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Instruction for use
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ORG 544 ENA-4-Profile

Immunometric Enzyme Immunoassay for the semi-quantitative determination of anti-ENA-Antibodies

CONTENTS

NAME AND INTENDED USE
SUMMARY AND EXPLANATION OF THE TEST
PRINCIPLE OF THE TEST
WARNINGS AND PRECAUTIONS
CONTENTS OF THE KIT
STORAGE AND STABILITY
MATERIALS REQUIRED
SPECIMEN COLLECTION AND HANDLING
PROCEDURAL NOTES
PREPARATION OF REAGENTS
TEST PROCEDURE
INTERPRETATION OF RESULTS
PERFORMANCE CHARACTERISTICS
LIMITATIONS OF PROCEDURE
INTERFERING SUBSTANCES
REFERENCES
INCUBATION SCHEME

NAME AND INTENDED USE

ENA-4-Profile is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for semi-quantitative determination of IgG class autoantibodies directed against the extractable nuclear antigens SS-A (Ro), SS-B (La), Sm and RNP/Sm 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

Rheumatoid autoimmune diseases are often associated with the occurrence of autoantibodies against several nuclear or cytoplasmic antigens. These so-called anti nuclear antibodies (ANA) can be divided into three groups:

1. true anti nuclear antibodies (ANA):
directed against dsDNA, ssDNA, histone, nucleolic RNA and DNP
2. extractable nuclear antibodies:
directed against Sm (Smith), n-RNP, Scl 70, Jo-1 and PM-1
3. cytoplasmatic antibodies:
directed against SS-A (Ro) and SS-B (La)

In patients with Sjögren-Syndrome antibodies against the two cytoplasmatic antigens often occur in combination. Due to their strong association of SS-A and SS-B antibodies to the HLA-DR3 and DR2 phenotypes a genetic predisposition is suspected. The anti SS-A protein passes the placenta and may cause the development of SLE in neonates.

Immunoreactive proteins may occur in various combinations and also bound to 'host proteins' of viral origin. They induce synthesis of polyclonal autoantibodies, of the IgG, IgM and IgA class of immunoglobulins. Especially for mixed connective tissue diseases a relation to viral infections by EBV (Eppstein-Barr-Virus) is indicated.

Each class of immunoglobulins causes specific immune fluorescent pattern. Basically immunofluorescence titres correlate with the quantitative determination of IgG antibodies but the concentrations may considerably vary within each titre. Quantitation of IgG class antibodies extensively correlate with the diseases' activity. This makes it superior to immunofluorescence using HEp 2 cells, which may give variable results depending on their degree of activity. Also IF with Crithidia lucillae sometimes gives discrepant results.

Today the best investigated immunoreactive antigens are double-stranded DNA (dsDNA), single stranded DNA (ssDNA), Sm (Smith), sn-RNP (small nuclear ribonucleoprotein particles), the complex RNP/Sm which is stabilised by ribonucleic acid as well as SS-A (Ro) and SS-B (La). The antigen Scl 70, a 70 kD molecular weight protein is associated with scleroderma.

In rheumatoid autoimmune diseases various profiles of autoantibodies to these antigens can be detected. In a high incidence they are related to active and inactive systemic Lupus erythematodes, mixed connective tissue diseases (Sharp Syndrome), rheumatoid arthritis, Sjögren-Syndrome, Sclerodermia, photosensitive dermatitis and drug-induced lupus.

In Lupus patients typically anti-dsDNA antibodies can be detected. Patients without these antibodies very often show anti-ssDNA antibodies and anti-SS-A and anti-SS-B are present. A strong correlation between antibody concentration and severity of the disease has been observed with higher antibody concentrations in active phases of the disease. Thus quantitation is more informative compared to simple titering by immunofluorescence.

Most of these parameters are not specific for just one disease but they occur in various combinations. The pattern of different antibody combinations and their concentration together with the whole clinical picture of the patient are helpful diagnostic tools in the assessment of rheumatoid autoimmune diseases.

The following graph gives brief information on the complexity of autoimmune diseases, occurring antibodies. It is not designed as a diagnostic schedule or program for ongoing diagnostic profiles:

Diseases	dsDNA	ssDNA	Histone	Anti-		Sm	RNP/Sm	Scl 70	Jo-1
				SS-A (Ro)	SS-B (La)				
Systemic Lupus erythematoses (SLE)	xxx	xx		x	x	xx	x		
Drug induced Lupus (LE)		xx	xxx						
Sharp-Syndrome / Mixed connective tissue diseases	x	x		x	x	xx	xxx		
Rheumatoid Arthritis	x	xx	x						
Sjögren-Syndrome	x	x		xx	xx				
Scleroderma	x		x			x		xx	x
Photosensitive Dermatitis Dermatomyositis	xx			x					xx

PRINCIPLE OF THE TEST

Highly purified antigens SS-A (Ro), SS-B (La), Sm and RNP/Sm are bound to microwells individually. Antibodies against these antigens, if present in diluted serum and plasma, bind to the respective antigen. 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. The amount of colour is directly proportional to the concentration of IgG 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 and 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 (NaN₃) 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	4 x 24 determ.
Qty.1	Divisible microplate consisting of 3 modules of 8 wells each, coated with individual highly purified antigens: SS-A, SS-B, Sm, RNP/Sm. Ready to use. The modules are colour coded: SS-A red SS-B blue Sm yellow RNP/Sm green
	Anti-ENA controls in a serum/buffer matrix (PBS, NaN ₃ <0,1% (w/w)). Ready to use.
1 vial, 2 ml	Positive Control (1) (100 U/ml).
1 vial, 2 ml	Negative Control (2) (0 U/ml).
4 vials, 1 ml each	Cut-off Calibrators (25 U/ml), specific for each individual antigen.
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.

	SS-A	SS-B	Sm	RNP/Sm
	1	2	3	4
A	NC	NC	NC	NC
B	CalC	CalC	CalC	CalC
C	PC	PC	PC	PC
D	P1	P1	P1	P1
E	P2	P2	P2	P2
F	P3	P3	P3	P3
G	P4	P4	P4	P4
H	P5	P5	P5	P5

NC Negative control
 CalC Calibration control
 PC Positive control
 P1, P2 Patient sample

- 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 and incubate for 5 minutes at room temperature.
- 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 Positive Control (1), Negative Control (2) and Cut-Off Calibrator 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 and interpretation of results

Evaluation of the ENA-4-Profile test is carried out by direct comparison of the optical density of each patient sample with the optical density of the cut-off calibrator.

Patient samples exhibiting optical densities higher than the optical density of the cut-off calibrator are considered to be positive.

Negative: OD Patient < OD cut-off Calibrator
 Positive: OD Patient > OD cut-off Calibrator

For detailed semi-quantitative results, each patient-OD value can be expressed as an "Index Value". The Index Value is calculated by dividing the sample-OD by the Cut-off-OD.

$$\text{Index value} = \frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Cut-off}}}$$

The calculation of Index Values is not influenced by variations of the sample-OD and/or Cut-off-OD. Index Values are recommended for long term validations (i.e. internal quality control samples).

Index Values are interpreted as follows:

	ENA-4-Profile ELISA (Index Value)
Negative:	<1.0
Borderline:	1.0-1.2
Positive:	>1.2

PERFORMANCE CHARACTERISTICS

Specificity

The microplate modules are coated with the individual antigens SS-A (Ro), SS-B (La), Sm and RNP/Sm. All antigen preparations are highly purified by affinity chromatography. The ENA-4-Profile test is specific only for autoantibodies directed to these antigens. No cross-reactivities 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

The ENA-4-Profile 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.

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.

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INCUBATION SCHEME

