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Instruction for use
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USA: PMS No. K100499

ORG 522 T Rheumatoid Factor Total

Immunometric Enzyme Immunoassay for the quantitative measurement of IgG, IgM and IgA Rheumatoid Factors in serum or plasma

SYMBOLS USED

IVD	In vitro diagnostic medical device
	Manufacturer
REF	Catalogue number
96	Sufficient for 96 determinations
LOT	Batch code
	Use by
	Temperature limitation
	Consult instructions for use
	Keep away from sunlight
	Do not reuse
	Date of manufacture

MICROPLATE	Microplate
CALIBRATOR A	Calibrator
CALIBRATOR B	Calibrator
CALIBRATOR C	Calibrator
CALIBRATOR D	Calibrator
CALIBRATOR E	Calibrator
CALIBRATOR F	Calibrator
CONTROL +	Control positive
CONTROL −	Control negative
DILUENT	Sample Buffer
CONJUGATE	Enzyme Conjugate
TMB	TMB Substrate
STOP	Stop solution
WASH	Wash Buffer
RTU	Ready to use

NAME AND INTENDED USE

Rheumatoid Factor Total is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG, IgM and IgA class rheumatoid factor antibodies in human serum or plasma (Citrate plasma, Na-heparin plasma, EDTA plasma). The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of rheumatoid arthritis (RA).

SUMMARY AND EXPLANATION OF THE TEST

The presence of IgM rheumatoid factor (RF) in the serum is the sole serological indicator included in the ACR list of criteria for the diagnosis of RA. RFs are a subset of antiglobulins directed against the Fc region of IgG. In this definition we do not include antibodies to the IgG Fab region and pepsin agglutinators, directed against neoantigens on IgG exposed by pepsin cleavage. It is claimed that the majority of antiglobulin activity in normal serum is Fab-specific, whereas antiglobulin from RA patients is mostly Fc-specific. RFs are present in the serum of 75-80% of patients with RA at some time during the disease course. However, RFs are also found in the serum of patients with infectious and autoimmune diseases, hyperglobulinemia, B-cell lymphoproliferative disorders and in the aged population. This suggests that RF may be a finding associated with B-cell hyperactivity.

Rheumatoid factors which have been found among the IgM, IgG and IgA classes of immunoglobulins, reacting only with xenogeneic Fc are not autoantibodies and are unlikely to be of pathological significance. However, RFs can bind IgG from many species, including autologous IgG, when immobilised on surfaces. Autologous binding is of higher affinity than xenogeneic binding. The here presented test systems for the determination of rheumatoid factors uses only human Fc fragments as coated antigen.

It is generally considered that high titers of RF are associated with more severe disease and the presence of extra-articular features and rheumatoid nodules. This conclusion may depend on the disease duration. Serum IgM RF may precede the onset of RA by several years. A high titer of RF in non-RA individuals is associated with increased risk of developing RA. In the first 2 years of RA (early RA), serum levels of IgM, IgG and IgA RF do not correlate with disease activity. Serum IgG and IgA RF in these years are prognostic of erosive joint disease.

In established RA, high titer serum IgM RF correlates with the presence of articular disease and nodules but not with systemic disease activity. The presence of either IgG or IgA RF in patients with long-standing RA may be a good prognostic indicator of systemic manifestations. IgG and IgM RF are associated with extra-articular RA including rheumatoid vasculitis and nodules. The presence of IgM RF containing immune complexes with bound complement (C1q) □ is also associated with extra-articular RA.

PRINCIPLE OF THE TEST

Fc fragments of highly purified human Immunoglobulin G are bound to microwells. Antibodies against this antigen, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG, anti-human IgM and anti-human IgA immunologically detect the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG, IgM and IgA antibodies present in the original sample.

WARNINGS AND PRECAUTIONS

1. All reagents of this kit are strictly intended for in vitro diagnostic use only.
2. Do not interchange kit components from different lots.

3. Components containing human serum were tested and found negative for HBsAg, HCV, HIV1 and HIV2 by FDA approved methods. No test can guarantee the absence of HBsAg, HCV, HIV1 or HIV2, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
4. Avoid contact with the TMB (3,3´,5,5´-Tetramethyl-benzidine). If TMB comes into contact with skin, wash thoroughly with water and soap.
5. Avoid contact with the Stop Solution which is acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.
6. Some kit components (i.e. Controls, Sample buffer and Buffered Wash Solution) contain Sodium Azide as preservative. Sodium Azide (NaN_3) is highly toxic and reactive in pure form. At the product concentrations (0.09%), though not hazardous. Despite the classification as non-hazardous, we strongly recommend using prudent laboratory practices (see 8., 9., 10.)
7. Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush drains with copious amounts of water to dilute the components below active levels.
8. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
9. Do not pipette by mouth.
10. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
11. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperature may initiate spontaneous combustion.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

CONTENTS OF THE KIT

Package size	96 determ.
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated Fc fragments of highly purified human Immunoglobulin G. Ready to use.
5 vials, 1.5 ml each	Combined calibrators with IgG, IgM and IgA class rheumatoid factor antibodies (A-E) in a serum/buffer matrix (PBS, BSA, NaN_3 <0.1% (w/w)) containing: 0; 15; 50; 150; 500 U/ml. Ready to use.
2 vials, 1.5 ml each	Rheumatoid factor controls in a serum/buffer matrix (PBS, BSA, NaN_3 <0.1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert. Ready to use.
1 vial, 20 ml	Sample buffer (Tris, NaN_3 <0.1% (w/w)), yellow, concentrate (5x).
1 vial, 15 ml	Enzyme conjugate solution (PBS, Proclin 300 <0.5% (v/v)), (light red) containing polyclonal rabbit anti-human IgG, polyclonal rabbit anti-human IgM and polyclonal rabbit anti-human IgA; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 ml	TMB substrate solution. Ready to use.
1 vial, 15 ml	Stop solution (contains hydrochloric acid). Ready to use.
1 vial, 20 ml	Wash solution (PBS, NaN_3 <0.1% (w/w)), concentrate (50x).

STORAGE AND STABILITY

1. Store the kit at 2-8 ℃.
2. Keep microplate wells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.

4. Do not expose test reagents to heat, sun or strong light during storage and usage.
5. Diluted sample buffer and wash buffer are stable for at least 30 days when stored at 2-8 ℃.
6. Stability of open kit calibrators and controls is at least 30 days when stored at 2-8 ℃.

MATERIALS REQUIRED

Equipment

- Microplate reader capable of endpoint measurements at 450 nm
- Multi-Channel Dispenser or repeatable pipette for 100 µl
- Vortex mixer
- Pipettes for 10 µl, 100 µl and 1000 µl
- Laboratory timing device

Preparation of reagents

- Distilled or deionised water
- Graduated cylinder for 100 and 1000 ml
- Plastic container for storage of the wash solution

SPECIMEN COLLECTION, STORAGE AND HANDLING

1. Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
2. Allow blood to clot and separate the serum by centrifugation.
3. Test serum should be clear and non-hemolysed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
4. Specimens may be refrigerated at 2-8 ℃ for up t o five days.
5. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity.
6. Testing of heat-inactivated sera is not recommended.

PROCEDURAL NOTES

1. Do not use kit components beyond their expiration dates.
2. Do not interchange kit components from different lots.
3. All materials must be at room temperature (20-28 ℃).
4. Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
5. Perform the assay steps only in the order indicated.
6. Always use fresh sample dilutions.
7. Pipette all reagents and samples into the bottom of the wells.
8. To avoid carryover contamination, change the tip between samples and different kit controls.
9. It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
10. All incubation steps must be accurately timed.
11. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
12. Do not re-use microplate wells.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

PREPARATION OF REAGENTS

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionised water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2-8 ℃ for at least 30 days after preparation.

Preparation of wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionised water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2-8 ℃ for at least 30 days after preparation.

Sample preparation

Dilute all samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well. Controls are ready to use and need not be diluted.

TEST PROCEDURE

1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
2. Pipette **100 µl** of calibrators, controls and prediluted patient samples into the wells.
   We recommend duplicate values for better accuracy.

	1	*2*	*3*	*4*	*5*	*6*
A	**SA**	**SE**	**P2**	**P6**		
B	**SA**	**SE**	**P2**	**P6**		
C	**SB**	**C1**	**P3**	**P..**		
D	**SB**	**C1**	**P3**	**P..**		
E	**SC**	**C2**	**P4**			
F	**SC**	**C2**	**P4**			
G	**SD**	**P1**	**P5**			
H	**SD**	**P1**	**P5**			

SA-SE: standards A to E
P1, P2... patient sample 1, 2
C1: positive control
C2: negative control

3. Incubate for 30 minutes at room temperature (20-28 ℃)
4. Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
5. Dispense **100 µl** of enzyme conjugate into each well.
6. Incubate for 15 minutes at room temperature.
7. Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
8. Dispense **100 µl** of TMB substrate solution into each well.
9. Incubate for 15 minutes at room temperature.
10. Add **100 µl** of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
11. Read the optical density at 450 nm and calculate the results.
    Single or dual wave length microplate reader with 450 nm filter. If dual wave length is used, set the reference filter to 620 nm.

Quality Control

The test results are only valid if the test has been performed following the instructions. The user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable local, state, and federal regulations. Positive and Negative Control sera are supplied with the kit. The Negative and Positive controls validate the assay at the critical absorbance levels to ensure test performance, test integrity and operator reliability. Good laboratory practice dictates running the positive and negative controls each time the kit is used. This test is only

valid if the optical density at 450 ± 10 nm for Positive Control (1) and Negative Control (2) as well as for the Calibrator A and E complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit! If any of these criteria is not fulfilled, the results are invalid and the test should be repeated. If the results of the positive and/or negative control are not within the range, the test results are invalid and the assay should be rerun. Warning: If QC results are “out of range” or invalid, the results must not be reported. Each laboratory should use known samples as further controls. Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations. It is recommended to participate in appropriate quality assessment programs.

INTERPRETATION OF RESULTS

Calculation of results

For Rheumatoid Factor Total a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice.

Recommended Lin-Log Plot

Use lin-log graph paper and plot the optical density (averaged optical density, if duplicate values were tested) of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Calculation example

The figures below show typical results for Rheumatoid Factor Total ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

Calibrators

No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl. Conc.	CV %
STA	A 1/B1	0.029	0.031	0.030	0	0	0	0	1
STB	C 1/D 1	0.232	0.214	0.223	16.4	14.9	15.6	15	4
STC	E 1/F 1	0.563	0.536	0.550	50.2	47.2	48.7	50	3
STD	G 1/H 1	1.263	1.181	1.222	160.6	143.8	152.2	150	5

Interpretation of results

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Rheumatoid Factor Total test:

	Rheumatoid Factor Total [U/ml]
Normal:	< 25
Elevated:	≥ 25

Samples falling within +/-10% of the cut-off (22 - 27 U/mL) should be retested and/or another sample drawn within 7 - 14 days and tested.
Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.
It is recommended that each laboratory establishes its own normal and pathological ranges of serum Rheumatoid Factors.

LIMITATIONS OF PROCEDURE

The absence of Rheumatoid Factor does not rule out rheumatoid arthritis. Rheumatoid Factor may appear transiently during various infections. The Rheumatoid Factor Total ELISA

is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated.

EXPECTED VALUES

A series of 200 assumed normal blood donor samples ages 18 to 67 years equally distributed by sex and age were collected from various geographically diverse blood banks. These 200 samples were tested in the Orgentec Rheumatoid Factor Total assay to determine a normal range and cut-off for the assay.

The mean concentration of RF Screen antibodies was 11.3 U/ml, and mean plus 3 S.D. and 98 percentile values resulted in 23.0 and 21.0 U/mL respectively. Two patients were positive yielding a 1 % positive rate.

Based on these results the cut-off was determined to be $\geq$ 25 U/ml.

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity (Limit of Blank - LOB)

Sample Buffer was diluted according to instructions for use and measured 20 times on one plate. Calibrators and Controls were analyzed in duplicate. The detection limit was calculated as the mean + 3 SD was 0.024 OD for the Sample Buffer, which corresponded to an analytical sensitivity of 0.4 U/mL.

Functional Sensitivity (Limit of Quantitation LOQ)

Functional sensitivity was determined from the coefficient of variation of four very low serum samples run in replicates of 16 in three assay runs. The lowest concentration which could be measured with a coefficient of variation below 20% is 8.4 U/mL

Interferences

Interference due to bilirubin, hemolysis and lipemia was evaluated using a negative serum, a low positive serum and a high positive serum spiked with the respective interfering substance in increasing concentrations. Hemolysis up to 1000 mg/dL, bilirubin up to 40 mg/dL, and lipemia (i.e. triglyceride concentration) up to 3000 mg/dL in human serum do not interfere with RF IgG ELISA results.

Cross-Reacting Conditions (different arthritic and autoimmune diseases) -

A series of 68 samples obtained from adult and juvenile patients diagnosed with various other arthritic and autoimmune disease conditions were collected from hospitals sent for routine testing. These 68 samples were tested in the Orgentec Rheumatoid Factor Total assay to identify RF positivity in these populations. The results of this testing are provided in the following Table.

Patient Group	No. of Samples	No. of RF Positive	% Positive
Juvenile Arthritis	14	0	0%
Other autoimmune	10	0	0%
Borreliose	2	0	0%
Rheumatological samples	11	3	27%
Psoriasis Arthritis	10	1	10%
Sjögren Syndrome	4	0	0%
SLE	17	8	47%

Precision (Reproducibility)

Statistics for Coefficients of variation (CV) were calculated for each of eight samples from the results of 20 determinations in a single run for Intra-Assay precision. Run-to-run precision

was calculated from the results of 20 different runs with double determinations of each sample. Inter-Lot precision was calculated from the results of three different kit lots with double determinations using seven samples. Results are shown in the below tables.

Intra-Assay		
Sample No.	Mean [U/ml]	CV [%]
1	5.2	6.7
2	10.5	7.6
3	23.8	3.4
4	29.0	5.5
5	32.0	6.4
6	34.0	4.8
7	89.5	5.5
8	317.0	7.2

Inter-Lot		
Sample No.	Mean [U/ml]	CV [%]
1	8.2	8.9
2	21.1	4.0
3	38.5	12.0
4	95.7	12.4
5	143.4	11.3
6	312.9	8.5
WHO	311.6	10.9

Inter-Assay		
Sample No.	Mean [U/ml]	CV [%]
1	26.1	5.9
2	92.2	5.5
3	320.5	8.0

LINEARITY

Three patient samples containing high levels of antibody were serially diluted in buffer to demonstrate the upper end of linearity and throughout the dynamic range of the assay. The calculated values together with the recovery and the linear regression coefficient (R^2) are shown in the table below.

	Sample 1	Sample 2	Sample 3
Concentration U/mL	629.7	691.2	497.6
Regression R2	0.9972	0.9981	0.9951
% Recovery	91-116	84-105	88-113

COMPARISON STUDIES

Studies were performed to evaluate the sensitivity and specificity of the RF Screen (ELISA) test when compared to the predicate assay using a mix of clinically diagnosed Rheumatoid Arthritis disease state samples and a presumed normal asymptomatic blood bank population and other arthritic and autoimmune patient samples obtained from a medical practice specializing in rheumatology and a rheumatology department of a hospital.

Specificity can be defined as the ability of a test to give a negative result for “normal” and control disease sera. The specificity performance of the ORGENTEC RF Screen assay was established using 181 “presumed normal” sera obtained from blood donor centers age 18 to 69 years.

Sensitivity can be defined as the ability of a test to give a positive result for sera from patients clinically diagnosed with an autoimmune disorder. The sensitivity performance of the ORGENTEC RF Screen assay was established using one hundred and sixty samples from clinically diagnosed patients as having Rheumatoid Arthritis disease that were obtained from a variety of clinical sources (hospitals and autoimmune clinics). The patients collectively included males and females. The age ranged from 40 to 87 years. An additional sixty-seven patients clinically diagnosed with other autoimmune and arthritic disorders were tested.

Four hundred sixty-nine (469) sera were tested by the ORGENTEC RF Screen ELISA to determine Clinical diagnostic agreement. Based on clinical diagnosis, three hundred seven of the 469 clinically diagnosed sera were positive in the ORGENTEC RF Screen assay and one hundred sixty-two tested as negative, thus yielding a clinical diagnostic sensitivity of 96.0% and a clinical specificity of 88.8%.

233 patient samples were compared to commercially available RF IgG, IgA, and IgM ELISA tests. The quantitative results were calculated from the calibration curves on the basis of tested calibrators. Based on comparison to the three predicate tests, the ORGENTEC RF

Screen found 170 of 173 samples positive yielding a positive percent agreement of 98.3%, and 59 of 60 negative yielding a negative percent agreement of 98.3%.

A summary analysis of the results are shown in the following Tables:

		Clinical Diagnosis Pos	Clinical Diagnosis Neg	
ORGENTEC Assay	Pos	288	19	307
	Neg	12	150	162
		300	169	469

Clinical Sensitivity:	**96.0%**	(95% C.I. = 91.2 - 98.2%)
Clinical Specificity:	**88.8%**	(95% C.I. = 84.0 - 93.5%)
Clinical Agreement:	**93.4%**	(95% C.I. = 88.6 - 94.8%)

		Comparative methods Pos	Comparative methods Neg	
ORGENTEC Screen	Pos	170	1	171
	Neg	3	59	62
		173	60	233

Positive Percent Agree	**98.3%**	(95% C.I. = 93.1 - 97.9%)
Negative Percent Agree	**98.3%**	(95% C.I. = 84.0 - 93.5%)
Overall Agreement	**98.3%**	(95% C.I. = 90.8 - 95.5%)

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INCUBATION SCHEME

1. Pipet **100 µl** calibrator, control or patient sample
   → Incubate for **30 minutes** at room temperature
   → Discard the contents of the wells and wash 3 times with **300 µl** wash solution
2. Pipet **100 µl** enzyme conjugate
   → Incubate for **15 minutes** at room temperature
   → Discard the contents of the wells and wash 3 times with **300 µl** wash solution
3. Pipet **100 µl** substrate solution
   → Incubate for **15 minutes** at room temperature
4. Add **100 µl** stop solution
   → Leave untouched for **5 minutes**
   → Read at **450 nm**

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