DIAGNOSTIKA GMBH

Instructions for Use

TSH Receptor Antibody ELISA

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

CE

IVD

REF EA101/96

12 x 8

2 – 8 °C

DLD Gesellschaft für Diagnostika und medizinische Geräte mbH
Adlerhorst 15 • D-22459 Hamburg • Tel.: +49/ 40/ 555 87 10 • Fax: +49/ 40/ 555 87 111
Internet: htpp://www.dld-diagnostika.de • e-mail: contact@dld-diagnostika.de

Contents

Symbols

I V D	In-Vitro-Diagnostic Device	CE	EC Declaration of Conformity
CONT	Contents		Expiry Date
LOT	Lot Number	+2 +8 °C	Store
	Manufactured by	Σ	Sufficient for
REF	Catalogue Number		Consult Instructions

Warning

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

Warning

4.3 **Standards A – D** **CAL A** – **CAL D** 4 vials
1 ml each, ready for use

Concentrations (NIBSC08/204):

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

Warning

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** **WASH**  Warning 1 vial
100 ml, 10 x concentrated

4.12 **Stop Solution** **STOP** 1 vial
10 ml, ready for use
0.5M sulphuric acid

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 for up to expiry of the kit.

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 expiry of the kit.

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 expiry of the kit.

Wash Buffer **WASH**

Dilute the contents of one bottle to 1 litre with pure water before use. Store at 2-8 °C for up to expiry of the kit.

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. Duplicate determinations are strongly recommended for test sera, calibrators and controls. Standards need not be included if results are to be expressed as inhibition of TSH binding. Do not perform the assay at temperatures above 25°C.

1. Add 75 µl of Start Buffer into each well to be used in the assay.

2. Pipette each 75 µl 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 µl 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 µl of reconstituted TSH-Biotin into each well and incubate for 25 min at room temperature (20 – 25 °C) without shaking.

6. After the 25-minute incubation with TSH-Biotin, aspirate or discard the reagent from the wells, add 300 µl 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 µl 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. Within 15 minutes 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. The concentrations in patients' sera can then be read off the calibration curve, plotted as a spline log/lin curve. The negative control can be assigned a value of 0.1 to assist in computer processing of assay results. Other data reduction procedures can also be used.

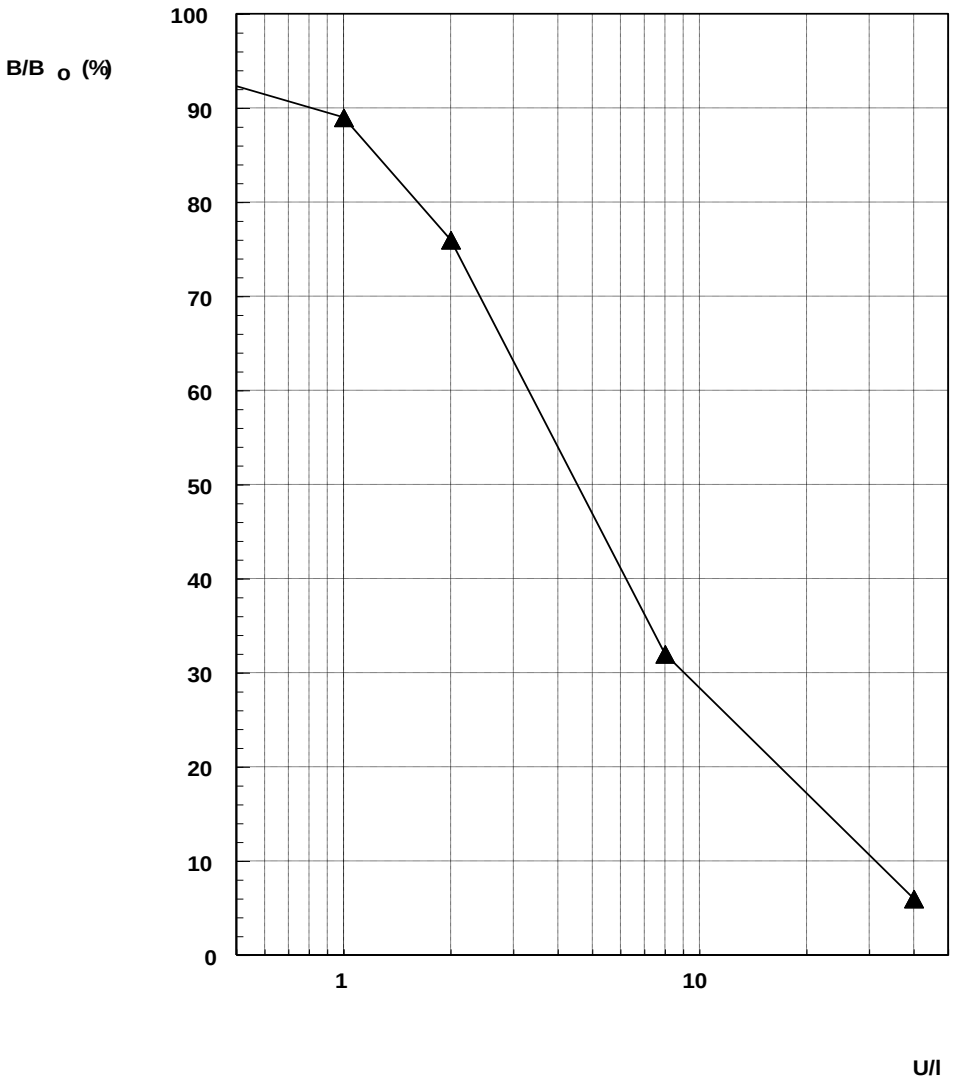
It is most important that the plate reader used has a linear relationship between concentration and absorbance in the region of the OD observed for the Negative Control, i.e. about 2.0 in the normal assay protocol and below. If the plate reader is an older machine, it may not be able easily to distinguish 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/l	1.766	89	11
Standard B	=	2 U/l	1.508	76	24
Standard C	=	8 U/l	0.624	32	68
Standard D	=	40 U/l	0.117	6	94
Positive Control	=	3.4 U/l	1.121	57	43

Note: Units are thyroid stimulating antibody first international standard08/204.



10. Expected Values

In a study of 154 individual healthy blood donor sera, 152/154 (99%) gave values of less than 1 Unit/l.

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 5 mg/ml nor bilirubin at 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 3 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.

50 Sera from patients diagnosed with Graves' disease were assayed. 49 (98%) were identified as being positive for TRAb. 1 sample (2%) was identified as being within the equivocal range.

11. Assay Precision

Typical coefficients of variation are

intra assay

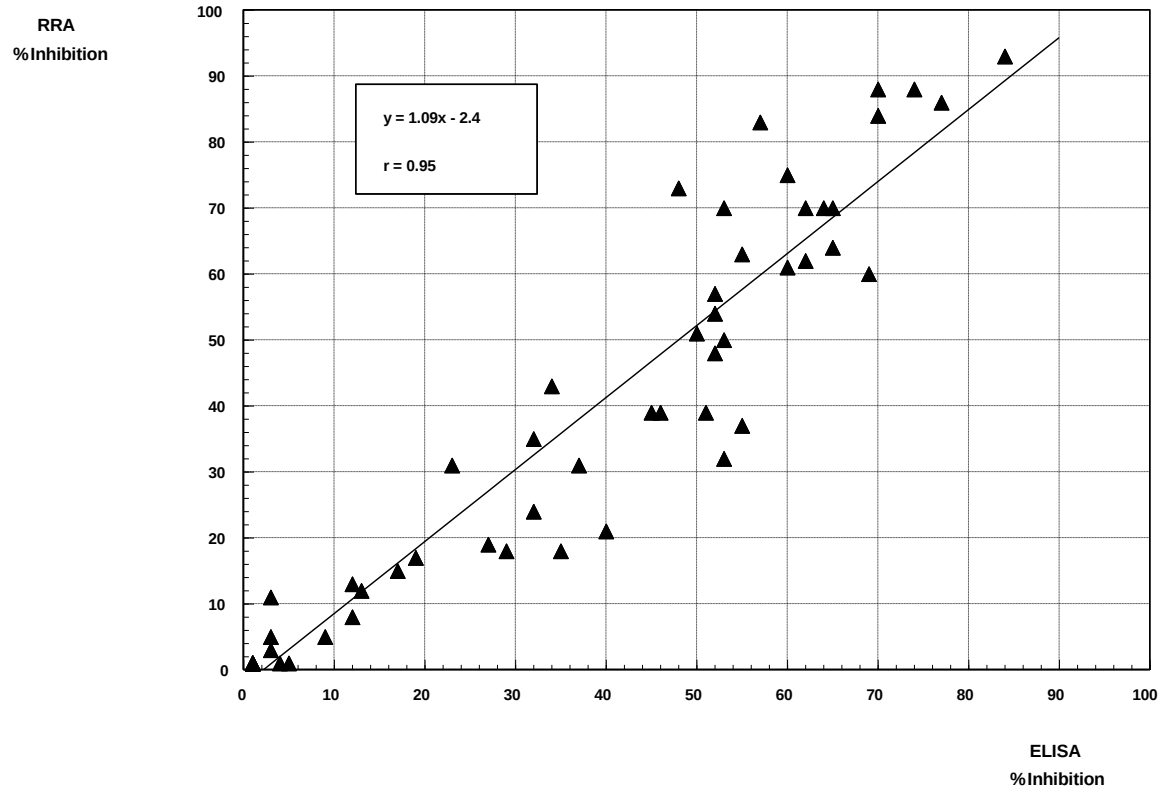
Sample	U/l (n=25)	CV (%)
1	1.8	7.1
2	7.8	2.2

inter assay

Sample	U/l (n=20)	CV (%)
1	3.9	12.9
2	5.4	10.9

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

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

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

B. Rees Smith et al
A new assay for thyrotropin receptor autoantibodies
Thyroid 2004 14: 830-835

Pipetting Scheme

Do not perform the assay at temperatures above 25°C.

		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

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

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

Incubate for 30 minutes at RT in the dark

Stop Solution	µl	50	50	50	50

5 sec shaking on an ELISA plate shaker

Reading of absorbance at 450 nm