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Instruction For Use
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ORG 539 ANAcombi

NAME AND INTENDED USE

ANAcombi assay is an ELISA test system for the qualitative measurement of ANA autoantibodies directed against RNP-70, RNP/Sm, Sm, SS-A (52 and 60 kDa), SS-B, Scl-70, Centromere B and Jo-1 in human serum or plasma. This product is intended for professional in vitro diagnostic use only.

SYMBOLS USED ON LABELS

IVD	In vitro diagnostic medical device	MICROPLATE	Microplate
Manufacturer		CALIBRATOR	Calibrator
REF	Catalogue number	CONTROL	Control negative
96	Sufficient for 96 determinations		
LOT	Batch code		
Use by			
Temperature limitation		DILUENT	Sample Buffer P
Consult instructions for use		CONJUGATE	Enzyme Conjugate
Keep away from sunlight		TMB	TMB Substrate
Do not reuse		STOP	Stop solution
Date of manufacture		WASH	Wash Buffer
		RTU	Ready to use

PRINCIPLE OF THE TEST

Purified antigens SS-A (52 and 60 kDa), SS-B, RNP-70, Sm, RNP/Sm, Scl-70, Centromere B and Jo-1 are bound to individual rows A to H of the microwell plate.

Antibodies against the coated antigen, if present in diluted patient sample, bind to the respective antigen. Washing of the microwells removes unbound unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human antibodies 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 antibodies present in the original sample.

SUMMARY AND EXPLANATION OF THE TEST

Connective tissue diseases (CTD) are a group of autoimmune disorders which are characterized by presence of antinuclear antibodies (ANA) in the blood of patients. ANA are a specific class of autoantibodies that have the capability of binding and destroying certain structures within the nucleus of the cells. These antibodies are involved in the disease pathogenesis, and they also constitute the basis for diagnosis and treatment of CTD.

ANA have been categorized into two main groups:

1. Autoantibodies to DNA and histones
2. Autoantibodies to extractable nuclear antigens (ENA): Sm, ribonucleoproteins (RNP), SSA/Ro, SSB/La, Scl-70, Jo-1 and PM1

Autoantibodies to DNA and histones include antibodies against single and double stranded DNA (ssDNA and dsDNA). Significant levels of anti-dsDNA antibodies are considered to be confirmatory in the diagnosis of systemic lupus erythematosus (SLE). Anti-histone antibodies are indicative of drug-induced lupus.

Besides DNA and histones, autoantibodies may also target other nuclear antigens. These nuclear antigens were named extractable nuclear antigens (ENA), as originally they were extracted from the nuclei with saline solution. Autoantibodies to Smith antigen (Sm) which is also considered to be highly specific for SLE were the first anti-ENA detected. Thereafter, further subtypes of ENA i.e. ribonucleoproteins (RNP), Sjögren antigen A or B (SSA/Ro or SSB/La), Scl-70, Jo-1 and PM1 were identified.

Although most of these ENA are disease specific, a significant overlap exists. Sensitivity and specificity may also vary depending upon the type of underlying CTD.

Presence of autoantibodies in the sera of patients constitutes one of the criteria used for diagnosis of CTD. Together with the clinical diagnosis ANA subtyping helps in identifying a specific CTD.

Indirect immunofluorescence tests (IF) and enzyme immunoassays (ELISA) are commonly used for ANA detection in day to day practice. Initially, screening is carried out by IF-ANA or a generic ELISA which detects ANA of a broad specificity similar to IF-ANA. If positive, more specific tests are performed based on clinical findings and the IF-ANA staining pattern.

These antigen specific ELISA assays react with single autoantigens e.g. dsDNA, SS-A/Ro, SS-B/La, Scl-70, Sm, Sm/RNP etc.

Autoantibodies to dsDNA are specific and diagnostic for SLE and levels are elevated during active disease. Recently published ACR Guidelines for Screening, Treatment, and Management of Lupus Nephritis recommend the testing of antibodies to dsDNA for monitoring of lupus nephritis, ranging from monthly intervals in pregnant patients with active glomerulonephritis at onset of treatment to every three months in patients with active nephritis at onset of treatment or pregnant patients with previous but not current nephritis, up to six-monthly testing in patients with previous active nephritis or no prior or current nephritis.

SLE-Patients without antibodies against dsDNA often produce antibodies against ssDNA. Similarly anti-Sm is highly specific for SLE but is present in only 10 % to 30 % of SLE cases.

Antibodies against dsDNA, histones, the 70 kD protein of the U1-snRNP complex (RNP70) and anti Sm are closely associated with SLE. Anti-SSA/Ro and anti-SSB/La antibodies are indicative for Sjögren's syndrome, but can also be found in up to 30 % cases of SLE with cutaneous involvement.

Anti-SS-A/Ro antibodies pass the placenta and may cause the development of SLE in neonates. Anti-SSA/Ro antibodies are almost always present in sera of mothers with babies with neonatal lupus syndrome and with complete congenital heart block.

Antinuclear antibodies are a group of autoantibodies which give a nucleolar IF-staining pattern. Most common of these are anti-PM-Scl, anti-RNA polymerase I-III and anti-U3-RNP. They are found in scleroderma and polymyositis (PM). Antibodies against RNP and the complex RNP/Sm are linked to mixed connective tissue disease (MCTD, Sharp syndrome) and to SLE. Serologically MCTD is characterized by the presence of autoantibodies directed against the 70 kD protein of the U1-snRNP-complex. Up to 100% of MCTD patients manifest high titers of Anti-RNP-70 antibodies.

Autoantibody prevalence to (values in %)

Diseases	dsDNA	ssDNA	Histone	SS-A	SS-B	Sm	RNP/ Sm	Scl-70	Jo-1
Systemic lupus erythematosus (SLE)	> 90	> 90	30-50	10-30	30-50	10-30	10-30		
Drug-induced lupus (DIL)		30-50	50-90						
Sharp-syndrome / mixed connective tissue disease	10-30	10-30					> 90		
Rheumatoid arthritis	10-30	30-50	30-50	10-30					
Sjögren's syndrome	10-30	10-30		> 90	> 90				
Scleroderma	10-30	10-30		10-30				> 90	
Photosensitive dermatitis, dermatomyositis	10-30	10-30							50-90

CONTENTS OF THE KIT

ORG 539	▽ 96	Sufficient for 96 determinations
MICROPLATE	1	One divisible microplate consisting of 12 modules of 8 wells each. Ready to use. Product code on module: Acb
CALIBRATOR	1x 2.5 ml	Calibrator, containing ANA antibodies in a serum/buffer matrix (PBS, BSA, NaN3 0.09%), yellow. Ready to use.
CONTROL	1x 2.5 ml	Control negative, containing ANA antibodies in a serum/buffer matrix (PBS, BSA, detergent, NaN3 0.09%), yellow. Ready to use.
DILUENT	20 ml	Sample Buffer P, containing PBS, BSA, detergent, preservative sodium azide 0.09%, yellow, concentrate (5 x).
CONJUGATE	15 ml	Enzyme Conjugate containing anti-human IgG antibodies, HRP labelled; PBS, BSA, detergent, preservative PROCLIN 0.05%, light red. Ready to use.
TMB	15 ml	TMB Substrate; containing 3,3', 5,5'- Tetramethylbenzidin, colorless. Ready to use.
STOP	15 ml	Stop solution; contains acid. Ready to use.
WASH	20 ml	Wash Buffer, containing Tris, detergent, preservative sodium azide 0.09%; 50 x conc.
i	1	Instruction for Use: ELISA Mini-CD
i	1	Certificate of Analysis

MATERIALS REQUIRED

- Microplate reader capable of endpoint measurements at 450 nm; optional: reference filter at 620 nm
- Data reduction software
- Multi-channel dispenser or repeatable pipette for 100 µl
- Vortex mixer
- Pipettes for 10 µl, 100 µl and 1000 µl
- Laboratory timing device
- Distilled or deionised water
- Measuring cylinder for 1000 ml and 100 ml
- Plastic container for storage of the wash solution

This ELISA assay is suitable for use on open automated ELISA processors. Each assay has to be validated on the respective automated system. Detailed information is provided upon request.

SPECIMEN COLLECTION, STORAGE AND HANDLING

- Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
- Allow blood to clot and separate the serum or plasma by centrifugation.
- Test serum should be clear and non-hemolyzed. Contamination by hemolysis or lipemia should be avoided, but does not interfere with this assay.
- Specimens may be refrigerated at 2-8°C for up to five days or stored at -20°C up to six months.
- Avoid repetitive freezing and thawing of serum or plasma samples. This may result in variable loss of antibody activity.
- Testing of heat-inactivated sera is not recommended.

STORAGE AND STABILITY

- Store test kit at 2-8°C in the dark.
- Do not expose reagents to heat, sun, or strong light during storage and usage.
- Store microplate sealed and desiccated in the clip bag provided.
- Shelf life of the unopened test kit is 18 months from day of production.
Unopened reagents are stable until expiration of the kit. See labels for individual batch.
- Diluted Wash Buffer and Sample Buffer are stable for at least 30 days when stored at 2-8°C.
We recommend consumption on the same day.

PROCEDURAL NOTES

- Do not use kit components beyond their expiration dates.
- Do not interchange kit components from different lots and products.
- All materials must be at room temperature (20-28°C) prior to use.
- Prepare all reagents and samples. Once started, perform the test without interruption.
- Double determinations may be done. By this means pipetting errors may become obvious.

- Perform the assay steps only in the order indicated.
- Always use fresh sample dilutions.
- Pipette all reagents and samples into the bottom of the wells.
- To avoid carryover or contamination, change the pipette tip between samples and different kit controls.
- Wash microwells thoroughly and remove the last droplets of wash buffer.
- All incubation steps must be accurately timed.
- Do not re-use microplate wells.

WARNINGS AND PRECAUTIONS

- All reagents of this kit are intended for professional in vitro diagnostic use only.
- 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.
- Bovine serum albumin (BSA) used in components has been tested for BSE and found negative.
- Avoid contact with the substrate TMB (3,3',5,5'-Tetramethyl-benzidine).
- Stop solution contains acid, classification is non-hazardous. Avoid contact with skin.
- Control, sample buffer and wash buffer contain sodium azide 0.09% as preservative. This concentration is classified as non-hazardous.
- Enzyme conjugate contains ProClin 300 0.05% as preservative. This concentration is classified as non-hazardous.

During handling of all reagents, controls and serum samples observe the existing regulations for laboratory safety regulations and good laboratory practice:

- First aid measures: In case of skin contact, immediately wash thoroughly with water and soap. Remove contaminated clothing and shoes and wash before reuse. If system fluid comes into contact with skin, wash thoroughly with water. After contact with the eyes carefully rinse the opened eye with running water for at least 10 minutes. Get medical attention if necessary.
 - Personal precautions, protective equipment and emergency procedures:
Observe laboratory safety regulations. Avoid contact with skin and eyes. Do not swallow. Do not pipette by mouth. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled. When spilled, absorb with an inert material and put the spilled material in an appropriate waste disposal.
 - Exposure controls / personal protection: Wear protective gloves of nitril rubber or natural latex.
Wear protective glasses. Used according to intended use no dangerous reactions known.
 - Conditions to avoid: Since substrate solution is light-sensitive. Store in the dark.
 - For disposal of laboratory waste the national or regional legislation has to be observed.
- Observe the guidelines for performing quality control in medical laboratories by assaying control sera.

PREPARATION OF REAGENTS

WASH

Dilute the contents of one vial of the buffered wash solution concentrate (50x) with distilled or deionised water to a final volume of 1000 ml prior to use.

DILUENT

Sample Buffer P: Prior to use dilute the contents (20 ml) of one vial of sample buffer 5x concentrate with distilled or deionised water to a final volume of 100 ml.

Preparation of samples

Dilute patient samples 1:100 before the assay: Put 990 µl of prediluted sample buffer in a polystyrene tube and add 10 µl of sample. Mix well. Note: Calibrators / Controls are ready to use and need not be diluted.

TEST PROCEDURE

Prepare enough microplate modules for all calibrators / controls and patient samples.

- Pipette **100 µl** of calibrators, controls and prediluted patient samples into the wells.
Incubate for **30 minutes** at room temperature (20-28 °C).
Discard the contents of the microwells and **wash 3 times** with **300 µl** of wash solution.
- Dispense **100 µl** of enzyme conjugate into each well.
Incubate for **15 minutes** at room temperature.
Discard the contents of the microwells and **wash 3 times** with **300 µl** of wash solution.
- Dispense **100 µl** of TMB substrate solution into each well.
Incubate for **15 minutes** at room temperature
- Add 100 µl** of stop solution to each well of the modules
Incubate for **5 minutes** at room temperature.
Read the optical density at 450 nm (reference 600-690nm) and calculate the results.
The developed colour is stable for at least 30 minutes. Read during this time.

Example for a pipetting scheme:

	1	2	3	4	5	6	7	8	9	10	11	12	Antigens coated in rows:
A	CAL	C-	P1	P2	P3								RNP-70
B	CAL	C-	P1	P2	P3								RNP/Sm
C	CAL	C-	P1	P2	P3								Sm
D	CAL	C-	P1	P2	P3								SS-A
E	CAL	C-	P1	P2	P3								SS-B
F	CAL	C-	P1	P2	P3								Sci-70
G	CAL	C-	P1	P2	P3								Centromer B
H	CAL	C-	P1	P2	P3								Jo-1

P1, ... patient sample CAL calibrator C- Control negative

VALIDATION

Test results are valid if the optical densities at 450 nm for calibrators / controls and the results for controls comply with the reference ranges indicated on the Certificate of Analysis enclosed in each test kit.

If these quality control criteria are not met the assay run is invalid and should be repeated.

CALCULATION OF RESULTS

First for each antigen-coated row optical density (OD) of cut-off is calculated by multiplying optical density of the calibrator by a lot-specific factor stated in the certificate of analysis:

$$\text{OD cut-off} = \text{OD Calibrator} * \text{lot-specific factor}$$

Then for each antigen-coated row the OD of a sample is compared to the OD of the cut-off:

Negative: OD sample < OD cut-off

Positive: OD sample ≥ OD cut-off

For detailed results: for each antigen-coated row the OD of a sample is expressed as Index value

$$\text{Index} = \text{OD sample} / \text{OD cut-off}$$

PERFORMANCE CHARACTERISTICS

Calibration

The assay system is calibrated against the internationally recognized reference sera from CDC, Atlanta USA.

Measuring range

not applicable

Expected values

In a normal range study with samples from healthy blood donors the following ranges have been established with this ELISA assay: Cut-off Index 1.0

Interpretation of results

Negative:	Index < 1.0
Borderline:	Index 1.0 - 1.2
Positive:	Index > 1.2

Linearity

Patient samples containing high levels of specific antibody were serially diluted in sample buffer. Activity for each dilution step was calculated as Index-Value.

Sample	Dilution	Observed Index	Expected Index	O/E [%]
RNP 70	1:100	3.6	3.6	100
.	1:200	1.8	1.8	102
.	1:400	0.9	0.9	104
Sm	1:100	3.3	3.3	100
.	1:200	1.6	1.6	98
.	1:400	0.8	0.8	98
RNP/Sm	1:100	2.6	2.6	100
.	1:200	1.3	1.3	97
.	1:400	0.6	0.7	85
SS-A	1:100	4.1	4.1	100
.	1:200	2.0	2.0	98
.	1:400	1.0	1.0	95
SS-B	1:100	3.6	3.6	100
.	1:200	1.9	1.8	102
.	1:400	0.9	0.9	102
Sci-70	1:100	4.4	4.4	100
.	1:200	2.4	2.2	106
.	1:400	1.0	1.1	95
Cen B	1:100	3.3	3.3	100
.	1:200	1.7	1.6	103
.	1:400	0.8	0.8	96
Jo-1	1:100	4.2	4.2	100
.	1:200	2.0	2.1	96
.	1:400	1.0	1.1	93

Limit of detection

not applicable

Reproducibility

Intra-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 24 determinations in a single run. Results for precision-within-assay are shown in the table below.

Inter-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 6 determinations in 5 different runs. Results for run-to-run precision are shown in the table below.

Intra-Assay		
Sample	Mean Index	CV %
RNP 70	1.3	5.5
Sm	2.4	3.8
RNP/Sm	1.9	4.5
SS-A	1.4	2.0
SS-B	1.6	2.5
Sci-70	1.1	2.9
Cen B	1.3	1.7
Jo-1	1.5	2.1

Inter-Assay		
Sample	Mean Index	CV %
RNP 70	1.4	6.7
Sm	2.4	4.5
RNP/Sm	2.1	4.8
SS-A	1.5	3.1
SS-B	1.5	2.9
Sci-70	1.2	3.3
Cen B	1.6	1.8
Jo-1	1.5	2.3

Interfering substances

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

Study results

Study population	n	n_Pos*	%
SLE	63	57	90.5
Sjogren's Syndrome	10	10	100.0
MCTD	10	10	100.0
Scleroderma	10	10	100.0
Poly- Dermatomyositis	8	7	87.5
CREST Syndrome	9	9	100.0
Rheumatoid arthritis	60	2	3.3
normal human sera	148	3	2.0

* positive for one or more antigens

		Clinical Diagnosis		
		Pos	Neg	
ORG 539	Pos	105	5	318
	Neg	5	203	
		110	208	

Sensitivity: 95.5 %
Specificity: 97.6 %
Overall agreement: 96.9 %

LIMITATIONS OF THE PROCEDURE

This assay 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 concerning the entire clinical picture of the patient. Also every decision for therapy should be taken individually.

The above pathological and normal reference ranges for antibodies in patient samples should be regarded as recommendations only. Each laboratory should establish its own ranges according to ISO 15189 or other applicable laboratory guidelines.

REFERENCES

- Alba P, Bento L, Cuadrado MJ, Karim Y, Tungekar MF, Abbs I et al. Anti-dsDNA, anti-Sm antibodies, and the lupus anticoagulant: significant factors associated with lupus nephritis. *Ann Rheum Dis* 2003; 62(6):556-560.
- Antico A, Platzgummer S, Bassetti D, Bizzaro N, Tozzoli R, Villalta D. Diagnosing systemic lupus erythematosus: new-generation immunoassays for measurement of anti-dsDNA antibodies are an effective alternative to the Farr technique and the Crithidia luciliae immunofluorescence test. *Lupus* 2010; 19(8):906-912.

- Brouwer R, Hengstman GJ, Vree EW, Ehrfeld H, Bozic B, Ghirardello A et al. Autoantibody profiles in the sera of European patients with myositis. *Ann Rheum Dis* 2001; 60(2):116-123.
- Castro C, Gourley M. Diagnostic testing and interpretation of tests for autoimmunity. *J Allergy Clin Immunol* 2010; 125(2 Suppl 2):S238-S247.
- Defendenti C, Atzeni F, Spina MF, Grosso S, Cereda A, Guercilena G et al. Clinical and laboratory aspects of Ro/SSA-52 autoantibodies. *Autoimmun Rev* 2011; 10(3):150-154.
- Eriksson C, Kokkonen H, Johansson M, Hallmans G, Wadell G, Rantapaa-Dahlqvist S. Autoantibodies predate the onset of Systemic Lupus Erythematosus in northern Sweden. *Arthritis Research & Therapy* 2011; 13(1):R30.
- Haugbro K, Nossent JC, Winkler T, Figenschau Y, Rekvig OP. Anti-dsDNA antibodies and disease classification in antinuclear antibody positive patients: the role of analytical diversity. *Ann Rheum Dis JID* - 0372355 2004; 63(4):386-394.
- Ippolito A, Wallace DJ, Gladman D, Fortin PR, Urowitz M, Werth V et al. Autoantibodies in systemic lupus erythematosus: comparison of historical and current assessment of seropositivity. *Lupus* 2011; 20(3):250-255.
- Isenberg DA, Manson JJ, Ehrenstein MR, Rahman A. Fifty years of anti-ds DNA antibodies: are we approaching journey's end? *Rheumatology (Oxford)* 2007; 46(7):1052-1056.
- Kattah NH, Kattah MG, Utz PJ. The U1-snRNP complex: structural properties relating to autoimmune pathogenesis in rheumatic diseases. *Immunol Rev* 2010; 233(1):126-145.
- Kumar Y, Bhatia A, Minz RW. Antinuclear antibodies and their detection methods in diagnosis of connective tissue diseases: a journey revisited. *Diagn Pathol* 2009; 4:1.
- Meroni PL, Schur PH. ANA screening: an old test with new recommendations. *Ann Rheum Dis* 2010; 69:1420-1422.
- Petri M, Magder L. Classification criteria for systemic lupus erythematosus: a review. *Lupus* 2004; 13(11):829-837.
- Poole BD, Schneider RI, Guthridge JM, Velte CA, Reichlin M, Harley JB et al. Early targets of nuclear RNP humoral autoimmunity in human systemic lupus erythematosus. *Arthritis Rheum* 2009; 60(3):848-859.
- Putova I, Dostal C, Becvar R. Prevalence of antinucleosome antibodies by enzyme-linked immunosorbent assays in patients with systemic lupus erythematosus and other autoimmune systemic diseases. *Ann N Y Acad Sci* 2007; 1109:275-286.
- Reveille JD. Predictive value of autoantibodies for activity of systemic lupus erythematosus. *Lupus JID* - 9204265 2004; 13(5):290-297.
- Simon JA, Cabiedes J, Ortiz E, Alcocer-Varela J, Sanchez-Guerrero J. Anti-nucleosome antibodies in patients with systemic lupus erythematosus of recent onset. Potential utility as a diagnostic tool and disease activity marker. *Rheumatology (Oxford)* 2004; 43(2):220-224.
- Sinclair D, Saas M, Williams D, Hart M, Goswami R. Can an ELISA replace immunofluorescence for the detection of anti-nuclear antibodies?--The routine use of anti-nuclear antibody screening ELISAs. *Clin Lab* 2007; 53(3-4):183-191.
- Tozzoli R, Bizzaro N, Tonutti E, Villalta D, Bassetti D, Manoni F et al. Guidelines for the laboratory use of autoantibody tests in the diagnosis and monitoring of autoimmune rheumatic diseases. *Am J Clin Pathol* 2002; 117(2):316-324.
- Maidhof W., Hilius O. Lupus: an overview of the disease and management options. *P T* 2012; 37(4):240-9.
- Hahn BH, McMahon MA, Wilkinson A, Wallace WD, Daikh DI, Fitzgerald JD et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res (Hoboken)* 2012; 64(6):797-808.

- 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**