ElisaRSR™ TRAb Fast™

Fast TSH Receptor Autoantibody ELISA Kit - Instructions for use

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RSR Limited
Parc Ty Glas, Llanishen,
Cardiff CF14 5DU United Kingdom
Tel.: +44 29 2068 9299 Fax: +44 29 2075 7770
Email: info@rsrltd.com Website: www.rsrltd.com

EC REP Advena Ltd. Tower Business Centre, 2nd Flr., Tower Street, Swatar, BKR 4013 Malta.

INTENDED USE

The RSR Fast 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

J. Sanders et al
"Human monoclonal thyroid stimulating autoantibody"
Lancet 2003 **362**:126-128

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

PATENTS

The following patents apply:
European Patents EP 1 456 234 B1, EP 1 565 493 B1, EP 2 383 290 B1, EP 2 383 291 B1 and EP 2 383 296 B1, US Patents US 8,309,693 B2, US 8,298,771 B2, US 8,298,769 B2, US 8,110,664 B2, US 8,753,637 B2, US 8,900,823 B2 and US 9,751,940 B2, Chinese patents CN 101799476 B, CN 103224562 B, CN 1622958 B and CN 1717418 B, Indian patents 219312 and 226719 and Japanese Patents 5576072, 5314234 and 5722927.

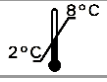
ASSAY PRINCIPLE

In RSR's Fast TRAb ELISA, TRAb in patients' sera, calibrators and controls are allowed to interact with TSHR coated onto ELISA plate wells. After a 1 hour incubation, the samples are discarded leaving TRAb bound to the immobilised TSHR. A thyroid stimulating human monoclonal autoantibody (M22, in the form of M22-Peroxidase) 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-Peroxidase bound to the plate is then determined in a third incubation step by the addition of the peroxidase substrate, 3,3',5,5'–tetramethyl-benzidine (TMB) resulting in the 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 M22-Peroxidase to TSHR coated plate wells.
The high sensitivity of the M22 based assay and use of M22-Peroxidase (rather than M22-Biotin followed by streptavidin peroxidase) allows a shorter first incubation and fewer steps giving a fast ELISA. The measuring range is 1 – 40 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 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 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
	Sufficient for
	Expiry Date
	Store
CONTROL +	Positive Control
CONTROL -	Negative Control

MATERIALS REQUIRED AND NOT SUPPLIED

Pipettes capable of dispensing 50 µL, 75 µL, and 100µL.
Means of measuring out various volumes to reconstitute or dilute reagents.
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 kits and all kit components (A-I) at 2–8°C.

A	**TSH Receptor Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in 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 tape. Then place foil bag in the self-seal plastic bag with desiccant provided, and store at 2-8°C for up to 12 weeks.
B	**Start Buffer** 10 mL Coloured yellow Ready to use
C1-4	**Calibrators** 1, 2, 8 and 40 IU/L (units are NIBSC 08/204) 4 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-Peroxidase** 2 vials Lyophilised
	Reconstitute each vial with 6 mL reconstitution buffer for M22-Peroxidase (F). Store at 2–8°C for up to shelf life of kit after reconstitution.
F	**Reconstitution Buffer for M22-Peroxidase** 15 mL Ready to use
G	**Peroxidase Substrate (TMB)** 15 mL Ready to use
H	**Concentrated Wash Solution** 100 mL Concentrated
	Dilute to 1 litre with pure water before use. Store at 2–8°C up to expiry date.
I	**Stop Solution** 10 mL Ready to use

ASSAY PROCEDURE

Allow all reagents 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 and 9. Duplicate determinations are strongly recommended for patients' sera, calibrators and controls.

1.	Pipette **75 µL** of start buffer (B) into respective wells (A), leaving the last well empty for a blank (see step 10).
2.	Pipette **75 µL** of patient s' sera, calibrators (C1-4) and controls (D1 and D2) into respective wells (start with the 40 IU/L calibrator and descend down the plate to the negative control and then patients' sera), leaving the last well blank. It is also recommended that the negative control is included again at the end of the patients' sera, at least in initial assay runs. Calibrators need not be included if results are to be expressed as inhibition of M22 binding (see result analysis).
3.	Cover the frame and shake the wells for 1 hour at room temperature on an ELISA plate shaker (500 shakes per min.).
4.	Aspirate the wells by use of a plate washing machine or discard the samples by briskly inverting the frame of wells over a suitable receptacle. Wash the wells once with diluted wash solution (H), and aspirate 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** of reconstituted M22-Peroxidase (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, washing 2 times with dilute wash solution and once with pure water, to remove any foam, before inverting and tapping dry. When using a plate washing machine dilute wash solution can be used for the third wash.
8.	Pipette **100 µL** of TMB (G) into each well (including blank) and incubate in the dark at room temperature for 25 minutes without shaking.
9.	Pipette **50 µL** stop solution (I) into each well (including blank), cover the frame and shake for approximately 5 seconds on an ELISA plate shaker. Ensure substrate incubation times are the same for each well.
10.	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 (G) and **50 µL** stop solution (I) **only**.

RESULT ANALYSIS

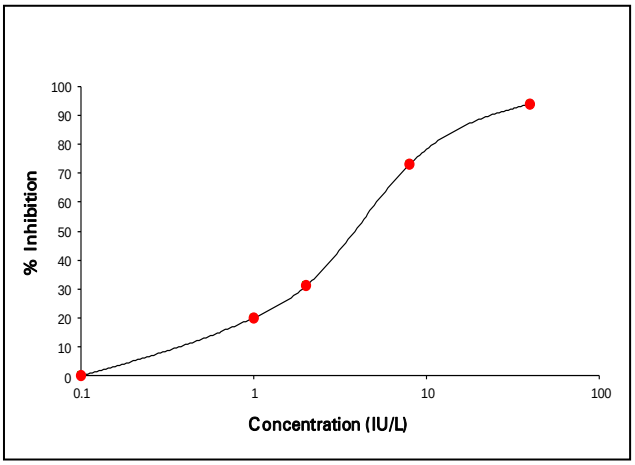
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The TRAb concentrations in patients' sera can then be read off the calibration curve [plotted at RSR as a spline log/lin curve (smoothing factor = 0)]. Other data reduction systems can be used. The negative control can be assigned a value of 0.1 to assist in computer processing of assay results. Results can also be expressed as inhibition (%I) of M22 binding calculated using the formula;

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

Samples with high TRAb concentrations can be diluted in kit negative control (D1). For example, 20µL 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	A450 (minus blank)	%I	IU/L
Control D1	2.096	0	0
C1	1.686	20	1
C2	1.439	31	2
C3	0.576	73	8
C4	0.135	94	40
Control D2	1.034	51	3.9



ASSAY CUT OFF

	IU/L
Negative	< 1 IU/L
Positive	≥ 1 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

104 samples from healthy blood donors (including 44 females) were assayed in the Fast TRAb ELISA kit. 104 (100%) were identified as being negative for TSH receptor autoantibodies.

Clinical Sensitivity

82 samples from patients diagnosed with Graves' disease (treated and untreated patients) were assayed using the Fast TRAb ELISA kit and 70 (85%) were identified as being positive for TSH receptor autoantibodies.

Lower Detection Limit

The kit negative control was assayed 54 times and the mean and standard deviation calculated. The lower detection limit at 2 standard deviations was 0.16 IU/L.

Inter Assay Precision

Sample	IU/L (n = 20)	CV (%)
1	4.6	3.3
2	18.6	7.6

Intra Assay Precision

Sample	IU/L (n = 20)	CV (%)
1	2.0	7.2
2	7.1	3.9

Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than Graves' disease indicated no interference from autoantibodies to thyroglobulin; thyroid peroxidase; 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 30 mu/mL.

SAFETY CONSIDERATIONS

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

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 shelf life for coated wells, diluted and reconstituted 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:	1 Hour 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-Peroxidase (reconstituted) into each well (except blank)
Incubate:	25 Minutes at room temperature **without shaking**
Aspirate/Decant:	Plate
Wash:	Plate three times as above
Pipette:	**100 µL** TMB into each well (including blank)
Incubate:	25 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	