




Instructions for Use


anti - TSH - Receptor ELISA

Enzyme Immuno Assay for the Quantitative Determination of TSH Receptor Autoantibodies (TRAb) in Serum



REF EA101/96

 12 x 8

 2 – 8 °C

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1. Introduction and Principle of the Test

The hyperthyroidism of Graves' disease is due to autoantibodies to the thyrotropin (TSH) receptor and measurement of these autoantibodies is valuable in the diagnosis and management of Graves' hyperthyroidism.

The enclosed kit provides a simple, sensitive and specific method for measuring TSH receptor autoantibodies (TRAb) in patients' serum samples. In the assay, TSH receptor autoantibodies in patients' sera are allowed to interact with TSH receptor coated onto ELISA plate wells. Bound TRAb are detected by their ability to inhibit the binding of TSH (in the form of TSH-biotin) to the receptor coated wells. The amount of TSH bound is then monitored by addition of streptavidin peroxidase and the peroxidase substrate tetramethyl benzidine. TRAb levels are expressed as an inhibition of TSH binding index or read off a standard curve.

2. Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative (<0.1%). Avoid skin contact.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.

3. Storage and Stability

On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.

4. Contents of the Kit

- 4.1 **MT strips** **STRIPS** 12 strips
8 wells per strip
coated with TSH receptor
- 4.2 **Start Buffer** **START** 1 vial
10 ml, ready for use
- 4.3 **Standards A – D** **CAL A** – **CAL D** 4 vials
1 ml each, ready for use
Concentrations (NIBSC 90/672):
- | Standard | A | B | C | D |
|----------|---|---|---|----|
| U/l | 1 | 2 | 8 | 40 |
- 4.4 **Positive Control** **CON +** 1 vial
1 ml, ready for use
values for the Control are given on the vial label
- 4.5 **Negative Control** **CON -** 1 vial
1 ml, ready for use
- 4.6 **TSH-Biotin** **TSH-BIOTIN** 3 vials
4.5 ml per vial, lyoph.
- 4.7 **Reconstitution Buffer** **RECONST** 1 vial
15 ml, coloured red, ready for use
for reconstituting TSH-biotin
- 4.8 **Streptavidin-peroxidase (SA-POD)** **SA-POD** 1 vial
0.75 ml, 20 x concentrated
- 4.9 **Diluent** **DIL** 1 vial
15 ml, ready for use
for diluting SA-POD
- 4.10 **Substrate** **SUB** 1 vial
15 ml tetramethyl benzidine (TMB), ready for use

4.11	Wash Buffer 100 ml, 10 x concentrated	WASH	1 vial
4.12	Stop Solution 10 ml, ready for use 0.5M sulphuric acid	STOP	1 vial

Additional materials and equipment required but not provided:

- Pipettes for 50 µl, 75 µl, 100 µl, and 4.5 ml
- ELISA plate shaker capable of 500 shakes per min
- Pure water
- Microtiter plate reader (450 nm)

5. Specimen Collection and Storage

Sera to be analysed should be assayed soon after separation or stored (preferably in aliquots) at or below -20 °C.

0.2 ml is sufficient for one assay. Subsequent freezing and thawing or increase in storage temperature must be avoided. Incorrect storage of serum samples can lead to loss of anti-TSH receptor autoantibodies. Do not use grossly haemolysed or lipaemic serum samples. Do not use plasma in the assay.

When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step.

6. Limitations

- Lipaemic or grossly haemolysed sera should not be used.
- Do not use plasma.
- Always centrifuge serum immediately before assay.
- Sera with unusually low or unusually high protein concentrations give erroneous results.
- Always store frozen serum samples carefully and do not allow increases in temperature above -20 °C. Incorrect storage can lead to loss of antibody activity.

7. Test Procedure

7.1. Preparation of Reagents

MT strips **STRIPS**

Before opening the packet of strip wells, allow it to stand at room temperature for at least 30 minutes. After opening, keep any unused wells in the original foil packet (reseal with adhesive tape) and in the self-seal plastic bag with the desiccant provided. Store at 2-8°C and use within 3 months.

TSH-Biotin **TSH-BIOTIN**

Reconstitute the contents of one vial with 4.5 ml Reconstitution Buffer (coloured red). If more than 1 vial of TSH-Biotin is going to be used, pool the contents of each vial after reconstitution and mix gently before use. Store at 2-8°C for up to 3 months after reconstitution.

Streptavidin-Peroxidase (SA-POD) **SA-POD**

Dilute the concentrate 1 in 20 with the Diluent provided (e.g. 0.5 ml SA-POD + 9.5 ml Diluent). Store at 2-8°C for up to four weeks after dilution.

Wash Buffer **WASH**

Dilute the contents of one bottle to 1 litre with pure water before use. Store at 2-8°C after dilution.

7.2. Assay Procedure

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips to reach room temperature (at least 30 min), remove the number of strip wells required and fit them firmly into the frame provided. Negative and Positive Controls in duplicate must always be included in each assay run. Standards need not be included if results are to be expressed as inhibition of TSH binding.

1. Add 75 μ l of Start Buffer into each well to be used in the assay.
2. Pipette each 75 μ l of Standards A - D, Negative and Positive Controls and test sera into the appropriate wells.
3. Cover the frame and incubate for 2 hours at room temperature on an ELISA plate shaker at 500 shakes per min.
4. After the 2-hour incubation, aspirate or discard the samples from the wells, add 300 μ l of Wash Buffer and aspirate or discard again. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
5. Pipette 100 μ l of reconstituted TSH-Biotin into each well and incubate for 25 min at room temperature (20 – 25 $^{\circ}$ C) without shaking.
6. After the 25-minute incubation with TSH-Biotin, aspirate or discard the reagent from the wells, add 300 μ l of Wash Buffer and aspirate or discard again. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
7. Pipette 100 μ l of ready-for-use diluted SA-POD into each well and incubate for 20 minutes at room temperature without shaking.
8. Aspirate or discard the reagent from the wells and wash twice with Wash Buffer followed by one wash with pure water to remove any foam from the wells. If a plate washing machine is used, wash 3 times with Wash Buffer only (i.e. omit water wash). Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

9. Pipette 100 µl of Substrate (TMB) into each well and incubate for 30 minutes at room temperature in the dark during which time a blue colour will develop.
10. Stop the substrate reaction by addition of 50 µl of Stop Solution to each well (this will cause the blue colour to turn yellow) and shake the plate for about 5 seconds on a plate shaker to ensure uniformity of the solution in each well.
11. Read the absorbance at 450 nm using an ELISA plate reader blanked against a well containing 100 µl Substrate plus 50 µl of Stop Solution.

8. Calculation of Results

If results are to be expressed as inhibition of TSH binding, this index is calculated as:

$$100 \times \left(1 - \frac{\text{O.D. test sample}}{\text{O.D. Negative Control}} \right)$$

Alternatively, if the kit Standards have been run in the assay, a standard curve can be plotted with absorbance at 450 nm at the vertical axis and \log_{10} Standards concentration on the horizontal axis. Other data reduction procedures can also be used.

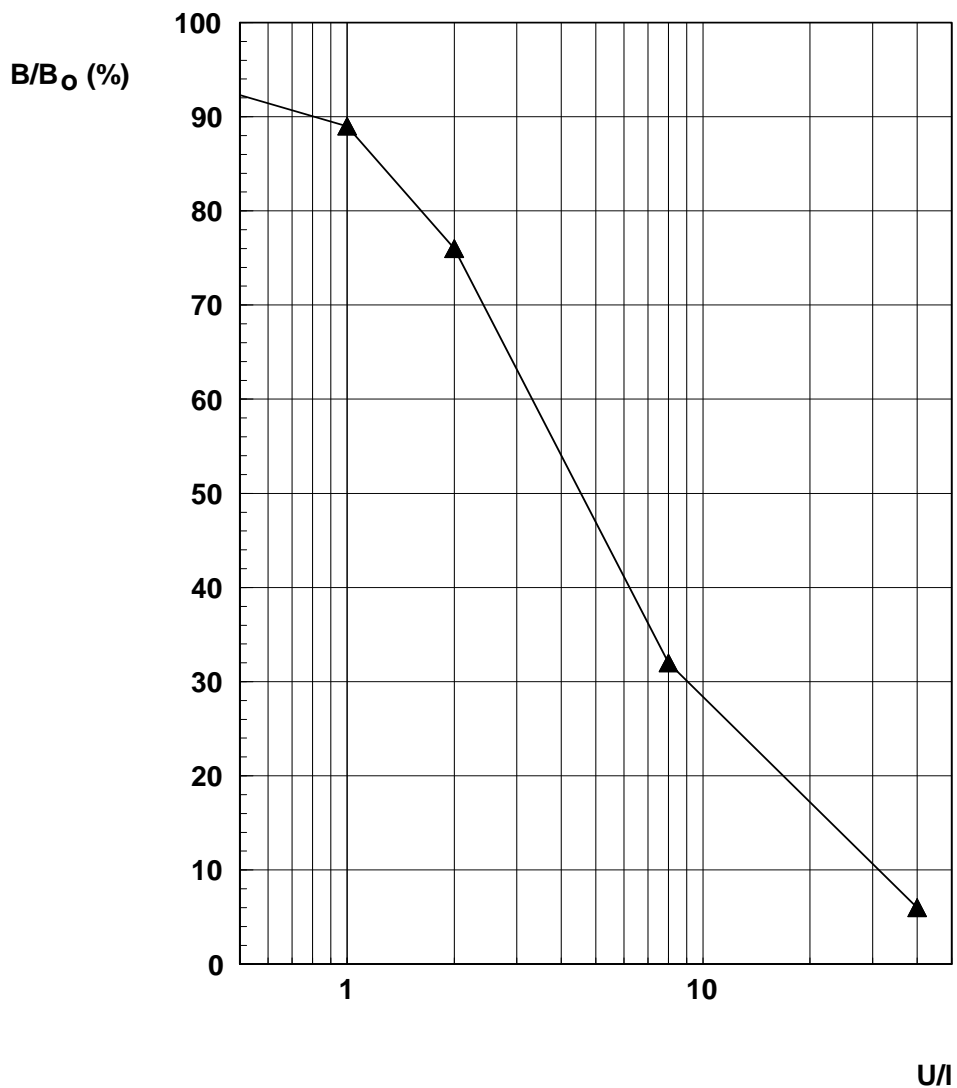
It is most important that the plate reader used has a linear relationship between concentration and absorbance in the region of the OD observed for the Negative Control, i.e. about 2.0 in the normal assay protocol and below. If the plate reader is an older machine, it may not be able easily to distinguish between changes in absorbance at ODs of about 2. Newer machines are usually linear up to ODs of 4. To deal with the problem of older plate readers, there are 2 possible solutions - shorten the substrate incubation time (e.g. to 10 min or 15 min) to reduce the OD values or read the absorbance at a non-optimum wavelength (e.g. 405 rather than 450 nm).

9. Typical Example

Typical results are shown in the following table.

Sample	OD ₄₅₀	% B/B ₀	% Inhibition of TSH binding
Negative Control (B ₀)	1.978	100	0
Standard A = 1 U/I	1.766	89	11
Standard B = 2 U/I	1.508	76	24
Standard C = 8 U/I	0.624	32	68
Standard D = 40 U/I	0.117	6	94
Positive Control = 3.4 U/I	1.121	57	43

Note: Units are thyroid stimulating antibody first international standard 90/672.



10. Expected Values

In the presence of the Negative Control, absorbance at 450 nm should be between 1.5 and 2 and this will be reduced to an absorbance of about 0.2 in the presence of sera with very high levels of TRAb.

In a study of 150 individual healthy blood donor sera, 147/150 (98%) gave values of less than 1 Unit/L (also less than 10% inhibition of TSH binding).

Analysis of sera from patients with autoimmune diseases other than Graves' disease indicated no interference from autoantibodies to thyroglobulin, thyroid peroxidase, glutamic acid decarboxylase, 21-hydroxylase, acetylcholine receptor, rheumatic factor or dsDNA. Furthermore, neither haemoglobin up to 0.5 mg/ml nor bilirubin up to 0.2 mg/ml had any influence in the assay. In addition, human LH up to 10 U/ml, hCG up to 160 U/ml, human FSH up to 70 U/ml and human TSH up to 30 U/l had no effect on TSH binding.

Assessment of results obtained with healthy blood donor sera, sera from different patient groups and the functional sensitivity of the assay indicate that values of 1 U/l or less can be considered TRAb negative, values of 1.1 to 1.5 U/l as borderline positive and values of greater than 1.5 U/l as positive. However, individual laboratories should establish their own reference ranges.

11. Assay Precision

Typical coefficients of variation are

intra assay

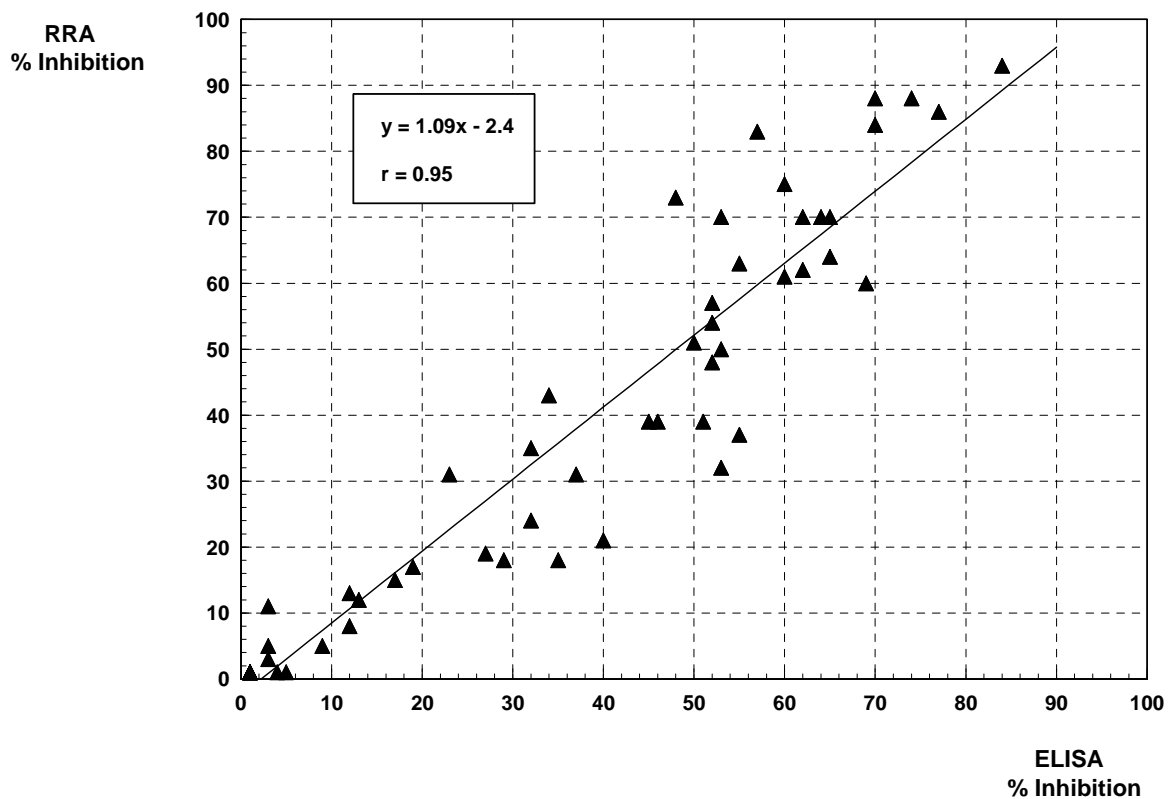
1 U/l (n=12)	1.7 U/l (n=12)	3 U/l (n= 12)	8 U/l (n=12)	22 U/l (n=12)
16%	14%	3.9%	2.4%	5.5%

inter assay

1.3 U/l (n=10)	1.8 U/l (n=10)	2.9 U/l (n= 10)	5.6 U/l (n=11)	9.0 U/l (n=8)
20%	15%	10%	6%	9.5%

12. Comparison Data

56 patient sera were compared using the conventional ^{125}I -labelled radio receptor assay with PEG precipitation and the ELISA test. Results are shown in the following figure. A good agreement with a correlation coefficient of $r = 0.95$ was found between the two methods.



13. Literature

1. Rees Smith B., McLachlan S.M. and Furmaniak J.
Autoantibodies to the thyrotropin receptor.
Endocrine Reviews (1988) 9:106-121.
2. Bolton J., Sanders J., Oda Y., Chapman C., Konno R., Furmaniak J. and Rees Smith B.
Measurement of thyroid-stimulating hormone receptor autoantibodies by ELISA.
Clinical Chemistry (1999) 45: 2285-2287
3. Sanders J., Oda Y., Roberts S., Kiddie A., Richards T., Bolton J., McGrath V., Walters S., Jaskolski D, Furmaniak J. and Rees Smith B.
The interaction of TSH receptor autoantibodies with ^{125}I -labelled TSH receptor.
Journal of Clinical Endocrinology and Metabolism (1999) 84: 3797-3802

Pipetting Scheme

		B ₀	Standard	Positive Control	Patients
Start Buffer	μl	75	75	75	75
Negative Control	μl	75			
Standards A - D	μl		75		
Positive Control	μl			75	
Patient sample	μl				75

Cover and incubate for 2 hours at RT on a shaker

Aspirate / discard and wash once with each 300 μl Wash Buffer

TSH-Biotin	μl	100	100	100	100
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Incubate for 25 min at RT (without shaking)

Aspirate / discard and wash once with each 300 μl Wash Buffer

SA-POD	μl	100	100	100	100
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Incubate for 20 min at room temperature (without shaking)

Aspirate / discard and wash twice with each 300 μl Wash Buffer
Wash once with 300 μl pure water

TMB-Substrate	μl	100	100	100	100
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Incubate for 30 minutes at RT in the dark

Stop Solution	μl	50	50	50	50
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5 sec shaking on an ELISA plate shaker

Reading of absorbance at 450 nm