



ORGENTEC Diagnostika GmbH
Carl-Zeiss-Straße 49
55129 Mainz
Tel.: 06131-9258-0
Fax: 06131-9258-58

ANAcombi

ORG 539

12 x 8 Tests

**Immunometric Enzyme Immunoassay
for the qualitative determination of
ANA-Autoantibodies**

Instruction for use

CE

August 2005

CONTENTS

<i>CONTENTS</i>	2
<i>NAME AND INTENDED USE</i>	3
<i>SUMMARY AND EXPLANATION OF THE TEST</i>	3
<i>PRINCIPLE OF THE TEST</i>	3
<i>WARNINGS AND PRECAUTIONS</i>	4
<i>CONTENTS OF THE KIT</i>	4
<i>STORAGE AND STABILITY</i>	5
<i>MATERIALS REQUIRED</i>	5
<i>SPECIMEN COLLECTION, STORAGE AND HANDLING</i>	5
<i>PROCEDURAL NOTES</i>	6
<i>PREPARATION OF REAGENTS</i>	6
<i>TEST PROCEDURE</i>	6
<i>INTERPRETATION OF RESULTS</i>	7
<i>PERFORMANCE CHARACTERISTICS</i>	9
<i>LIMITATIONS OF PROCEDURE</i>	9
<i>INTERFERING SUBSTANCES</i>	9
<i>REFERENCES</i>	9
<i>INCUBATION SCHEME</i>	10

NAME AND INTENDED USE

The ORGENTEC ANAcombi assay is an indirect solid phase enzyme immunometric assay (ELISA) for the qualitative determination ANA autoantibodies directed against RNP-70, RNP/Sm, Sm, SS-A, SS-B, Scl-70, Centromer B and Jo-1 in human serum or plasma. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of certain systemic rheumatic diseases.

SUMMARY AND EXPLANATION OF THE TEST

Inflammatory connective tissue diseases are characterized by idiopathic genesis along with disturbances in terms of cellular and humoral immunity, systemic organ failure and a chronic course of disease. Additionally, connective tissue diseases exhibit overlapping symptomatic features that render an accurate diagnosis difficult [1].

Considering the diversity of mixed connective tissue diseases, such disorders exhibit a common serological characteristic; the presence of anti-nuclear antibodies [2]. These antibodies are directed against parts of the cell nucleus and the cytoplasm, and many rheumatic diseases are characterized by the presence of one or more of these ANAs [3].

Antibodies to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), histone, nuclear ribonucleoprotein (RNP) and Smith antigen (Sm) are associated with SLE [4], while antibodies to Sjogren's Syndrome A (SSA/Ro) and Sjogren's Syndrome B (SSB/La) can occur in both SLE and Sjogren's Syndrome (SS) [5, 6]. Antibodies to Jo-1 may be observed in polymyositis and dermatomyositis [6], while antibodies to scleroderma-associated antigen (Scl-70) and centromere can occur in patients with progressive systemic sclerosis (PSS). Anti-histone antibodies are associated with SLE and drug-induced lupus [7], while anti-RNP antibodies are linked with mixed connective tissue disease (MCTD) and with SLE [2]. Antibodies directed against centromere are associated with CREST syndrome [3].

PRINCIPLE OF THE TEST

Purified antigens SS-A/Ro, SS-B/La, RNP 70, Sm, RNP/Sm, centromere B and Jo-1 are bound to microwells in row A to H separately. Antibodies against these antigens, if present in diluted serum or plasma, bind to the respective antigens. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically detects 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 color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450nm.

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 hydrochloric acid (1 M). 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	12 x 8 determ.
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified antigens RNP-70 (row A), RNP/Sm (row B), Sm (row C), SS-A (row D), SS-B (row E), Scl-70 (row F), Centromer B (row G), Jo-1 (row H). Ready to use.
2 vials, 2,5 ml each	Anti-ANA controls in a serum/buffer matrix (PBS, NaN_3 <0,1% (w/w)). Negative Control (NC), Calibrator control (CC). 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; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 ml	TMB substrate solution. Ready to use.
1 vial, 15 ml	Stop solution (1 M 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 °C.
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 °C.

MATERIALS REQUIRED

Equipment

- Microplate reader capable of endpoint measurements at 450 nm
- Multi-Channel Dispenser or repeatable pipet for 100 µl
- Vortex mixer
- Pipets for 10 µl, 100 µl and 1000 µl
- Laboratory timing device
- Data reduction software

Preparation of reagents

- Distilled or deionized 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-hemolyzed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
4. Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months.
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 °C).
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 deionized water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionized water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Sample preparation

Dilute all patient 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. Pipet 100 µl of controls and prediluted patient samples in duplicate into the wells.

	1	2	3	4	5	6	
RNP-70	A	CC	NC	P1	P2	P..	
RNP/Sm	B	CC	NC	P1	P2	P..	
Sm	C	CC	NC	P1	P2	P..	
SS-A	D	CC	NC	P1	P2	P..	
SS-B	E	CC	NC	P1	P2	P..	
Scl-70	F	CC	NC	P1	P2	P..	
Centromer B	G	CC	NC	P1	P2	P..	
Jo-1	H	CC	NC	P1	P2	P..	

CC Calibrator control
NC negative control
P1, P2 patient samples

3. Incubate for 30 minutes at room temperature (20-28 °C).
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. Bi-chromatic measurement with a reference at 600-690 nm is recommended.

The developed colour is stable for at least 30 minutes. Read optical densities during this time.

INTERPRETATION OF RESULTS

Quality Control

This test is only valid if the optical density at 450 nm for Negative Control (NC) and Calibrator Control (CC) 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.

Calculation of results

Evaluation of the ANAcombi test is carried out by comparison of the optical densities of the patient sample with the optical density of the Calibrator Control.

For each antigen the OD-Cut-off value should be calculated according to the following equation:

$$OD_{\text{Cut-Off}} = OD_{\text{CalibratorControl}} \times \text{SpezificFactor}$$

For each patient sample 8 Index-values should be calculated according to the following equation:

$$\text{Index-Value} = \frac{OD_{\text{Sample}}}{OD_{\text{Cut-Off}}}$$

Calculation Example

The chart below shows typical results for ANAcombi. These data are intended for illustration only and should not be used to calculate results from another run.

Sample No.	Anti-	Row	ODCalibrator	Factor	ODCut-off	ODSample	Result
1	RNP-70	A	0,48	1,10	0,53	0,083	negative
1	RNP/Sm	B	0,50	1,00	0,50	0,420	negative
1	Sm	C	0,40	1,25	0,50	0,138	negative
1	SS-A	D	0,42	0,85	0,36	0,133	negative
1	SS-B	E	0,53	0,75	0,39	0,505	positive
1	Scl-70	F	0,57	1,00	0,57	0,820	positive
1	Centromer B	G	0,49	0,80	0,39	0,212	negative
1	Jo-1	H	0,50	0,80	0,40	0,189	negative

Interpretation of results

Qualitative Interpretation

A sample is positive for one ANA parameter if OD-Sample is $>$ OD Cut-off:

	ANACombi (OD-Sample)
Negative:	$<$ OD Cut-off
Positive:	\geq OD Cut-off

For detailed quantitative analyses we recommend to use the individual ANA-ELISA test systems.

Semi-quantitative interpretation

A sample is positive for one ANA parameter if its Index-value is ≥ 1.2 :

	ANACombi (Index-value)
Negative:	< 1.0
Borderline:	$1.0 - 1.2$
Positive:	≥ 1.2

Expected Values

The approximate incidence of positive ANA is 5% in the general normal population, 40% in normal old age and 25% in healthy relatives of SLE patients. ANA positivity has been reported in:

SLE (systemic Lupus erythematosus)	$>95\%$
SS (Sjogren's syndrome)	50-65%
PSS (progressive systemic sclerosis)	40-60%
RA (rheumatoid arthritis)	12-24%
juvenile RA (juvenile rheumatoid arthritis)	20%

PERFORMANCE CHARACTERISTICS

Specificity

The microplate is coated with the antigens RNP-70, RNP/Sm, Sm, SS-A, SS-B, Scl-70, Centromer B and Jo-1, respectively. All antigen preparations, native and recombinant, are highly purified by affinity chromatography. The ANAcombi test is specific only for autoanti-bodies directed to these antigens. No crossreactivities have been observed.

Calibration

The assay system is calibrated against the internationally recognised reference sera from CDC, Atlanta USA, since no other international standards are available.

LIMITATIONS OF PROCEDURE

1. The ANAcombi ELISA is a diagnostic aid and by itself is not diagnostic. 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.
3. Positive ANA may be found in apparently healthy people.
4. SLE patients undergoing steroid therapy may have negative test results.
5. Commonly prescribed drugs may induce ANA.

INTERFERING SUBSTANCES

No interference has been observed with haemolytic (up to 1000 mg/dL), lipemic (up to 3 g/dL triglycerides) or bilirubin (up to 40 mg/dL) containing sera. Nor have any interfering effects been observed with the use of anticoagulants. However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

REFERENCES

1. Hiepe F., Burmester G.R.. Klinik und Diagnostik des systematischen Lupus erythemadodes. Dtsch.med.Wschr.; Vol.121; 1129-1133; 1996.
2. Nakamura R.M., Tan E.M.. Update on autoantibodies to intracellular antigens in systemic rheumatic diseases. Clin.Lab.Med.; Vol.12; 1-23; 1992.
3. Barland P., Lipstein E.. Selection and use of laboratory test in the rheumatic diseases. Am.J.Med.; Vol.100 (suppl 2A); 16s-23s.
4. Isenberg D.A., Ravirajan C.T., Rahman A., Kalsi J.. The role of antibodies to DNA in systemic lupus erythematosus - A review and introduction to an international workshop on DNA antibodies held in London, May 1996. Lupus; 6; 290-304; 1997.
5. Alexander E, Buyon J.P., Provost T.T., Guarnieri T. Anti-Ro/SS-A antibodies in the pathophysiology of congenital heart block in neonatal lupus syndrome, an experimental model. Arthritis and Rheumatism; Vol. 35 No.2; 176-189; 1992.
6. Hietarinta M., Lassila O.. Clinical significance of antinuclear antibodies in systemic rheumatic disease. Ann.Med.; Vol.28; 283-291; 1996.

7. Fritzler M.J.. Clinical relevance of autoantibodies in systemic rheumatic diseases. Mol.Biol.Rep.; Vol.23; 133-145; 1996.
8. Feltkamp T.E.W.. Antinuclear antibody determination in a routine laboratory. Ann.Rheum.Dis.; Vol.55; 723-727; 1996.
9. Amoura Z., Koutouzov S., Chabre H., Cacoub P., Amoura I., Musset L., Bach J.-F., Piette J.-C.. Presence of antinucleosome autoantibodies in a restricted set of connective tissue diseases. Arthritis and Rheumatism; Vol. 43 (1); 76-84; 2000.
10. Lundberg U.N., Hedfors E., Pettersson I.. Recombinant 70-kD protein for determination of autoantigenic epitopes by anti-RNP sera. Clin.Exp.Immunol.; 81; 52-58; 1990.
11. Sanchez-Guerrero J., Lew R.A., Fossel A.H., Schur P.H.. Utility of anti-Sm, anti-RNP, anti-Ro/SS-A, and anti-La/SS-B (extractable nuclear antigens) detected by enzyme linked immunosorbent assay for the diagnosis of systemic lupus erythematoses. Arth.Rheum.; Vol.39; 1055-1061; 1996.
12. Shen G.Q., Shoenfeld Y., Peter J.B.. Anti-DNA, anti-histone and anti-nucleosome antibodies in systemic Lupus erythematosus and drug-induced Lupus. Clin. Reviews in Allergy and Immunology; Vol.16; 321-334; 1998.

INCUBATION SCHEME

