



INSTRUCTION MANUAL

IVD

(March 28th, 2007)

Medizym[®] T.R.A.

- 96 determinations -

REF 4003



Enzyme immuno assay for the determination
of autoantibodies to TSH receptor
in human serum



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IFU symbols non-radioactive assays MEDIPAN GMBH

	In vitro diagnostic device		EC Declaration of Conformity
	Catalogue number		Batch code
	Expiry date		Manufactured by
	Consult accompanying documents		Consult operating instruction
	Store at		Biological risk
	Coated microtiterplate (96 wells)		Optical density
	Wash buffer		Substrate
	Calibrators		Conjugate
	Stop solution		Control serum
	TSH complex		Incubation buffer
	Conjugate diluent		TSH-dilution buffer

INTENDED USE

Medizym[®] T.R.A. is used for the quantitative determination of Thyrotropin (TSH) receptor autoantibodies in human serum.

Whereas thyrotoxicosis is caused by thyroid autonomy, hyperthyroidism of Graves' disease is due to TSH receptor autoantibodies (TRAb). These autoantibodies mimic TSH effects on the thyroid cell and thus increase blood levels of T₄ and T₃.

Consequently, the measurement of TRAb is valuable for the differential diagnosis of hyperthyroidism as well as for the follow-up of Graves' disease, both during and after its treatment by antithyroid drugs, 131-radioiodine or surgery.

LITERATURE

- Kakinuma A, Morimoto I, Kuroda T, Fujihira T, Eto S, McLachlan SM, and Rapoport B. Comparison of recombinant human thyrotropin receptors versus porcine thyrotropin receptors in the thyrotropin binding inhibition assay for thyrotropin receptor autoantibodies. Thyroid 1999; 9 (9): 849 – 855.
- Zimmermann-Belsing T, Nygaard B, Rasmussen ÅK, and Feldt-Rasmussen U. Use of the 2nd generation TRAK human assay did not improve prediction of relapse after antithyroid medical therapy of Graves' disease. Eur J Endocrinol 2002; 146: 173 – 177.
- Kamijo K. 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 J 2003; 50 (1): 113 – 116.

PRINCIPLE of the TEST

Medizym[®] T.R.A. is a competitive enzyme immunoassay with a step by step incubation.

During the first incubation the TSH receptor antibodies of the patient samples and calibrators bind to the immobilized receptor on the solid phase of the microtiter plate. Following an incubation period of 120 min, antibodies not bound are separated from the solid-phase immune complexes. In a second incubation step of 20 min the TSH complex binds to the free epitopes of the receptor.

The absence of autoantibodies against TSH receptor results in a complete saturation of the provided receptor by the TSH complex. Thus, the more autoantibodies (TRAb) are present in the sample, the less complex is bound by the TSH receptor immobilized.

The bound receptor-TSH complexes then react specifically with horseradish peroxidase (HRPO) conjugate. After the incubation period of 20 min, excessive conjugate is separated from the solid-phase immune complexes by the following washing step.

Horseradish peroxidase converts the colorless substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) added into a blue product. This enzyme reaction is stopped by dispensing an acidic solution (H₂SO₄) into the wells after 20 min at room temperature turning the solution from blue to yellow.

The optical density (OD) of the solution at 450 nm is indirectly proportional to the amount of specific antibodies bound.

The standard curve is established by plotting the concentrations of the antibodies of the standards (x-axis) and their corresponding OD values (y-axis) measured. The concentration of antibodies of the specimen is directly read off the standard curve.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Do not use lipaemic or hemolytic samples. Plasma should not be employed.

Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20 °C.

Preparation before use

Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

The samples may be kept at 2 - 8 °C for up to three days. Long-term storage requires - 20 °C.

Note: Before assayed the sera have to be free of any particulate matter (centrifuge, if necessary and use the **clear supernatants** only).

TEST COMPONENTS for 96 DETERMINATIONS

A	Microtiter plate , 12 breakable strips per 8 wells (total 96 individual wells) coated with ab (mouse)/TSH receptor	1 frame vacuum sealed with desiccant
MP		
B	Wash buffer 10-fold for 1000 ml	100 ml concentrated
WASHB		
G	TSH complex (biotinylated)	3 vials lyophilized
TSH		
K	Dilution buffer for TSH complex	1 x 15 ml ready for use
BUF G		
D	SA-POD conjugate for 15 ml	1 x 0.75 ml concentrated
CONJ		
J	Conjugate diluent	15 ml ready for use
BUF D		
E	Substrate TMB 3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide	15 ml ready for use
SUB		
F	Stop solution 0.25 M sulfuric acid	10 ml ready for use
STOP		
H	Incubation buffer	10 ml ready for use
START		
1 - 4	T.R.A. calibrators (serum) conc.: see leaflet enclosed	1.0 ml, each ready for use
CAL		
CI - II	Controls (serum) conc.: see leaflet enclosed	1.0 ml, each ready for use
CONTROL		

Materials required

- micropipette 100 - 1000 µl
- micropipette 10 - 100 µl
- multi-channel pipette 50 - 200 µl
trough for multi-channel pipette
- 8-channel wash comb with vacuum pump and waste bottle or microplate washer
- microplate reader with optical filters for 450 nm and 620 nm or 690 nm
- horizontal shaker
- graduated cylinders
- very pure distilled water
- foil to seal microtiterplate

Size and storage

Medizym® T.R.A. has been designed for 96 determinations. This is sufficient for the analysis of 42 unknown samples as well as for calibrators and controls assayed in duplicates.

The expiry date of each component is reported on its respective label that of the complete kit on the box labels.

Upon receipt, all components of the Medizym® T.R.A. have to be kept at 2 - 8 °C, preferably in the original kit box.

Preparation before use

Allow sera to reach room temperature prior to use in the assay.

The kit includes the frame for the microtiter plate.

Allow the sealed plate to reach room temperature for at least 30 min before opening.

A Microtiter plate

After opening a bag of strip wells keep any unused wells in the foil packet (reseal with adhesive tape) and place in the bag provided with desiccant. Wells can be stored this way at 2 - 8 °C up to 6 months.

B Wash buffer

Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled water. For example, dilute 50 ml of the concentrate with 450 ml distilled water. B 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.

G TSH complex

Reconstitute each vial with 4.5 ml dilution buffer (K). Remaining reconstituted TSH complex can be stored at 2 - 8 °C up to 20 weeks. Pool the TSH complex solution if more than 1 vial should be used.

D SA-POD conjugate

Dilute SA-POD concentrate (D) 21-fold (1+20) with conjugate diluent (J) **prior to use**.

For example, 0.5 ml SA-POD concentrate + 10 ml conjugate diluent = 10.5 ml conjugate ready for use. For 96 determinations: 96 x 100 µl = 9.6 ml conjugate are necessary.

The diluted SA-POD conjugate is stable at 2 - 8 °C up to 4 weeks.

E Substrate TMB

Avoid exposure to light!

ASSAY PROCEDURE

- Use neat patient sera
- Duplicates are recommended

1. Pipette **50 µl** incubation buffer (H) in the provided wells.
2. Dispense
100 µl control (CI)
100 µl calibrators (1 - 4)
100 µl control (CII)
100 µl neat patient samples
into the respective wells, shake for 5 sec.
3. Seal plate and incubate **120 min** at room temperature while shaking vigorously (>500 rpm). An increase of the incubation time to 160 min will improve the accuracy of the standard curve. Without any shaking the incubation time is at least 180 min.
4. Aspirate or "flick out" by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash once with 300 µl washing solution (diluted from B) with 5 seconds soaking time.
5. Add **100 µl** of reconstituted TSH complex (prepared from G and K) to each well, shake for 5 sec.
6. Seal plate, incubate **20 min** at room temperature.
7. Repeat step 4.
8. Add **100 µl** of diluted conjugate (prepared from D and J) to each well, shake for 5 sec.
9. Seal plate, incubate **20 min** at room temperature.
10. Aspirate or "flick out" by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash 3 times with 300 µl washing solution (diluted from B) with 5 seconds soaking time each.
11. Add **100 µl** of substrate (E) to each well, shake for 5 sec.
12. Incubate **20 min in the dark** at room temperature.
13. Add **50 µl** of stop solution (F) to each well and mix gently.
14. Read the optical density at **450 nm** versus 620 or 690 nm within 20 min after adding the stop solution.

Please note that the washing procedure is crucial. Insufficient washing will result in poor precision and falsely elevated OD readings.

DATA PROCESSING

The standard curve is established by plotting the mean OD-values of the calibrators 1 - 4 and the negative control CI on the ordinate, y-axis, versus their respective T.R.A.-concentrations on the abscissa, x-axis.

TSH receptor antibody concentrations of the unknown samples are directly read off in IU/l (WHO NIBSC 90/672) against the respective OD values.

Medizym® T.R.A. used with Computer Assisted Analysis using software able to curves with 4 parameter / sigmoid.

TYPICAL EXAMPLE

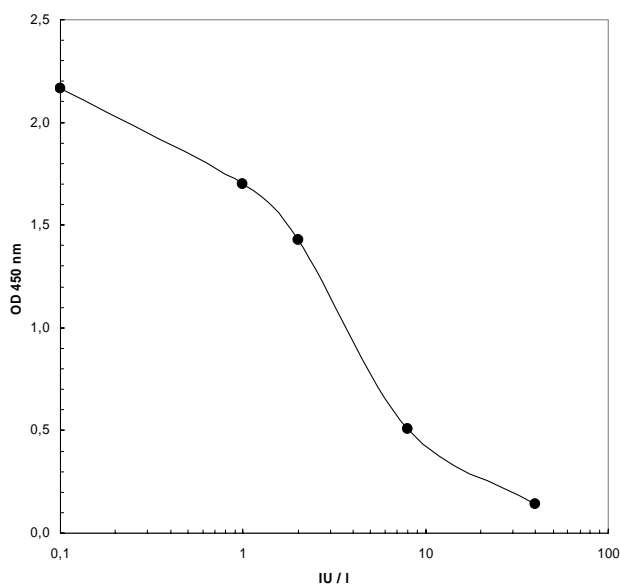
Do not use for evaluation!

well	OD (a)	OD (b)	OD (mean)	IU/l
Control I	2.225	2.105	2.165	0.1
Calibrator 1	1.724	1.675	1.700	1
Calibrator 2	1.411	1.443	1.427	2
Calibrator 3	0.520	0.489	0.505	8
Calibrator 4	0.145	0.135	0.140	40
Control II	1.146	1.051	1.099	2.9

The above mentioned standard concentrations and control values are only an example for a typical standard curve. They can change from lot to lot, see leaflet enclosed. Medizym® T.R.A. is strictly (1:1) calibrated against the WHO standard NIBSC 90/672.

STANDARD CURVE

Typical example



Criteria of validation

Specimens with an OD lower than the OD of calibrator 4 should be diluted by antibody-free serum and tested again. The results have to be multiplied with the dilution factor chosen.

REFERENCE VALUES

Medizym® T.R.A.	IU/l
negative	≤ 1
grey zone	> 1 - 1.5
positive	> 1.5

It is recommended that each laboratory establish its own normal and pathological reference ranges for serum anti-TSH receptor antibody levels, as usually done for other diagnostic parameters, too. Therefore, the above mentioned data only provide a guide to values which might be expected.

CHARACTERISTIC ASSAY DATA

Linearity of Medizym® T.R.A.

Dilutions of specimens in TRAb-free human serum are determined according to their expected theoretical values with Medizym® T.R.A. On the basis of the heterogeneous nature of the autoantibody population and in view of epitope specificity and affinity of the autoantibodies exceptions are possible in some cases.

Specificity

Human TSH levels up to 100 mIU/l did not show any significant cross reactivity in Medizym® T.R.A.

Sensitivity

The lower detection limit or **analytical sensitivity** (0 ± 3 S.D.) can regularly be determined at **0.5 IU/l**.

The most appropriate and statistically reasonable characteristic of any assay is at present the so-called **functional assay sensitivity**. This functional assay sensitivity generally represents that concentration which corresponds to the 10 % (within-assay) and to the 20 % (between assay) coefficient of variation in the respective precision profiles of the assay in the lower concentration range. Upon correct and thorough performance of Medizym® T.R.A., this value is found at **0.8 IU/l**.

Medizym® T.R.A. values below this defined level of functional assay sensitivity do not meet the statistical criteria for reliability according to GLP (Good Laboratory Practice) and therefore can not be distinguished from zero due to the statistically necessary certainty.

Medizym® T.R.A. concentrations above 0.8 IU/l, however, fulfill these criteria and are consequently assessed as valid.

Intra - and inter-assay variation

Intra-assay (n = 12)			Inter-assay (n = 5)		
Sample no.	Mean Concentration (IU/l)	CV (%)	Sample no.	Mean Concentration (IU/l)	CV (%)
1	0.6	13	5	0.2	39
2	1.4	6	6	2.8	6
3	2.8	6	7	4.1	9
4	14.0	4	8	13.7	12

At a cut-off value of 1.5 IU/l the sensitivity was found to be 95 % and the specificity is 100 % for patients with untreated Graves' disease.

LIMITATIONS of the METHOD

Healthy individuals should be tested negative by the Medizym® T.R.A..

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

Medizym[®] T.R.A.

ASSAY SCHEME

Bring all reagents to room temperature. Gently mix all reagents to ensure homogeneity.

Step	Activity	Material	CI / CAL	CII	Patients 1, 2, ...
1	Pipette	Incubation buffer (H)	50 µl	50 µl	50 µl
2	Pipette	Samples	100 µl	100 µl	100 µl
3	Incubate	Plate	120 minutes (room temperature) while shaking vigorously >500rpm		
4	Aspirate or decant	Microtiter plate	put sharply onto absorbent tissue		
	Pipette	Washing solution (made from B)	1 x 300 µl	1 x 300 µl	1 x 300 µl
5	Pipette	TSH solution (made from G and K)	100 µl	100 µl	100 µl
6	Incubate	Plate	20 minutes (room temperature) *		
7	Aspirate or decant	Microtiter plate	put sharply onto absorbent tissue		
	Pipette	Washing solution (made from B)	1 x 300 µl	1 x 300 µl	1 x 300 µl
8	Pipette	Conjugate (made from D and J)	100 µl	100 µl	100 µl
9	Incubate	Plate	20 minutes (room temperature) *		
10	Aspirate or decant	Microtiter plate	put sharply onto absorbent tissue		
	Pipette	Washing solution (made from B)	3 x 300 µl	3 x 300 µl	3 x 300 µl
11	Pipette	Substrate (E)	100 µl	100 µl	100 µl
12	Incubate	Plate	20 minutes (room temperature) in the dark *		
13	Pipette	Stop solution (F)	50 µl	50 µl	50 µl
14	Measure OD	Plate	at 450 nm versus 620 (690) nm within 20 min		

- Only clear sera should be assayed (centrifuge, if necessary)
- * After adding reagents and before any incubation shake the plate for 5 seconds

SAFETY PRECAUTIONS

- **This kit is for in vitro use only.** Follow the working instructions carefully. This instruction manual is valid only for the present kit with the given composition. An exchange of single components is not in agreement with CE regulations.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts (< 0.1 % w/w) of sodium azide as a preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
 - Do not smoke, eat or drink while handling kit material,
 - Always use protective gloves,
 - Never pipette material by mouth,
 - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.