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ANA-9-Line

ORG 710-08 8 strips

ORG 710-16 16 strips

Membrane based immunoblot for the semiquantitative determination of antinuclear autoantibodies

Instruction for use

February 2004

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NAME AND INTENDED USE

ANA-9-Line is membrane based enzyme immunoassay for the semi-quantitative determination of IgG class autoantibodies to extractable nuclear antigens (ENAs) SS-A 52, SS-A 60, SS-B, RNP, Sm, Centromere B, Jo-1, Scl-70 and ribosomal P proteins. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of rheumatological autoimmune diseases.

SUMMARY AND EXPLANATION OF THE TEST

Systemic autoimmune diseases are multifactorial in their clinical presentation. They also express considerable overlap. For diagnosis of rheumtological autoimmune diseases to be meaningful it should be restricted to patients with evidence of autoimmunity. The presence of autoantibodies against normally inaccessible antigens (cytoplasm, nucleoplasm, nuclear matrix and nucleolus) is in fact one of the hallmarks for the systemic autoimmune diseases (Tan, 1988; Sturgess, 1992).

It has become increasingly clear that systemic diseases can be distinguished on the basis of their antinuclear antibody (ANA) profiles. There is a close correlation between ANA and a specific disease making them ideal diagnostic markers (Mongey and Hess,1991; Von Muhlen, 1995).

In addition, to the detection of ANA using indirect immunofluorescence assay (IFA) on HEp-2 cells, further immunological differentiation is recommended or required (Pollock, 1999; Tan et al., 1997) due to:

- multiple specificity of 50% IFA positive sera;
- 'healthy' individuals being IFA positive but negative for the diagnostically significant autoantibodies
- negative IFA not excluding the presence of some extractable nuclear antigen (ENA) specificities.
- the interpretation variability of IFA between laboratories ranging from 36-51% coefficient of variance (Tan et al., 1997).

The superior sensitivity and specificity of the immunoblot sytem is achieved by using purified native or recombinant antigens and makes it an important diagnostic tool in the clinical laboratory for ANA detection (Carey, 1997).

Disease association of the most common Anti-Nuclear Antibodies (ANAs):

Antibody Specificity	Disease Association
SS-A	SS, SLE (20-30%), NL
SS-B	SLE, SS
U1-RNP	SLE
Ribosomal P protein	SLE (sometimes associated with neuropsychiatric diseases)
Centromere B	CREST
Jo-1	Polymyositis/dermatomyositis
Scl-70	Scleroderma

SLE - systemic lupus erythematosus; MCTD- mixed connective tissue disease; RA- rheumatoid arthritis; SS- Sjögren's syndrome; NL- neonatal lupus; CREST - calcinosis, Raynaud's phenomenon, oesophagyl dysfunction, sclerodactyly and telangiectasiae.

PRINCIPLE OF THE TEST

Highly purified extractable nuclear antigens are bound to nitrocellulose membrane strips. Antibodies to these antigens, if present in diluted serum, bind on the membrane. Washing of the membrane strips removes unbound serum antibodies. Alkaline phosphatase conjugated anti-human IgG immunologically bind to the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the membrane strips removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydolyzes to form an insoluble blue-violet product. Washing of the membrane strips removes unhydrolyzed substrate. The amount of color 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 and HIV by FDA approved methods. No test can guarantee the absence of HBsAg or HIV, and so all human serumbased reagents in this kit must be handled as though capable of transmitting infection.
4. Some kit components (i.e. Dilution 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, though not hazardous. Despite the classification as non-hazardous, we strongly recommend using prudent laboratory practices (see 6., 7., 8.)
5. 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.
6. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
7. Do not pipette by mouth.
8. Do not Eat, Drink, Smoke or Apply Makeup in areas where specimens or kit reagents are handled.

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	8 or 16 determ.
Qty. 8 or 16	nitrocellulose strips, loaded with highly purified native or recombinant nuclear antigens. Ready to use.
1 vial, 20 ml	Sample buffer. Ready to use
1 vial, 20 ml	Wash buffer, concentrate (50x)
1 vial, 20 ml	Enzyme conjugate solution (PBS, NaN3 <0.1 % (w/w)), (pink) containing polyclonal rabbit anti-human-IgG; labelled with alkaline phosphatase. Ready to use
1 or 2 vials, 5 ml	Substrate solution. Ready to use
Qty. 1 or 2	Pre-developed nitrocellulose calibration strip (labbeled CAL) for semiquantitative evaluation. Ready to use
Qty. 1 or 2	incubation tray
Qty. 1 or 2	Documentation sheet. Ready to use

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

- Pipets for 10 µl, 500 µl and 1000 µl
- Laboratory timing device
- rocking platform
- tweezers

Preparation of reagents

- distilled or deionized water
- graduated cylinder for 1000 ml

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. To avoid carryover contamination change the tip between samples and different kit controls
8. Nitrocellulose strips must be handled with gloves or tweezers
9. All incubation steps must be accurately timed
10. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.

PREPARATION OF REAGENTS

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.

TEST PROCEDURE

1. Insert an ANA-9-Line strip using tweezers then add 1.0 ml sample buffer to each chamber of the incubation tray. Allow to equilibrate for 5 minutes with gentle rocking.
2. Add 10 µl of patient serum directly to the chamber (effective dilution 1:101).
3. Incubate for 60 minutes with gentle rocking at room temperature.
4. Carefully remove the diluted serum, completely, from the strips.
5. Add 1.0 ml wash buffer, incubate for 5 minutes, then remove as in step 4. Repeat this procedure twice.
6. Add 1.0 ml enzyme conjugate to each chamber..
7. Incubate for 30 minutes with gentle rocking at room temperature.
8. Remove the diluted conjugate completely from the strips.
9. Add 1.0 ml wash buffer, incubate for 5 minutes, then remove as in step 4. Repeat this procedure twice.
10. Add 500 µl substrate to each strip.
11. Incubate for 10 minutes with gentle rocking at room temperature.
12. Remove the substrate and wash the strips with distilled water three times 5 minutes each to stop the reaction.
13. Carefully blot the strips dry with a paper towel.
14. Allow strips to air dry before evaluating.

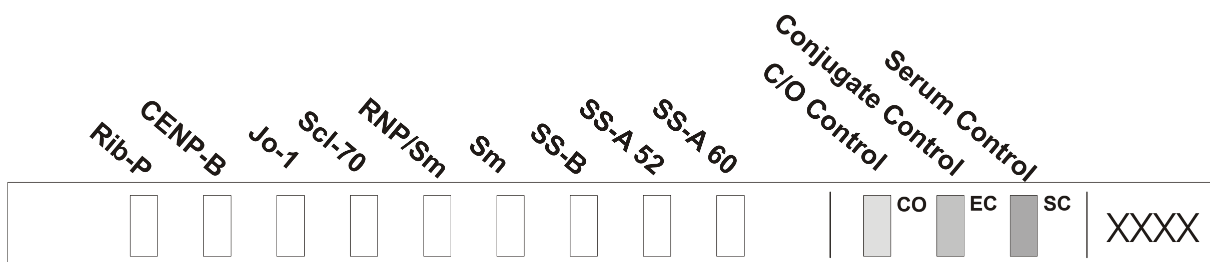
INTERPRETATION OF RESULTS

Quality Control

This test is only valid if the Serum Control (first line), Conjugate Control (second line) and Cut-Off Control (Third line) show a turn-over of substrate in terms of developed bands! If this criteria is not met, the result is invalid and the test should be repeated.

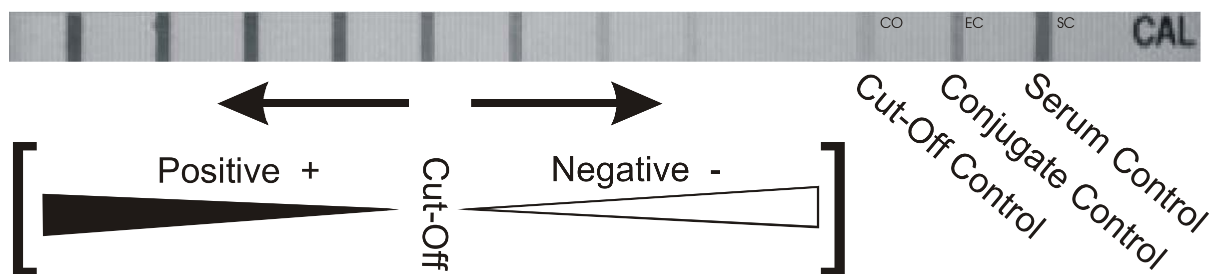
Interpretation of results

The antigens are coated onto the membrane in the order illustrated in the figure below.



The developed lines are compared with the calibration strip as follows:

1. Compare the Cut-Off control line of the patient strip to the calibration lines on the calibration strip to adjust.
2. Compare the patient lines to the calibration lines adjusted to the Cut-Off control for semiquantitative determination



Notes to interpretation of patient results:

1. This is a semiquantitative assay for the determination of the specificity of autoantibodies in patient serum, allowing a discrimination between negative, borderline, weak positive, positive, and strong positive. Borderline samples should be repeated or tested using an alternative procedure.
2. Patient sera in many rheumatological autoimmune diseases often contain multiple autoantibody specificities. Such individual sera may show a positive reaction with more than one antigen line.

PERFORMANCE CHARACTERISTICS

Specificity

The ANA-9 Line test was evaluated by testing patient sera of known specificity and blood donor sera using the general test procedure. All blood donors gave negative lines for all antigen specificities.

Calibration

Testing of CDC sera using the protocol devised by Tan et al. (1999) Arthritis & Rheumatism 42, 455-464.

The sensitivity, specificity and dose response of the ANA-9-Line immunoblot was evaluated using CDC sera and in house quality control sera containing varying relative amounts of sera with known specificity.

LIMITATIONS OF PROCEDURE

The ANA-9-Line immunoblot assay 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.

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 be avoided.

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INCUBATION SCHEME

1. Add **blot strip** into the incubation tray
   → Add **1000 µl** sample buffer per strip into the incubation tray
   → Shake **5 minutes** while incubating

2. Add **10 µl** patient sample and resuspend
   → Shake **60 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with**1000 µl** wash buffer, discard wash

3. Add **1000 µl** enzyme conjugate solution per strip
   → Shake **30 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **1000 µl** wash buffer, discard wash

4. Add **500 µl** substrate per strip
   → Shake **10 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **1000 µl distilled water**, dry blot strips. Read after complete drying, only