TSH RECEPTOR Ab
Quantitative determination of thyrotropin receptor autoantibodies (TRAb) in human serum

INTENDED USE
TSH Receptor Ab (TRAb) ELISA kit is intended for use by professional persons only for the quantitative determination of thyrotropin receptor autoantibodies in human serum.
TSH Receptor Ab kit is intended for laboratory use only.

1. CLINICAL SIGNIFICANCE
The hyperthyroidism is a disease that produce an increase in blood circulating levels of thyroid hormones. In some cases, as in Graves’ disease is caused by autoantibodies against the TSH receptor (TSH). Autoantibodies mimic the effect of TSH on the thyroid gland causing a rise in blood levels of T3 and T4. The determination of these autoantibodies (TRAb) may be useful for the diagnosis of the disease and its treatment. The main treatment for Graves’ disease is represented by use of antithyroid drugs (propylthiouracil or methimazole), I\(^{131}\) and surgery. The dosage of TRAb is therefore useful in the course or at the end of therapy.

2. PRINCIPLE
In TSH Receptor Ab (TRAb) ELISA kit the TSH receptor autoantibodies in the patient sera, calibrators and controls are allowed to interact with TSH receptor coated into ELISA plate wells. After a 2 hours incubation, the samples are discarded leaving TRAb bound to the immobilized TSH receptor. TSH Biotin, added in a 2nd incubation step, interacts with the immobilised TSH receptors, which have not been blocked by the bound TRAb from patient sera, calibrators or controls. The amount of TSH Biotin bound to the plate is then determined in a third incubation step by addition of streptavidin peroxidase, which binds specifically to biotin. Excess unbound streptavidin peroxidase is then washed away and the addition of tetramethylbenzidine (TMB) substrate results in formation of a blue colour. This reaction is stopped by the addition of stop solution causing the well contents to turn from blue to yellow. The absorbance of the yellow reaction mixture at 450nm is then read using an ELISA plate reader. A lower absorbance indicates the presence of TRAb in the test sample as TRAb inhibits the binding of TSH biotin to TSH receptor coated plate wells.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

1. Calibrators (4 vials, 1 mL each)
   CAL1 REF DCE002/8507-0
   CAL2 REF DCE002/8508-0
   CAL3 REF DCE002/8509-0
   CAL4 REF DCE002/8510-0

2. Controls (2 vials, 1mL each, ready to use)
   Negative Control REF DCE045/8501-0
   Positive Control REF DCE045/8502-0

3. TSH biotin (3 vials, lyophilized)
   REF DCE019/8519-0

4. TSH biotin buffer (1 vial, 15 mL)
   REF DCE047/8547-0

5. 20X Conc. Streptavidin-Peroxidase (1 vial, 0.75 mL)
   REF DCE041/8541-0

6. Streptavidin-Peroxidase Diluent (1 vial) 15 mL
   REF DCE048/8548-0

7. Coated Microplate (1 breakable microplate)
   Microplate coated with TSH receptor
   REF DCE002/8503-0

8. Start Buffer (1 vial, 10 mL)
   REF DCE046-0

9. TMB Substrate (1 vial, 15 mL)
   0.25 M sulfuric acid
   REF DCE004/8504-0

10. Stop Solution (1 vial, 10 mL)

11. 10X Conc. Wash Solution (1 vial, 100 mL)

3.2. Reagents necessary not supplied
Distilled or deionized water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm, 620-630 nm)
5. PRECAUTIONS

- Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- **WARNING:** the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips. For this purpose, Meridian supplies a separate decontamination reagent for cleaning needles.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- 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 minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

4. WARNINGS

- This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Calibrators and Controls should be handled in the same manner as potentially infectious material.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents contain small amounts of Sodium Azide (NaN₃) or Proclin 300® as preservatives. Avoid the contact with skin or mucosa.
- Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.

6. PROCEDURE

### 6.1. Preparation of Calibrators (C₁...C₄)

The Calibrators are ready to use, are calibrated against the WHO NIBSC 90/672 and have the following concentration:

<table>
<thead>
<tr>
<th>U/L</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
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<td>1</td>
<td>2</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at 2-8°C.
6.2. Preparation of the Sample
The sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at –20°C or below.
Repeated freeze thawing or increases in storage temperature must be avoided.
Incorrect storage of serum samples can lead to loss of TRAb activity. Do not use highly lipaemic or haemolysed serum samples. Do not use plasma in the assay. When required, thaw test sera at room temperature and mix gently to ensure homogeneity. The Controls are ready to use.

6.3. Preparation of the TSH biotin
Reconstitute each vial with 4.5 mL of TSH Biotin buffer (reagent 4).
When more than one vial is to be used, pool the vials and mix gently before use.
Store at 2-8°C for up to 6 months after reconstitution.

6.4. Preparation of the Streptavidin Peroxydase
Dilute the Streptavidin Peroxidase 1:20 with Streptavidin Peroxydase Diluent (for example, 0.5 mL of Streptavidin-Peroxidase 20X concentrate + 9.5 mL of Streptavidin-Peroxidase Diluent).
Store at 2-8°C for up to the expiry date of the kit.

6.5. Preparation of the Wash Solution
Prepare a sufficient amount of washing solution by diluting the 10X Concentrated Wash Solution 1 + 9 with distilled or deionized water. For example, dilute 50 mL of the Concentrate Wash with 450 mL of distilled water. The solution should be free of crystals before dilution, otherwise dissolve by warming up to max 37°C. The diluted washing solution can be stored at 2-8°C up to 30 days.

6.6. Procedure
- Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.
  At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C1-C4), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Calibrator</th>
<th>Sample/ Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start buffer</td>
<td>75 μL</td>
<td>75 μL</td>
<td></td>
</tr>
<tr>
<td>Calibrator</td>
<td>75 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample or Controls</td>
<td>75 μL</td>
<td></td>
<td></td>
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</table>

Cover the plate and incubate 120 minutes at room temperature (22-28°C) while shaking at >500 rpm. Alternatively incubate without shaking for at least 180 minutes at room temperature (22-28°C).
Remove the contents from each well and wash the wells with 300 μL of diluted Wash Solution, drain the wash completely.

<table>
<thead>
<tr>
<th>TSH Biotin</th>
<th>100 μL</th>
<th>100 μL</th>
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</table>

Cover the plate and incubate 25 minutes at room temperature (22-28°C)
Remove the contents from each well and wash the wells with 300 μL of diluted Wash Solution, drain the wash completely.

<table>
<thead>
<tr>
<th>Streptavidin-Peroxidase</th>
<th>100 μL</th>
<th>100 μL</th>
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</table>

Cover the plate and incubate 20 minutes at room temperature (22-28°C)
Remove the contents from each well and wash the wells with 300 μL of diluted Wash Solution. Repeat the washing procedure other two times by draining the wash completely.

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

Incubate at room temperature (22-28°C) for 30 minutes in the dark.

<table>
<thead>
<tr>
<th>Stop Solution</th>
<th>50 μL</th>
<th>50 μL</th>
<th>50 μL</th>
</tr>
</thead>
</table>

Shake the microplate gently.
Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.

7. QUALITY CONTROL
It is recommended that each laboratory include its own panel of control samples in the assay. Each laboratory should establish its own normal and pathological reference ranges for TRAb levels.

8. RESULTS

8.1. Calibration curve
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The TRAb concentrations in patient sera can then be read off the calibration curve. Other data reduction systems can be used. Results can also be expressed as inhibition (%I) of TSH binding calculated using the formula;
Samples with high TRAb concentrations can be diluted in kit negative control (D1). For example, 20 µL of sample plus 180 µL of negative control to give a 10x dilution. Other dilutions (e.g. 100x) can be prepared from a 10x dilution or otherwise as appropriate. Some sera will not dilute in a linear way and we suggest that the dilution giving a value closest to 50% inhibition is used for calculation of TRAb concentration.

Typical Results (example only, not for use in calculation of actual results)

<table>
<thead>
<tr>
<th>Sample</th>
<th>A450</th>
<th>% Inhibition</th>
<th>U/L</th>
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<tbody>
<tr>
<td>Control D1</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>1.70</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>C2</td>
<td>1.50</td>
<td>25</td>
<td>2</td>
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<tr>
<td>C3</td>
<td>0.65</td>
<td>68</td>
<td>8</td>
</tr>
<tr>
<td>C4</td>
<td>0.15</td>
<td>93</td>
<td>40</td>
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<tr>
<td>Control D2</td>
<td>1.26</td>
<td>37</td>
<td>3.5</td>
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8.2. Reference Values

<table>
<thead>
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<th>TSH Receptor Ab</th>
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<tr>
<td>Negative</td>
<td>≤ 1.0 U/L</td>
</tr>
<tr>
<td>Grey zone</td>
<td>1.1 – 1.5 U/L</td>
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<tr>
<td>Positive</td>
<td>&gt; 1.5 U/L</td>
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Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

9. PERFORMANCE AND CHARACTERISTICS

9.1. Clinical Specificity

154 samples from healthy blood donors were assayed in the TSH Receptor Ab ELISA kit. 152 (99%) were identified as being negative for TSH Receptor autoantibodies.

9.2. Clinical Sensitivity

50 samples from patients diagnosed with Graves disease were assayed using the TSH Receptor Ab ELISA kit. 49 (98%) were identified as being positive for TSH Receptor autoantibodies. 1 sample (2%) was identified as being within the equivocal range.

9.3. Lower Detection Limit

The kit negative control was assayed 32 times and the mean and standard deviation calculated. The lower detection limit at 2 standard deviations was 0.21 U/mL.

9.4. Intra and inter-assay variations

9.4.1 Intra-Assay

Within run variation was determined by replicate 16 times two different sera with values in the range of calibration curve. The within assay variability is ≤ 7.6%.

9.4.2 Inter-Assay

Between run variation was determined by replicate the measurements of one control serum with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤ 6.5%.

9.5. Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than Graves disease indicated non interference from autoantibodies to: thyroglobulin, thyroid peroxidise, glutamic acid decarboxylase, 21-hydroxylase, acetylcholine receptor, dsDNA or from rheumatoid factor.

9.6. Interference

No interference was observed when samples were spiked with the following materials: haemoglobin (5 mg/mL), bilirubin (20 mg/dL), intralipid (10 mg/mL).

10. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.
BIBLIOGRAPHY

1. J. Bolton et al Measurement of thyroid stimulating hormone receptor autoantibodies by ELISA
   Clin. Chem 1999 45: 2285-2287

2. K Kamijo TSH receptor antibody measurement in patients with various thyrotoxicosis and Hashimoto’s thyroiditis: a comparison of two two-step assays, coated plate ELISA using porcine TSH receptor and coated tube radioassay using human recombinant TSH receptor Endocrine Journal 2003 50:113-116

3. B. Rees Smith et al A new assay for thyrotropin receptor autoantibodies Thyroid 2004 14: 830-835
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SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORE CAUSE POSSIBILI/ SUGGERIMENTI

Nessuna reazione colorimetrica del saggio
- mancata dispensazione del coniugato
- contaminazione del coniugato e/o del Substrato
- errori nell’esecuzione del saggio (es. Dispensazione accidentale dei reagenti in sequenza errata o provenienti da flaconi sbagliati, etc.)

Reazione troppo blanda (OD troppo basse)
- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo breve, temperatura di incubazione troppo bassa

Reazione troppo intensa (OD troppo alte)
- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo lungo, temperatura di incubazione troppa alta
- qualità scadente dell’acqua usata per la soluzione di lavaggio (basso grado di deionizzazione,)
- lavaggi insufficienti (coniugato non completamente rimosso)

Valori inspiegabilmente fuori scala
- contaminazione di pipette, puntali o contenitori- lavaggi insufficienti (coniugato non completamente rimosso)
  - CV% intrasaggio elevato
- reagenti e/o strip non portate a temperatura ambiente prima dell’uso
- il lavatore per micropiastre non lava correttamente (suggerimento: pulire la testa del lavatore)
  - CV% intersaggio elevato
- condizioni di incubazione non costanti (tempo o temperatura)
- controlli e campioni non dispensati allo stesso tempo (con gli stessi intervalli) (controllare la sequenza di dispensazione)
- variabilità intrinseca degli operatori

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation