1. INTRODUCTION

Thyroid peroxidase (TPO) present at the apical plasma membrane of human thyroid cells catalyzes the first step in thyroid hormone synthesis. TPO oxidizes iodide-anions and iodinates some tyrosyl residues of thyreoglobulin (TG), leading to the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT). Coupling of MIT and DIT yields triiodothyronine (T₃) and tetra-iodothyronine (T₄). TPO (previously known as thyroid microsomal antigen) is a frequent epitope of autoantibodies in autoimmune thyroid disease. TPO autoantibodies (TPOAb) are detectable in over 90 % of patients with Hashimoto's thyroiditis and over 70 % of those with Graves' disease (autoimmune hyperthyroidism), irrespective of the functional state of the thyroid gland. Postpartum thyroiditis is a special form of autoimmune thyroiditis observed following pregnancy and may involve temporary hyperthyroidism or hypothyroidism. It affects about 5 % of all women within a year after giving birth. The risks are higher for women with autoimmune disorders (such as type 1 diabetes), positive anti-thyroid antibodies or a history of previous thyroid dysfunction. Moderately elevated TPOAb values are sometimes found in patients without other evidence of thyroid disease, especially in the elderly. The clinical significance of low levels of TPOAb in euthyroid subjects is still unknown. Whether these individuals should be considered normal remains in question until long-term follow-up studies show that they do not have an increased risk for developing thyroid dysfunction.

<table>
<thead>
<tr>
<th>Autoantigen</th>
<th>Disease</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid peroxidase (TPO)</td>
<td>Hashimoto's thyroiditis Graves' disease (= Morbus Basedow)</td>
<td>initially hyperthyroidism: nervousness, weight loss, insomnia, tachycardia, heat intolerance etc. later gradual transition to chronic hypothyroidism: fatigue, decreased concentration, weight gain, constipation, cold intolerance etc. hyperthyroidism: exophthalmos, struma, tachycardia (Merseburg triad)</td>
</tr>
</tbody>
</table>

The presence of autoantibodies to TPO may be detected by indirect immunofluorescence and several quantitative immunoassays, e.g. radioimmunoassays and Enzyme-linked Immunosorbent Assay (ELISA).

2. INTENDED USE

The GenWay anti-TPO ELISA is intended for the quantitative determination of autoantibodies to TPO (thyroid peroxidase) in human serum or plasma (citrate).

3. PRINCIPLE OF THE ASSAY

The quantitative immunoenzymatic determination of autoantibodies to TPO is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Microtiter strips are precoated with recombinant thyroid peroxidase (TPO) to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgG conjugate is added. This conjugate binds to the captured TPO-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of TPO specific IgG antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.
4. MATERIALS

4.1. Reagents supplied

TPO Coated Wells (IgG): 12 breakapart 8-well snap-off strips coated with thyroid peroxidase; in resealable aluminium foil.

IgG Sample Diluent***: 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2, coloured yellow; ready to use; white cap.

Stop Solution: 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.

Washing Solution (20x conc.)*: 1 bottle containing 50 ml of a 20-fold concentrated buffer for washing the wells; pH 7.2 ± 0.2; white cap.

TPO anti-IgG Conjugate**: 1 bottle containing 20 ml of peroxidase labelled antibodies to human IgG; coloured blue; ready to use; black cap.

TMB Substrate Solution: 1 bottle containing 15 ml 3,3',5,5'-tetramethylbenzidine (TMB); ready to use; yellow cap.

Negative Control***: 1 bottle containing 2 ml control solution; coloured yellow; blue cap.

Positive Control***: 1 bottle containing 2 ml control solution; coloured yellow; red cap.

Anti-TPO Standards***: 6 vials, each containing 2 ml; coloured yellow; ready to use; yellow cap.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration (AU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard A</td>
<td>0 AU/ml</td>
</tr>
<tr>
<td>Standard B</td>
<td>5 AU/ml</td>
</tr>
<tr>
<td>Standard C</td>
<td>10 AU/ml</td>
</tr>
<tr>
<td>Standard D</td>
<td>20 AU/ml</td>
</tr>
<tr>
<td>Standard E</td>
<td>80 AU/ml</td>
</tr>
<tr>
<td>Standard F</td>
<td>320 AU/ml</td>
</tr>
</tbody>
</table>

* contains 0.1 % Bronidox L after dilution ** contains 0.2 % Bronidox L *** contains 0.1 % Kathon

4.2. Materials supplied

1 Strip holder
1 Cover foil
1 Test protocol
1 Distribution and identification plan

4.3. Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
- Incubator 37 °C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Timer

5. STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2…8 °C.

6. REAGENT PREPARATION

It is very important to bring all reagents and samples to room temperature (20…25 °C) before starting the test run!

6.1. Coated Snap-off Strips

The ready to use breakapart snap-off strips are coated with thyroid peroxidase (TPO). Store at 2…8 °C. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2…8 °C; stability until the expiry date.

6.2. TPO anti-IgG Conjugate

The bottle contains 20 ml of a solution with anti-human IgG horseradish peroxidase, buffer, stabilizers, preservatives and an inert blue dye. The solution is ready to use. Store at 2…8 °C. After first opening stability until the expiry date when stored at 2…8 °C.

6.3. Controls

The bottles labelled with Negative and Positive Control contain 2 ml of a ready to use control solution.

Negative Control: < 25 AU/ml Positive
Control: > 40 AU/ml
It contains 0.1 % Kathon and has to be stored at 2...8 °C. After first opening stability until the expiry date when stored at 2...8 °C.

6.4. Standards
The vials labelled with Standard A, B, C, D, E and F contain a ready to use standard solution. The standards have the following concentrations in arbitrary units (AU):
- Standard A: 0 AU/ml
- Standard B: 5 AU/ml
- Standard C: 10 AU/ml
- Standard D: 20 AU/ml
- Standard E: 80 AU/ml
- Standard F: 320 AU/ml

The solutions have to be stored at 2...8 °C and conta in 0.1 % Kathon. After first opening stability until the expiry date when stored at 2...8 °C.

6.5. IgG Sample Diluent
The bottle contains 100 ml phosphate buffer, stabilizers, preservatives and an inert yellow dye. It is used for the dilution of the patient specimen. This ready to use solution has to be stored at 2...8 °C. After first opening stability until the expiry date when stored at 2...8 °C.

6.6. Washing Solution (20x conc.)
The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute Washing Solution 1 + 19; e. g. 10 ml Washing Solution + 190 ml fresh and germ free redistilled water. The diluted buffer will keep for 5 days if stored at room temperature. Crystals in the solution disappear by warming up to 37 °C in a water bath. After first opening stability until the expiry date when stored at 2...8 °C.

6.7. TMB Substrate Solution
The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2...8 °C, away from the light. The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away. After first opening stability until the expiry date when stored at 2...8 °C.

6.8. Stop Solution
The bottle contains 15 ml 0.2 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8 °C. After first opening stability until the expiry date.

7. SPECIMEN COLLECTION AND PREPARATION
Use human serum or plasma (citrate) samples with this assay. If the assay is performed within 5 days after sample collection, the specimens should be kept at 2...8 °C; otherwise they should be aliquoted and stored deep-frozen (-20... -70 °C). If samples are stored frozen, mix thawed s amples well before testing. Avoid repeated freezing and thawing. Heat inactivation of samples is not recommended.

7.1. Sample Dilution
Before assaying, all samples should be diluted 1 + 100 with IgG Sample Diluent. Dispense 10 µl sample and 1 ml IgG Sample Diluent into tubes to obtain a 1 + 100 dilution and thoroughly mix with a Vortex.

For patients with expected anti-TPO concentrations greater than Standard F (320 AU/ml) a second 1 + 1 dilution of this 1 + 100 diluted patient sample should be performed; e. g. 100 µl of first sample dilution + 100 µl of IgG Sample Diluent (mix well). Dilution factor: 2.

8. ASSAY PROCEDURE

8.1. Test Preparation
Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. The following test procedure is only validated for manual procedure. If performing the test on ELISA automatic systems we recommend to increase the washing steps from three to five and the volume of Washing Solution from 300 µl to 350 µl to avoid washing effects. Prior to commencing the assay, the distribution and identification plan for all specimens, standards and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

Please allocate at least:
- 1 well (e. g. A1) for the substrate blank, 6 wells (e. g. B1, C1, etc.) for
Standard A, B, C, D, E and F, 1 well (e. g. H1) for the Negative Control and 1 well (e. g. A2) for the Positive Control

It is recommended to determine standards, controls and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each standard, control and sample.

Adjust the incubator to 37 ± 1 °C.

1. Dispense 100 µl of each Standard (A, B, C, D, E and F), controls and diluted samples into their respective wells. Leave well A1 for the substrate blank.
2. Cover wells with the foil supplied in the kit.
3. Incubate for 30 min at 37 ± 1 °C.
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be > 5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!
   a. Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.
5. Dispense 100 µl TPO anti-IgG Conjugate into all wells except for the blank well (e. g. A1). Cover with foil.
6. Incubate for 30 min at room temperature (20…25 °C). Do not expose to direct sunlight.
7. Repeat step 4.
8. Dispense 100 µl TMB Substrate Solution into all wells.
9. Incubate for exactly 15 min at room temperature (20…25 °C) in the dark.
10. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.
   a. Any blue color developed during the incubation turns into yellow.
   b. Note: Highly positive patient samples can cause dark precipitates of the chromogen! These precipitates have an influence when reading the optical density. Predilution of the sample -as described under 7.1. Sample Dilution -is recommended.
11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

8.2. Measurement

Adjust the ELISA Microwell Plate Reader to zero using the substrate blank in well A1.

If – due to technical reasons – the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

Measure the absorbance of all wells at 450 nm and record the absorbance values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the mean absorbance values of all duplicates.

9. RESULTS

9.1. Assay Validation Criteria

In order for an assay to be considered valid, the following criteria must be met:

- Substrate blank in A1: Absorbance < 0.100
- Standard A in B1: Absorbance < 0.200
- Standard B in C1: Absorbance > Standard A
- Standard C in D1: Absorbance > Standard B
- Standard D in E1: Absorbance > 0.100
- Standard E in F1: Absorbance > 0.400
- Standard F in G1: Absorbance > 1.000
- Negative Control in H1: Result < 25 AU/ml
- Positive Control in A2: Result > 40 AU/ml


If these criteria are not met, the test is not valid and must be repeated.

9.2. Calculation of Results

In order to obtain quantitative results in AU/ml plot the (mean) absorbance values of the Standards A – F (y-axis, linear) on graph
paper in a system of coordinates against their corresponding concentrations (x-axis, logarithmic) and draw a standard calibration curve. Read results from this standard curve employing the (mean) absorbance values of each patient specimen and control.

Note: Readings of additionally (1+1) diluted patient samples must be multiplied by the appropriate dilution factor in order to obtain correct results! Dilution: 1+1 = Dilution factor: 2. (See section “Sample Dilution”).

All suitable computer programs available can be used for automated result reading and calculation.

9.3. Typical Calibration Curve

![Typical Calibration Curve](image)

9.4. Interpretation of Results

Normal value ranges for this ELISA should be established by each laboratory based on its own patient populations in the geographical areas serviced.

The following values should be considered as a guideline:

- **normal**: < 30 AU/ml
- **grey zone**: 30 – 35 AU/ml
- **elevated**: > 35 AU/ml

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (OD)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraassay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pos. Serum</td>
<td>20</td>
<td>1.334</td>
<td>3.8</td>
</tr>
<tr>
<td>Pos. Serum</td>
<td>20</td>
<td>1.265</td>
<td>4.8</td>
</tr>
<tr>
<td>Neg. Serum</td>
<td>20</td>
<td>0.209</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Interassay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pos. Serum</td>
<td>12</td>
<td>113.3</td>
<td>7.1</td>
</tr>
<tr>
<td>Pos. Serum</td>
<td>12</td>
<td>176.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

10.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. It is > 97 %.

10.3. Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. It is > 97 %.

10.4. Analytical Sensitivity

The analytical sensitivity – defined as the apparent concentration of the analyte that can be distinguished from the zero calibrator – is < 0.5 AU/ml.
10.5. Interferences
Interferences with hemolytic, lipemic or icteric sera are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.5 mg/ml bilirubin.

11. LIMITATIONS OF THE PROCEDURE
Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values. Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data. In immunocompromised patients and newborns serological data only have restricted value.

12. PRECAUTIONS AND WARNINGS
Only for laboratory research use.
All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious.
Do not interchange reagents or strips of different production lots.
No reagents of other manufacturers should be used along with reagents of this test kit.
Do not use reagents after expiry date stated on the label.
Use only clean pipette tips, dispensers, and lab ware.
Do not interchange screw caps of reagent vials to avoid cross-contamination.
Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.
To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.
The Genway ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

WARNING: In the used concentration Bronidox L has hardly any toxicological risk upon contact with skin and mucous membranes!
WARNING: Sulphuric acid irritates eyes and skin. Keep out of the reach of children. Upon contact with the eyes, rinse thoroughly with water and consult a doctor!

12.1. Disposal Considerations
Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

13. ORDERING INFORMATION
SCHEME OF THE ASSAY

Anti-TPO

Assay Preparation

Prepare reagents and samples as described. Establish the distribution and identification plan for all specimens, standards and controls on the result sheets supplied in the kit.

Select the required number of microtiter strips or wells and insert them into the holder.

Assay Procedure

<table>
<thead>
<tr>
<th>blank</th>
<th>Standard A-F</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Sample (diluted 1+100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard A -F</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative Control</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Control</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample (diluted 1+100)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Cover wells with foil supplied in the kit. **Incubate for 30 min at 37 °C.** Wash each well three times with 300 µl diluted Washing Solution.

<table>
<thead>
<tr>
<th>conjugate</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
</tr>
</thead>
</table>

Cover wells with foil supplied in the kit. **Incubate for 30 min at 20….25 °C.** Wash each well three times with 300 µl diluted Washing Solution.

<table>
<thead>
<tr>
<th>TMB Substrate</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
</tr>
</thead>
</table>

**Incubate for exactly 15 min at room temperature in the dark.**

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
<th>100 µl</th>
</tr>
</thead>
</table>

Photometric measurement at 450 nm (reference wavelength: 620 nm)