



ANA LineBlot

Immunoassay for the detection of antinuclear (ANA)
antibodies

Code:

DB 100 E

10Tests

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PRECAUTIONS

The reagents supplied in this pack are for in vitro diagnostic use only. Do not change anything of the recommended protocol!

Store all kit components at 2 – 8 °C in a refrigerator! After first opening of the vial with the strips, store remaining strips at room temperature protected from light.

Do not touch the nitrocellulose-strips with fingers. Use always clean tweezers.

The components supplied in this kit are intended for use as an integral unit. Therefore, the components of different lots should not be mixed.

Do not use kits or components after the expiry date printed on the outer carton.

Do not cross-contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles. Always use clean, preferably disposable, glassware for all reagent preparations.

The chromogen is potentially cancerogenic and can irritate skin and mucous membranes. Any substrate coming into contact with skin should be rinsed off with running tap water immediately.

MATERIALS PROVIDED

Test unit	10 Tests
Nitrocellulose-strips loaded with specific nuclear antigens.	10 strips
Template	1 piece
Blocking-buffer (cloudy) Contains PBS, blocking protein and 0,1 % azide, Ready for use	1 vial 20 ml
Goat-anti-human-IgG-Conjugate (conjugated with alkaline phosphatase), Concentrated	1 vial 120 µl
Substrate (BCIP), ready for use	1 vial 15 ml
Stop solution (3 % acetic acid), Ready for use	1 vial 20 ml
Serum dilution- and wash-buffer (SDW-buffer); contains PBS, proteins and 0,1 % azide 10x concentrated	1 vial 20 ml

TECHNICAL DATA

Specimen:	Serum
Required sample size:	20 µl per test
Incubation time:	approx. 1,5 hours at room temperature
Storage:	at 2 to 8 °C (refrigerator)
Test unit	10 strips

PRINCIPLE OF THE TEST

Specific nuclear antigens are applied (sprayed) to nitrocellulose (NC) strips at equal distances.

The required number of strips is placed to the respective row of the incubation tray. To rehydrate and to block free binding sites against unspecific binding, the strips are incubated with buffer, containing blocking protein. After discarding the blocking buffer, the membrane strips are incubated with prediluted serum samples. According to their specificity, autoantibodies, if present in the sample, will bind to the antigens during the 30 minutes of incubation. Unbound serum components are eliminated by a washing step.

During the next incubation step all specifically bound autoantibodies are traced by alkaline phosphatase conjugated anti-human-IgG antibodies. After a second wash to remove excess enzyme label, specifically bound autoantibodies are detected by the use of the chromogenic reaction with BCIP.

Labelled antigens and their respective autoantibodies appear as blue stained bands on the strips. Using the evaluation template the specificity of the autoantibodies is identified.

SUMMARY AND EXPLANATION OF THE TEST

Autoimmune diseases are serologically characterized by the detection of several autoantibodies and typical antibody profiles. The majority of these autoantibodies is directed against antigens localized in the nucleus, the nuclear plasma, the nuclear matrix and the nucleolus, but also against mitochondrial and microsomal antigens in the cytoplasm.

Rheumatoid diseases are mainly associated with the occurrence of anti-nuclear antibodies (ANA). Some of these ANA are disease-specific and therefore useful serological markers in the diagnosis. They include antibodies against:

- Double-stranded DNA (ds-DNA) and the Sm antigen in systemic Lupus erythematosus (SLE)
- Scl-70 (Topoisomerase I) associated with diffuse scleroderma
- Centromere (ACA) for CREST syndrome
- Histidyl-tRNA-synthetase (Jo-1) in polymyositis/dermatomyositis
- PM-Scl in polymyositis and scleroderma overlap syndromes.

ANA with different prevalence are found in several diseases, including:

- anti-histone in SLE, drug-induced Lupus and nutritive toxic chronic liver diseases;
- anti-RNP in SLE and mixed connective tissue disease (MCTD, Sharp syndrome) and
- anti SS-A (Ro) and anti SS-B (La) associated with SLE and Sjögren's Syndrome (1,2).

The earliest method for the detection of ANA and AMA has been the immunofluorescence test (IFT), applying cryostat tissue sections or cells as substrate. Distinguishable immunofluorescence staining patterns allow the differentiation of several antibodies. Many of today's known antibody specificities to soluble cellular antigens have been defined by using Ouchterlony tests, based on radial immunodiffusion (RID). Antinuclear and anti-mitochondrial antibodies have been further characterized by the use of complement fixation tests (CFT) and immunoblotting assays.

REFERENCES

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Autoantibodies to U-RNP Particles relationship to clinical diagnosis and nephritis.
Clin. Exp. Med. 1991; 83:286 - 90.
3. Buyon, J.P., Winchester, R.
Congenital complete Heartblock.
Arthritis and Rheumatism 1990; Vol. 33, No.5: 609 - 14.
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Association between Lupus Psychosis and anti -ribosomal P Protein Antibodies.
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INSTRUCTIONS FOR USE

MATERIALS REQUIRED BUT NOT PROVIDED

- Disposable tip micropipettes to dispense volumes of 10, 20 und 1000 µl
- Range of standard, clean volumetric laboratory glassware
- Freshly distilled water
- Whirlmix
- Clean disposable glass or plastic tubes for serum dilution
- Absorbent paper towels
- Shaker
- Tweezers

SPECIMEN COLLECTION AND PREPARATION

Autoantibody detection with the ANA LineBlot is carried out with serum.

All serum samples are prediluted 1:51 with serum-dilution- and wash-buffer (SDW-buffer). Therefore, 20 µl of serum sample may be diluted with 1.000 µl SDW-buffer.

Serum samples may be stored refrigerated at 2 – 8 °C for up to 5 days. For longer storage the samples should be stored frozen at –20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Alternative procedure for liquor

Alternatively to the antibody determination in serum, autoantibodies to nuclear antigens may be detected in liquor, too.

Since the antibody concentration in liquor is much lower than in serum samples, different predilutions have to be prepared. Serial predilutions of 1:5, 1:10 and 1:20 with SDW-buffer are recommended.

INSTRUCTIONS FOR REAGENT PREPARATION AND STORAGE

All not opened components can be utilized until expiry date, printed on the outer box, presumed, they are stored at 2 - 8 °C. The strips should be stored at room temperature after first opening.

Reconstitution of the serum-dilution-wash-buffer (SDW)

Add 20 ml concentrated SDW-buffer to 180 ml distilled water (= 200 ml). Mix well. This working solution is stable up to two weeks at 2 – 8 °C.

Conjugate

While the first incubation is running dilute the anti-IgG-conjugate:

number of strips needed	serum-dilution-wash-buffer (ml)	concentrated conjugate (µl)
1	1,0	10
2	2,0	20
3	3,0	30
4	4,0	40
5	5,0	50
etc.	etc.	etc.

It is not necessary to bring the conjugate to room temperature prior to dilution. It is recommended to take the conjugate out of the refrigerator and to pipet the needed volume from the vial. Put back the remaining conjugate into the refrigerator immediately. This working solution is stable for one hour at room temperature (22°C).

TECHNICAL NOTES AND QC-CRITERIA

Do not change steps of the recommended protocol! Temperatures below 18 °C and above 28 °C may in part affect the development of the bands. The recommended time for substrate incubation has to be followed exactly

Store all kit components at 2 – 8 °C in a refrigerator!

The components supplied in this kit are intended for use as an integral unit. Therefore, the components of different lots should not be mixed. **This is especially true for the reference (template) delivered in the kit.**

Do not use kits or components exceeding the expiry date, printed on the outer box.

Efficient rinsing of uncomplexed serum components is a fundamental requirement of the LineBlot procedure. Therefore, the washing procedure should be performed particularly careful. Insufficient washing will cause aberrant results and high background staining.

QC-criteria

These LineBlots are manufactured in lots of 12 strips. Two of these strips are developed with control sera and are documented at the manufacturer's. Using these two marker-strips, the reference (template) is drawn. Running of controls therefore is not necessary for the analysis of the band pattern.

To control the proper performance of each strip, a control band is lined at the top of the bands. This band **must** develop in any case. If this band does not appear, or only very faintly, one of the components was not added or is deteriorated.

ASSAY PROCEDURE

1. Take the required number of strips out of the tube with tweezers and put them, the right side up, into their respective channels of the incubation tray. The upper side of the strips (with the strip number and the front marker line) must never turn down during all incubations. If it happens while adding reagents or buffers, turn the strip back the right way up immediately with tweezers.
2. Add 1 ml blocking-buffer (mix well prior to use) to each strip. Take care that all strips are completely covered with fluid. It is important for all other steps of the procedure, too.
3. Incubate for 15 min. on a shaker. Take care that the fluid is mixed well by shaking but it must not contaminate the adjacent channel. This is especially important for the following serum incubation!
4. Dilute serum specimens 1 : 51 with SDW buffer (add 20 µl specimen to 1.000 µl SDW buffer and mix well).
5. Discard the blocking-buffer by decanting from the incubation tray. The strips are completely rehydrated now and will stay adhesive to the bottom of the channels during decanting. Add 1 ml diluted serum specimen to their respective channels and incubate for 30 minutes on the shaker.

6. Wash all strips 4 times for 3 min with SDW buffer. Sufficient washing is very important:
 - a) Inclining the tray carefully, aspirate the channel contents using a vacuum line, fitted with a trap. Avoid cross-contamination!
 - b) Add 1 ml SDW buffer, shake gently for some seconds and aspirate.
 - c) Add 1 ml SDW buffer, shake for 3 minutes and aspirate. Repeat this step two times. For the last two steps, aspirating can be replaced by decanting.
 - d) Check that there is no residual wash-buffer in the channels.
7. Add 1,0 ml freshly prepared conjugate-dilution to each strip.
8. Incubate for 25 minutes on the shaker.
9. Wash all strips 4 times by decanting and shaking for 3 minutes.
10. Add 1 ml substrate to each strip.
11. Incubate for exactly 5 minutes on the shaker.
12. Immediately add 1 ml stop solution to each channel , shake gently and decant.
13. Add aqua dest., shake for a short time and decant. Repeat this washing-step with water. Then add water again and keep strips in the tray for up to 15 min..
14. Remove the strips with tweezers from the tray and dry them on absorbent paper. Analyse the band pattern according to the procedure described on the next page. For documentation store the strips protected from light.

ANALYSIS OF BAND PATTERN AND INTERPRETATION OF THE RESULTS

After complete drying of the strips (please note, minimum drying 1 hour) put them on white paper with the front line and the number at the bottom. Put the template above the strip, placed in such a way, that the white rim with lot number etc. is at the top. Fit the strip into the long blank space marked on the template. The bottom end line of the blank space must be fitted exactly upon the front line of the strip. At the rim of the blank space the possible band pattern and the positions of the antigens are marked. Now inspect the ready developed strip to see whether and how strong there are bands, identical with the positions on the template.

Using LineBlot strips, coated with bands of purified antigens, the detection and characterization of many ANA autoantibodies is possible:

MATERIALS PROVIDED

Table of detectable autoantibodies

Type of Antibody	Molecular weight (kD or bp)
anti-dsDNA	3400bp
anti-Sm-B'B	30 kD
anti-U1 -snRNP 68/70	68 kD
anti-RNP-A	34 kD
anti-RNP-C	25 kD
anti-rib. RNP	35
anti-Ro/SS-A1	60
anti-Ro/SS-A2	52
anti-La/SS-B	52
anti-Scl-70 (Topoisomerase)	102
anti-PM-Scl	n.d.
anti-Centromere (CENP-B)	80
anti-Jo-1 (Histidyl-tRNA-Synthetase)	58

Specificity of the antigens

Anti-dsDNA

Antibodies against double stranded DNA are specific markers for the diagnosis of systemic lupus erythematosus (SLE).

Anti-Sm

The specific „common“ proteins of the Sm antigen are associated with the snRNP complexes and thus involved in multiple molecular processes, that take place in the nucleus.

Sm consists of three proteins: B' (29 kD), B (28 kD) and D (19 kD), which share some epitopes.

Anti-RNP

The antigenic structures are specific proteins of the snRNP called "small nuclear ribonucleoprotein complex". This complex is involved in various molecular processes, that take place in the nucleus. It contains proteins with molecular weights of about 68-70, 33(34) (protein A) and 22(25)kD (protein C).

In total six snRNP particles (U1 to U6) can be distinguished.

These particles are complexes consisting of messenger-RNA (mRNA) and proteins. The main proteins of the particles U1, U2, U5 and U4/U6 contain the protein units B', B, D, E, F and G.

The U1-snRNP 68-70 additionally contains the proteins A and C. All RNP particles are involved in the splicing of mRNA precursors.

The occurrence of isolated anti-snRNP antibodies in the absence of anti-Sm is a pathognomic marker for mixed connective tissue diseases (MCTD) and shows a clinical sensitivity of 95-100%.

Bands at 33 kD may occur in parallel with the 68-70kD- or the Sm-bands. Acc. to the literature (2) this band is indicative for the SLE and nephritis, existing or developing.

In combination with the 68-70kD RNP they are indicative for the SLE or MCTD.

Anti-rib.RNP

These antibodies are directed against three subunit ribosomal phospho-proteins, called PO(38 resp 35kD), P1(19kD) and P2(17kD). They occur in approx. 12% of patients with SLE.

Clinically, anti-ribosomal RNP antibodies are associated with lupus psychosis (4).

Anti-SS-A (Ro)

These autoantibodies are directed against proteins of RNA-protein particles located in the nucleus. They may also be present in the cytoplasm. Antibodies to SS-A bind to a particular antigen that consists of two proteins with molecular weights of 60 and 52 kD, respectively, and small RNA particles of 80 to 112 nucleotides. Antibodies to Ro-antigens are indicative for the SLE or Sjögren's Syndrome (5)

Anti-SS-B (La)

SSB is a nucleus-based particular antigen. It consists of a 52 kD phosphoprotein, which is associated with Polymerase III transcripts. In addition to human RNA, the protein may also bind to virus-encoded RNA.

In general, the SS-B bands appear - with very few exceptions- only in combination with the Ro 52 kD-band.

Anti-SS-B antibodies can be demonstrated in 45% of SLE patients and in 90 % of patients with Sjögren's Syndrome. Clinically, these antibodies are indicative for SLE or Sjögren's Syndrome with existing Sicca symptoms.

Anti-Scl 70

This 100(102) kD protein has been characterized as DNA-Topoisomerase I.

Antibodies against the Scl 70 antigen are a highly specific marker for progressive systemic sclerosis (PSS). Occasionally, Scl 70 antibodies are detected in patients with circumscribed or linear scleroderma. Diagnostic sensitivity of Scl 70 determination in progressive scleroderma is more than 70 % at a diagnostic specificity of about 100 %.

Anti-Pm-Scl

Antibodies against PM-Scl are nearly in complete association with the disease of polymyositis and dermatomyositis.

Pm-Scl is a complex consisting of 10 nucleolar antigens with an overall molecular weight of approx. 120 kD.

Anti-Pm-Scl is a very rare, but highly specific marker for the scleroderma-polymyositis overlap syndrome.

Anti-Centromere (ACA)

The centromere is a special domain of eucaryotic DNA and adhesion point of the mitotic spindle fibres. Antibodies are directed against specific DNA associated proteins (CENP-A, -B and -C), most frequently against the CENP-B antigen.

Clinically ACA are relevant for patients with CREST syndrome and less frequent with scleroderma.

Anti-Jo-1

The antigen called Jo-1 recently has been characterized as Histidyl-tRNA-Synthetase. This antigen has a molecular weight of 55(58) kD.

Jo-1 antibodies are highly specific markers for dermatomyositis or polymyositis. A correlation between disease activity and antibody titers has been discussed.

LIMITATIONS OF USE

As for many other serological tests the results of this blots should only be interpreted in context with all available clinical and laboratory dates.

SUMMARY OF THE PROCEDURE

ANA LineBlot

