ElisaRSR™ TRAb 3rd Generation

TSH Receptor Autoantibody 3rd Generation ELISA Kit - Instructions for use

CE

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INTENDED USE

RSR's TSH receptor (TSHR) autoantibody (TRAb) ELISA is intended for use by professional persons only for the quantitative determination of TRAb in human serum. Hyperthyroidism in Graves' disease is due to the presence of autoantibodies to the TSHR and measurement of these autoantibodies can be useful in disease diagnosis and management.

REFERENCES

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

K. Kamijo et al
Clinical Evaluation of 3rd Generation assay for Thyrotropin Receptor Antibodies: The M22-biotin-based ELISA initiated by Smith
Endocrine Journal 2005 52: 525-529

A. Theodoraki et al
Performance of a third-generation TSH-receptor antibody in a UK clinic
Clinical Endocrinology 2011 75: 127-133

PATENTS

The following patents apply:
US Patents US 8,110,664 B2.

ASSAY PRINCIPLE

In the ELISA, TRAb in patients' sera, calibrators and controls are allowed to interact with TSHR coated onto ELISA plate wells. After a 2 hour incubation, the samples are discarded leaving TRAb bound to the immobilised receptor. A human monoclonal autoantibody to the TSHR, labelled with biotin, (M22-Biotin) is added in a second incubation step, where it interacts with immobilised TSHR which have not been blocked by bound TRAb. The amount of M22-Biotin bound to the plate is then determined in a third incubation step by addition of Streptavidin Peroxidase (SA-POD) which binds specifically to Biotin. Excess unbound SA-POD is then discarded and addition of the peroxidase substrate 3,3',5,5'–tetramethyl-benzidine (TMB) results in the formation of a blue colour. This reaction is stopped by 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 a test sample (as TRAb inhibit the binding of M22-Biotin to TSHR coated plate wells). The measuring interval is 0.4 – 30 IU/L (NIBSC 08/204).

STORAGE AND PREPARATION OF TEST SERUM SAMPLES

Sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at or below –20°C. 150 µL is sufficient for one assay (duplicate 75 µL determinations). 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 lipaemic or haemolysed serum or samples containing particulates. 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 minutes at 10-15,000 rpm in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step for sera that are cloudy or contain particulates.

SYMBOLS

Symbol	Meaning
CE	EC Declaration of Conformity
IVD	In Vitro Diagnostic Device
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured by
Σ	Sufficient for
	Expiry Date
8°C 2°C	Store
CONTROL +	Positive Control
CONTROL -	Negative Control

MATERIALS REQUIRED AND NOT SUPPLIED

Pipettes capable of dispensing 50 µL, 75 µL, 100 µL and appropriate volumes for diluting SA-POD (F).
Means of diluting concentrated wash (I).
Pure water.
ELISA Plate reader suitable for 96 well formats and capable of measuring at 450nm.
ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).
ELISA Plate cover.

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and all kit components (A–J) at 2–8 °C

A	**TSH Receptor Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Allow to stand at room temperature (20-25 °C) for at least 30 minutes before opening.
	Ensure wells are fitted firmly into frame provided. After opening, return any unused wells to the original foil packet and seal with adhesive tape. Place foil bag in the self-seal plastic bag with desiccant provided, at 2-8°C for up to 15 weeks.
B	**Start Buffer** 10 mL Coloured yellow Ready to use
C1-5	**Calibrators** 0.4, 1.0, 2.5, 10 and 30 IU/L (units are NIBSC 08/204) 5 x 1.0 mL Ready to use
D1	**Negative Control** 1.0 mL Ready to use
D2	**Positive Control** **(See label for concentration range)** 1.0 mL Ready to use
E	**M22-Biotin** 15 mL Coloured red Ready to use
F	**Streptavidin Peroxidase (SA-POD)** 0.75 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (G). For example, 0.5 mL (F) + 9.5 mL (G). Store at 2–8°C after dilution for up to kit expiry date.
G	**Diluent for SA-POD** 15 mL Ready to use
H	**Peroxidase Substrate (TMB)** 15 mL Ready to use
I	**Concentrated Wash Solution** 100 mL Concentrated
	Dilute to 1 litre with pure water before use. Store at 2–8°C up to kit expiry.
J	**Stop Solution** 10 mL Ready to use

ASSAY PROCEDURE

Allow all reagents and test samples to stand at room temperature (20-25°C) for at least 30 minutes before use. A repeating Eppendorf type pipette is recommended for steps 1, 5, 8, 10 & 11. Duplicate determinations are strongly recommended for test sera, calibrators and controls.

1.	Pipette **75 µL** of start buffer (B) into respective wells, leaving the last well for a blank (see step 12).
2.	Pipette **75 µL** of calibrators (C1-5), controls (D1 and D2) and test sera into respective wells (start with the 30 IU/L calibrator and descend down the plate to the negative control and then test sera, in duplicate is recommended), leaving the last well blank.
3.	Cover the frame and shake the wells for 2 hours at room temperature (20–25°C) on an ELISA plate shaker (500 shakes per min.).
4.	Aspirate well contents by use of a plate washing machine or discard by briskly inverting the frame of wells over a suitable receptacle. Wash the wells once by addition of diluted wash solution (I) and aspirating the wash by use of a plate washing machine, or discard the wash by briskly inverting the frame of wells over a suitable receptacle. Tap the inverted wells gently on a clean, dry, absorbent surface to remove excess wash solution (only necessary if washing plate by hand).
5.	Pipette **100 µL** M22-Biotin (E) into each well (except blank). Avoid splashing the material out of the wells during addition.
6.	Cover the frame, and incubate at room temperature for 25 minutes without shaking.
7.	Repeat wash step 4.
8.	Pipette **100 µL** of diluted SA-POD (F) into each well (except blank) and incubate at room temperature for 20 minutes without shaking.
9.	Aspirate well contents by use of a plate washing machine or discard by briskly inverting the frame of wells over a suitable receptacle. Wash the wells twice with diluted wash solution (I) followed by once with pure water (to remove any foam) and tap the inverted wells gently on a clean dry absorbent surface to remove excess wash solution (if a plate washing machine is used, the plate can be washed 3 times with diluted wash solution (I) only).
10.	Pipette **100 µL** of TMB (H) into each well (including blank) and incubate in the dark at room temperature for 30 minutes without shaking.
11.	Pipette **50 µL** stop solution (J) into each well (including blank), cover the frame and shake for approximately 5 seconds on a plate shaker. Ensure substrate incubation times are the same for each well.

12.	Within 15 minutes, read the absorbance of each well at 450nm using an ELISA plate reader, blanked against the well containing **100 µL** of TMB (H) and **50 µL** stop solution (J) only.

RESULT ANALYSIS

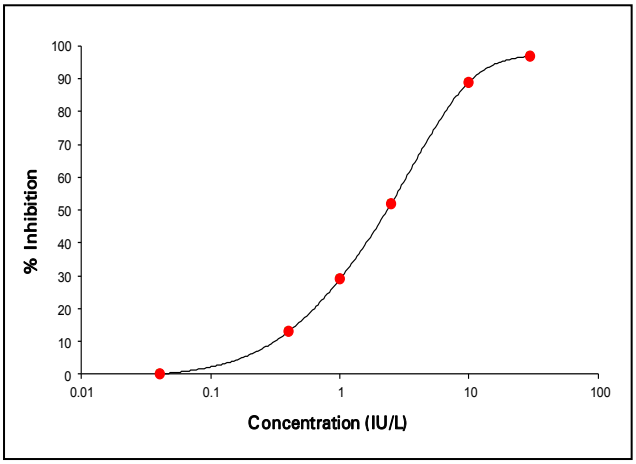
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The TRAb concentrations in patients' sera can then be read off the calibration curve) [plotted at RSR as a spline log/lin curve (smoothing factor = 0)]. Other data reduction systems can be used. The negative control can be assigned a value of 0.04 to assist in computer processing of assay results. Results can also be expressed as inhibition (%I) of M22 binding calculated using the formula:

$$100 \times \left(1 - \frac{\text{test sample absorbance at 450nm}}{\text{negative control (D1) absorbance 450nm}}\right)$$

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)

Sample	Absorbance at 450nm (minus blank)	%I	IU/L
Control D1	2.289		
C1	1.910	17	0.4
C2	1.553	32	1
C3	1.008	56	2.5
C4	0.278	88	10
C5	0.077	97	30
Control D2	1.231	46	1.8



ASSAY CUT OFF

	IU/L
Negative	< 0.4 IU/L
Positive	≥ 0.4 IU/L

This cut off has been validated at RSR. However each laboratory should establish its own normal and pathological reference ranges for TRAb levels. Also it is recommended that each laboratory include its own panel of control samples in the assay.

CLINICAL EVALUATION

Clinical Specificity

139 Sera from healthy blood donors were assayed in the 3rd generation TRAb ELISA kit. All 139 were found to be negative for TRAb.

Clinical Sensitivity

108 Sera from patients with Graves' disease (treated and untreated patients) were assayed and 103 (95%) were identified as being positive for TRAb.

Lower Detection Limit

The kit negative control was assayed 50 times and the mean and standard deviation calculated. The lower detection limit at 2 standard deviations was 0.08 IU/L.

Inter Assay Precision

Sample	IU/L (n = 20)	CV (%)
1	5.5	8.7
2	1.6	8.9

Intra Assay Precision

Sample	IU/L (n = 21)	CV (%)
3	1.3	5.5
4	5.1	4.2

Clinical Accuracy

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; dsDNA or from rheumatoid factor.

Interference

No interference was observed when samples were spiked with the following materials; haemoglobin at 5 mg/mL; bilirubin at 0.2 mg/mL; Intralipid up to 30 mg/mL; 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 mu/mL.

SAFETY CONSIDERATIONS

<u>Streptavidin Peroxidase (SA-POD)</u>

Signal word: Warning

Hazard statement(s)

H317: May cause an allergic skin reaction

Precautionary statement(s)

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P302 + P352: IF ON SKIN: Wash with plenty of soap and water

P333 + P313: If skin irritation or rash occurs: Get medical advice/attention
P362 + P364: Take off contaminated clothing and wash it before reuse

Peroxidase Substrate (TMB)

Signal word: Danger

Hazard statement(s)
H360: May damage fertility or the unborn child
Precautionary statement(s)
P280: Wear protective gloves/protective clothing/ eye protection/face protection
P308 + P313: IF exposed or concerned: Get medical advice/attention

Diluent for SA-POD and Concentrated Wash Solution

Hazard statement(s)
EUH208: Contains 2-Chloroacetamide. May produce an allergic reaction

This kit is intended for *in vitro* use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified stability for coated wells and diluted reagents. Refer to Safety Data Sheet for more detailed safety information. Material of human origin used in the preparation of the kit has been tested and found non-reactive for HIV1 and 2 and HCV antibodies and HBsAg but should, none-the-less, be handled as potentially infectious. Wash hands thoroughly if contamination has occurred and before leaving the laboratory. Sterilise all potentially contaminated waste, including test specimens before disposal. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious. Some components contain small quantities of sodium azide as preservative. With all kit components, avoid ingestion, inhalation, injection and contact with skin, eyes and clothing. Avoid formation of heavy metal azides in the drainage system by flushing any kit component away with copious amounts of water.

ASSAY PLAN

Allow all reagents and samples to reach room temperature (20-25 °C) before use	
Pipette:	**75 µL** Start buffer into each well (except blank)
Pipette:	**75 µL** Calibrators (starting with the highest concentration and descending to the lowest), controls, patient sera (except blank)
Incubate:	2 Hours at room temperature on an **ELISA plate shaker at 500 shakes/min**
Aspirate/Decant:	Plate
Wash:	Plate once on automatic washer (or wash once, invert and tap dry on absorbent material for manual washing)
Pipette:	**100 µL** M22-Biotin into each well (except blank)
Incubate:	25 minutes at room temperature **without shaking**
Aspirate/Decant:	Plate
Wash:	Plate once as above
Pipette:	**100 µL** SA-POD (diluted 1:20) into each well (except blank)
Incubate:	20 Minutes at room temperature **without shaking**
Aspirate/Decant:	Plate
Wash:	Plate three times on automatic washer (or wash twice, rinse once with pure water and dry on absorbent material for manual washing)
Pipette:	**100 µL** TMB into each well (including blank)
Incubate:	30 Minutes at room temperature **in the dark without shaking**
Pipette:	**50 µL** Stop solution into each well (including blank) and shake for 5 seconds
Read absorbance at 450 nm, within 15 minutes of adding stop solution	
Do not perform the assay at temperatures above 25 °C	