TPO Concen. (U/ml)
CAL 0	0		0.020	0.034	0.024	0.026	
CAL 1	50		0.393	0.386	0.420	0.400	
CAL 2	150		1.047	0.974	1.169	1.063	
CAL 3	300		1.941	2.002	2.162	2.035	
CAL 4	1000		3.882	4.188	3.759	3.943	
CAL 5	3000		4.626	4.734	4.689	4.683	
CONTROL	303±60	U/ml	1.682	1.829	1.975	1.829	257.8
P1			0.755	0.759	0.760	0.758	96.1
P2			1.368	1.247	1.532	1.382	183.5
P3			3.597	3.900	3.864	3.787	1026

TYPICAL CALIBRATION CURVE



NORMAL VALUES

The normal values determined on 83 samples, are only indicative since they may be affected by various agents (climate, geographic area, diet, etc.). We recommend that each laboratory establishes its own normal range.

Lower than 40 U/ml:	negative for anti-TPO antibodies.
Borderline >40- ≤80 U/ml	
Higher than 80 U/ml:	positive for anti-TPO antibodies

PERFORMANCES OF THE ASSAY

SPECIFICITY

No cross-reactions have been observed with anti-Tg autoantibodies present in human serum.

SENSITIVITY

The sensitivity was calculated based upon the calibration curve and expressed as the minimal dose showing a significant difference from the Zero calibrator (mean value + 2 S.D.). This dose is 1.4 U/ml.

PRECISION

Precision was evaluated upon intra- and inter-assay variability, in 3 sera at different anti-TPO autoantibody concentrations.

Intra-assay

Serum	Mean	± (U/ml)	S.D.	C.V. %	Replicates No.
a	74.1	±	5.1	6.9	20
b	538.1	±	45.4	8.4	20
c	1417	±	220.9	15.6	20

Inter-assay

Serum	Mean	± (U/ml)	S.D.	C.V. %	Assays No.
a	105.9	±	14.2	13.4	9
b	214.3	±	35.1	16.4	9
c	1115	±	243.1	21.8	9

ACCURACY

Accuracy of the method has been checked by the recovery and parallelism tests:

Recovery Test

Two prediluted (1:301) samples, mixed with equal volumes of each calibration point, were tested.

Samples	Expected (U/ml)	Measured (U/ml)	Recovery %
S1	-	266.8	-
S1+0	133.4	135.9	101.9
S1+50	158.4	172.8	109.1
S1+150	208.4	206.1	98.9
S1+300	283.4	300.1	105.9
S1+1000	633.4	448.9	70.9
S1+3000	1633.4	1367.1	83.7
S2	-	168.4	-
S2+0	84.2	39.0	46.3
S2+50	109.2	104.8	96.0
S2+150	159.2	163.7	102.8
S2+300	234.2	225.0	96.1
S2+1000	584.2	482.9	82.7
S2+3000	1584.2	1669.1	105.4

Parallelism Test

Two sera with high anti-TPO autoantibody concentrations were tested at different dilutions with the Zero calibrator.

Dilution	Expected (U/ml)	Measured (U/ml)	Recovery %
S1 undiluted	-	908	-
1/2	454	435	95.8
1/4	227	217	95.6
1/8	114	116	101.8
1/16	57	48	84.2
S2 undiluted	-	1267	-
1/2	633	522	82.5
1/4	317	270	85.2
1/8	158	142	89.9
1/16	79	61	77.2






N.B. Because of the heterogeneity of autoantibodies, for some patient samples a nonlinear dilution is possible.

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Bio-Line aTPO-Elisa Kit
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ISO15223 MEDICAL DEVICES SYMBOL

	storage temperature limitation
LOT	batch code
	use by
	consult operating instructions
IVD	<i>in vitro</i> diagnostic device
	manufactured by
REF	catalogue number
SYMBOLS (EDMA recommendations)	
	number of determinations (96)
CAL	Calibrators
CONTROL	Control serum
SORB MTP	Microtiterplate
CONJ HRP	Enzyme tracer
DIL 10X	Diluent to be diluted ten-fold
CHROM	Chromogen
SUBS	Substrate buffer
BUF WASH 10X	Washing solution to be diluted ten-fold
H₂SO₄ 0.5 M	Blocking reagent