AESKU.BION
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ANTI-NUCLEAR ANTIBODY TEST SYSTEM

CE

NOTE: Changes highlighted

SYMBOL DEFINITIONS

= Consult Directions for Use
= Store Away From Direct Light
= Storage Temperature
= Expiration Date
= Number of Tests
= Amount
= Single Use Only
= Contains human blood or plasma derivatives

IVD = *In Vitro* Diagnostic Reagent
CONTROL + = Positive Control
CONTROL − = Negative Control
TITER = Endpoint Titer
REF = Code Number
LOT = Lot Number
= Contains biological material of animal origin

Bion ANA KITS AND REAGENTS

KITS and KIT COMPONENTS	CODE NO.
ANA (HEp-2) 60 Test Kit	ANK-60
ANA (HEp-2) 120 Test Kit	ANK-120
ANA (HEp-2) Substrate Slide, six wells	AN-1006
ANA (HEp-2) Substrate Slide, twelve wells	AN-1012
IFA Diluent, 60 ml bottle	DIL-9993
IFA Diluent, 125 ml bottle	DIL-9994
ANA Positive Control Serum (Homogeneous), 0.5 ml	ANP-1040
ANA Negative Control Serum, 0.5 ml	ANN-1010
Conjugate, Immunoglobulin with Counterstain, 3.5 ml	CCP-9970
Mounting Medium, 3.5 ml	MM-9985
PBS Packet, 1 liter	PBS-9990

Additional Available ANA Control Sera	CODE NO.
ANA Nucleolar Control	NUC-1042
ANA Anti-Centromere Control, (ACA)	ACA-1043
ANA Ribonucleoprotein Control, (RNP)	RNP-1044
ANA Sjogren's Syndrome A Control, (SS-A)	SSA-1045
ANA Sjogren's Syndrome B Control, (SS-B)	SSB-1047
ANA Scleroderma-70 Control, (Scl-70)	SCL-1046

INTENDED USE

The Bion ANA (Anti-Nuclear Antibody) TEST SYSTEM is an indirect fluorescent antibody assay utilizing HEp-2 tissue culture cells as a substrate for the qualitative and/or semi-quantitative determination of antinuclear antibodies in human serum. The Bion ANA TEST SYSTEM is intended for use as an aid in the diagnosis of certain autoimmune diseases.

SUMMARY AND EXPLANATION

Antinuclear antibodies (ANA) are a group of autoantibodies characterized by specificity for numerous antigenic determinants of cell nuclei. While the role of ANA's in the pathogenesis of autoimmune disease is controversial, they are quite useful as disease markers, primarily for diagnostic screening and also to monitor the course of connective tissue diseases.[1,2,3]

Because of the high correlation of positive antinuclear antibodies with SLE a negative ANA essentially rules out this disease.[4] Although antibodies specific to DNA have a high correlation with SLE,[5] antibodies to a number of other nuclear antigens appear to be of diagnostic and/or prognostic significance in diseases such as Progressive Systemic Sclerosis,[6,7] Mixed Connective Tissue Disease,[8] Sjogren's Syndrome,[9] and Polymyositis;[10] making ANA testing useful not only for SLE, but as a general screening tool for connective tissue diseases.[11]

Among the methodologies available to detect ANA's are EIA, ELISA, Dot Blot and the Indirect Fluorescent Antibody (IFA) technique. The antigen for the first three methods can either be a spectrum of clinically significant, specific autoantigens, a single mixture of autoantigens from a cell lysate, or a combination of the two. These methods are not as sensitive as IFA, nor can they detect the variety of autoantibodies. They also do not have the pattern recognition quality of the IFA. The IFA test is sensitive, screens for a wide variety of known and unknown autoantibodies and, through pattern recognition, offers insights into the probable identity of the antigen and associated autoimmune disorder. It is the dominant methodology in clinical laboratories at this time[2] and the method of choice for ANA screening and semi-quantitation.

The antigen of the Bion ANA substrate is a human epithelial cell (HEp-2) line established by Moore, Sabachensky and Toolan.[12] HEp-2 cells have been shown to have greater sensitivity than tissue sections and yield sharper pattern recognition.[13] The presence of mitotic figures aids in differential pattern recognition as well as detecting previously unreported nuclear antibodies.[14,15] Antinuclear antibodies can be found in all major immunoglobulin classes (IgG, IgA or IgM), therefore, antihuman gammaglobulin conjugate that detects all classes is recommended for use in routine ANA testing.[16]

PRINCIPLE OF THE IFA PROCEDURE

The Bion ANA TEST SYSTEM utilizes the indirect fluorescent antibody assay method first described by Weller and Coons,[17] and applied to ANA detection by Friou,[18] and Holman and Kunkel.[19] The procedure is carried out in two basic reaction steps:

Step 1 - Human serum is reacted with the antigen substrate. Antibodies, if present, will bind to the antigen forming stable antigen-antibody complexes. If no antibodies are present, the complex will not be formed and serum components will be washed away.

Step 2 - Fluorescein labeled antihuman antibody is added to the reaction site which binds with the complexes formed in step one. This results in a positive reaction of bright apple-green fluorescence when viewed with a properly equipped fluorescence microscope. If no complexes are formed in step one, the fluorescein labeled antibody will be washed away, exhibiting a negative result.

REAGENTS

SUBSTRATE SLIDES

Ten individually foil-wrapped slides of six or twelve wells, with HEp-2 tissue culture cells fixed onto each well. Stable in sealed foil pouch at 8ºC, or lower, until labeled expiration date.

POSITIVE CONTROL SERUM

One bottle containing 0.5 ml of ANA positive human control serum (homogeneous pattern) with protein stabilizer and 0.005% thimerosal. Stable at 2-8ºC until labeled expiration date.

When used undiluted as provided, specific fluorescent intensity of 3+ or greater should be seen. Optionally, the positive control can be titered to endpoint. If titered, the control should be serially diluted in PBS. When the control has been tested for the endpoint titer by Bion, an endpoint titer is printed on the positive control vial. Due to variations within each laboratory (fluorescent microscope, etc.), each laboratory should establish its own mean titer for each lot of positive control (generally ± one dilution from stated endpoint).

NEGATIVE CONTROL SERUM

One bottle containing 0.5 ml of ANA negative human control serum with protein stabilizer and 0.005% thimerosal. Stable at 2-8ºC until labeled expiration date.

The control is intended to be used undiluted as provided. The staining should exhibit less than 1+ fluorescence.

MOUNTING MEDIUM

One or two dropper bottles containing 3.5 ml phosphate buffered glycerol of pH 7.4 ± 0.2. Stable at 2-8ºC until labeled expiration date

IFA DILUENT (Optional)

One bottle containing 60 ml or 125 ml of sample diluent, withless than 0.1% thimerosal, formulated to reduce nonspecific staining. Stable at 2-8ºC until labeled expiration date. Do not use if solution turns cloudy or if a precipitate forms.

FLUORESCENT ANTIBODY CONJUGATE

One, two or four ready to use dropper bottles, each containing 3.5 ml fluorescein isothiocyanate labeled goat antihuman immunoglobulins with 0.001% Evans Blue counterstain, protein stabilizer, less than 0.1% sodium azide and 0.001% thimerosal added. Stable at 2-8ºC away from direct light until labeled expiration date.

PHOSPHATE BUFFERED SALINE (PBS)

Two or four one-liter packets of dry PBS. Stable in sealed packet at 25ºC, or lower, until labeled expiration date.

BUFFER PREPARATION

Place contents of a one-liter PBS packet into a one-liter volumetric flask, add *distilled water to the one-liter mark, mix and leave several hours or overnight to dissolve. Reconstituted buffer should have a pH of 7.4 ± 0.2. Adjust with 1N NaOH or 1N HCL if pH value is outside the stated range. Store in a clean screw capped bottle at 25°C or lower. Stable until labeled expiration date provided no gross contamination is seen. Do not use if pH changes, if the solution turns cloudy, or if a precipitate forms.

* Use deionized water with caution, as pH of this type of water may vary causing the pH of PBS to become unstable upon prolonged storage.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use. Thus, only staff trained in methods of in vitro diagnostics may perform the test.
2. Substrate slides are for single use only and must not be used more than once.
3. Care should be taken when handling substrate slides due to sharp edges.
4. Do not remove slides from pouch until ready for testing. Do not use if pouch has been punctured, as indicated by a flat pouch.
5. All reagents should be brought to room temperature (20-25ºC) prior to use.
6. Abnormal test results may be seen if the antigen substrate slides are allowed to dry during the staining procedure.
7. Refrigeration (2-8ºC) of kit immediately upon arrival will insure stability until labeled expiration date.
8. Reagents should not be used beyond stated expiration date.
9. Substitution of components other than those provided may yield inconsistent results.
10. Do not expose conjugate to strong light during storage or use.
11. Avoid microbial contamination of all reagents involved in the testing procedure or incorrect results may occur.
12. Deviation from the defined test procedure, such as incubation times or temperatures, may give erroneous results.
13. Lipemic, hemolyzed or contaminated sera may yield erroneous results.
14. Previously frozen specimens after thawing should be thoroughly mixed prior to testing. It is recommended that sera freeze thawed no more than one time. If repeated testing is required, it is suggested that specimen be aliquoted.
15. Care should be taken to avoid splashing and generation of aerosols.
16. Reusable glassware must be washed and thoroughly rinsed free of detergents.
17. Patient samples, as well as all materials coming into contact with them, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual “Biosafety in Microbiological and Biomedical Laboratories”, 1984 Edition. Never pipette by mouth. Avoid contact with skin and mucous membranes.
18. Sera used to prepare positive and negative controls have been tested by an FDA approved method and found to be negative (were not repeatedly reactive) for the presence of Hepatitis B surface Antigen (HBsAg) and antibodies to Hepatitis C (HepCAb) and HIV 1 & 2. However, because no test method can offer complete assurance of the absence to these or other infectious agents, these reagents should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories," 1984 Edition.
19. The preservatives used in conjugates, controls and diluent are toxic if ingested. Azides may react with copper or lead plumbing to form explosive metal azides. When disposing, flush drains with water to minimize buildup of azide and metal compounds.
20. Bion IFA diluent should be used ONLY as a diluent for patient specimens. Do NOT prepare serial dilutions for endpoint titer with the IFA diluent. Do NOT use in any of the wash steps.

SPECIMEN COLLECTION

Blood should be collected fasting or at least one hour after meals to avoid lipemic serum, as excess lipids may produce a "film" over the substrate. Aseptically collect 5-8 ml of blood by venipuncture. Allow the blood to clot at room temperature (20-25°C) before separating serum to avoid hemolysis which could interfere with test results. Specimens should be stored refrigerated at 2-8°C and tested within one week of collection. Long term storage should be at -20°C in aliquots to avoid repeated freezing and thawing. Do not store in self-defrosting freezer.

Avoid using contaminated sera as they may contain proteolytic enzymes, which will digest the substrate. It is unnecessary to heat inactivate serum specimens prior to testing; however, sera that have been heat inactivated may be used.

When testing paired samples to look for a significant change in antibody titer, the two specimens must be tested simultaneously in the same assay.

PROCEDURE

MATERIALS PROVIDED

1. ANA Antigen Substrate Slides
2. Fluorescent Antibody Conjugate
3. Positive Control Serum
4. Negative Control Serum
5. Phosphate Buffered Saline (PBS)
6. Mounting Medium

MATERIALS REQUIRED BUT NOT PROVIDED

1. One liter volumetric flask or one liter graduated cylinder
2. Distilled water - CAP Type one or equivalent
3. One liter screw capped container
4. Disposable test tubes and rack
5. Disposable serological pipettes
6. Calibrated pipettes to deliver 10 µl and 100 µl, with disposable pipette tips
7. Pasteur pipettes and bulbs
8. Moist chambers
9. Plastic squeeze wash bottle
10. Coplin jars or staining dishes with slide racks
11. Coverslips, No. 1, 24 x 60 mm
12. Permanent black felt tip marking pen
13. Fluorescence microscope equipped with a mercury or tungsten-halogen light source, a 390-490nm excitation filter and 515-520nm barrier filter, and optics to give a total magnification of 400X. The excitation wavelength of FITC is 490nm and the emission wavelength is 520nm

TEST PROCEDURE

NOTE: Bring slides and reagents to room temperature (20-25°C) before use.

1. **SPECIMEN PREPARATION**

   Screening:

   Prepare a 1:40 dilution of each patient's serum by adding .01 ml (10 µl) of patient's serum to 0.39 ml of PBS or IFA diluent.

   Semi-quantitation:

   The significance of a positive screening result should be confirmed by repeating the test with dilutions of the serum. Each laboratory should establish its own titering protocol; however, the following fourfold serial titration is suggested:

   a. Prepare a 1:40 dilution of each patient's serum by adding .01 ml (10 µl) of patient's serum to 0.39 ml of PBS or IFA diluent.

   b. Add 0.3 ml PBS to tubes #2, #3, #4 and #5. (DO NOT use IFA diluent for serial dilutions.)

   c. Using a 100 µl pipette, transfer 0.1 ml (100 µl) from tube #1 to tube #2. Mix. Using a new tip for each dilution, transfer 0.1 ml (100 µl) from the second tube to the third, from the third tube to the fourth, and from the fourth tube to the fifth, mixing after each transfer. This will give a fourfold titration with the following dilutions:

      Tube #1 = 1:40
      Tube #2 = 1:160
      Tube #3 = 1:640
      Tube #4 = 1:2560
      Tube #5 = 1:10240

   NOTE: The Positive and Negative Controls are intended to be used undiluted. However, if performing a semi-quantitative test, the Positive Control should be diluted as suggested above.

2. **SLIDE PREPARATION**

   Remove as many slides as are required from the refrigerator and allow to equilibrate to room temperature (20-25°C) for at least five minutes. Remove slides from sealed foil pouches, being careful not to touch antigen surface. Identify each slide using a permanent black marking pen.

3. **SPECIMEN APPLICATION**

   Using separate Pasteur pipettes, apply one drop (20-30 µl) of each control, and one drop (20-30 µl) of each patient serum dilution to individual wells of the slide. Do not touch the antigen surface with the pipette while dropping. Do not allow drops to mix, as cross contamination of samples between wells could cause erroneous results.

TEST PROCEDURE (continued)

4. **INCUBATION 1**
   Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes.

   NOTE: THE ANTIGEN MUST NOT BE ALLOWED TO DRY DURING ANY OF THE FOLLOWING STEPS. Nonspecific binding may occur if the reagent is allowed to dry on the slide.

5. **RINSE 1**
   Remove slides from moist chamber one at a time and rinse GENTLY with PBS using a squeeze wash bottle. Do not focus the PBS stream directly onto the wells. To prevent cross contamination tilt slide first toward wells 1-6 and, running PBS stream along the midline of the slide, allow the PBS to run off the top edge of the slide. Then tilt the slide toward wells 7-12, and repeat this procedure, allowing the PBS to run off the bottom edge of the slide. For six well slides, tilt slide down and run the PBS stream across the slide above the wells, allowing the PBS to run off the bottom edge of the slide.

6. **WASH 1**
   Place slides in Coplin jars or staining dishes and wash in two changes of PBS for 5 to 6 minutes each wash, agitating gently at entry and prior to removal.

7. **CONJUGATE APPLICATION**
   Remove slides from the wash one at a time, shake off excess PBS, dry around outside edges if necessary and return each slide to the moist chamber. Using dropper bottle provided, apply one drop of conjugate (30-50 µl) to each well of each slide, making sure each well is completely covered.

8. **INCUBATION 2**
   Incubate in a moist chamber at room temperature (20-25°C) for 30 minutes. Protect slides from excessive light.

9. **RINSE 2**
   Remove slides from moist chamber and rinse GENTLY with PBS using a squeeze wash bottle. As suggested in step 5., do not focus PBS stream directly onto the wells.

10. **WASH 2**
    Place slides in Coplin jars or staining dishes and wash in two changes of PBS for 5 to 6 minutes each wash, agitating gently at entry and prior to removal.

11. **COVERSLIP**
    Remove slides one at a time from last PBS wash, shake off excess PBS and immediately add two to four drops of mounting medium across the slide. Tilt slide and rest the edge of the coverslip against the bottom of the slide allowing the mounting medium to form a continuous bead between the coverslip and slide. Gently lower the coverslip from the bottom of the slide to the top, being careful to avoid air bubbles. Drain excess mounting medium by holding the edge of the slide against absorbent paper. Wipe off back of slide.

12. **READ**
    Examine stained slides as soon as possible using a properly equipped fluorescence microscope. It is recommended that slides be examined on the same day they are stained. If any delay is anticipated, store slides in the refrigerator (2-8°C) away from direct light and read the following day. Do not allow mounting medium to dry between slide and coverslip. If drying should occur, add additional mounting medium or recoverslip slide.

FLUORESCENT INTENSITY GRADING

Fluorescent intensity may be semi-quantitated by following the guidelines established by the Centers for Disease Control, Atlanta, Georgia.[20]

4+ = Maximal fluorescence; brilliant yellow-green.
3+ = Less brilliant yellow-green fluorescence.
2+ = Definite but dull yellow-green fluorescence.
1+ = Very dim subdued fluorescence.

The degree of fluorescent intensity is not clinically relevant and has only limited value as an indicator of titer. Differences in fluorescence microscope optics, filters and light sources may result in differences of 1+ or more fluorescent intensity when observing the same slide using different microscopes.

QUALITY CONTROL

SPECIFICITY CONTROL

Both a positive and negative antibody control must be included with each run. These controls must be examined prior to reading test samples and should demonstrate the following results:

Negative Control

Using the Bion ANA NEGATIVE CONTROL SERUM as provided with the Bion ANA TEST SYSTEM, the cells should exhibit less than 1+ fluorescence and appear reddish-orange due to the counterstain.

Positive Control

Using the Bion ANA POSITIVE CONTROL SERUM as provided with the Bion ANA TEST SYSTEM, the cells should exhibit a homogeneous staining pattern with a fluorescent intensity of 3+ to 4+.

Each control must demonstrate the expected reaction in order to validate the test. If the controls fail to appear as described above, the test results should not be reported and the test should be repeated. If upon repeat testing the controls still fail to show the proper reaction, do not report test results.

The specificity of the antigen substrate can further be tested by running a panel of various types and patterns of antinuclear antibodies (These are available separately from Bion).

SENSITIVITY CONTROL

A titered control included with each run tests substrate sensitivity, as well as, checks technique, conjugate quality and the microscope optical system. The endpoint titer of each lot of Bion ANA POSITIVE CONTROL SERUM must be determined. There must not be more than a twofold difference (+/-) in titer from the stated endpoint. Each run should include the endpoint dilution, one fourfold dilution above and one fourfold dilution below the endpoint dilution. The more concentrated dilution should be positive and the less concentrated negative. If the control does not behave as described, the test results are invalid and the test should be repeated. If the control fails to show the proper reaction upon repeat testing, do not report the test results.

READING OF TEST RESULTS

NEGATIVE

A serum dilution is considered negative for antinuclear antibodies if the cells exhibit less than 1+ fluorescence and lack a clearly discernible pattern. Cells will appear reddish-orange due to the Evans Blue counterstain.

A sample is considered negative for antinuclear antibodies if it exhibits less than 1+ fluorescence at a serum dilution of 1:40 and all greater dilutions, or if the fluorescence observed is not a discernible ANA pattern.

.... Negative samples may exhibit fluorescent staining slightly greater than the Negative Control, but less than 1+.

.... Some sera may show a low degree of nuclear or cytoplasmic fluorescence with no clearly discernible staining pattern. This phenomenon is generally due to heterophile antibodies and should be reported as negative.[21]

.... Intense non-nuclear staining may be observed in some sera containing Anti-Mitochondrial, Anti-Smooth Muscle or other cytoplasmic antibodies.

POSITIVE:

A serum dilution is considered positive for antinuclear antibodies if the fluorescent staining is at an intensity of 1+ or greater with a clearly discernible pattern of fluorescence.

A sample is considered positive for antinuclear antibodies if it exhibits a characteristic ANA staining pattern with a fluorescent intensity of 1+ or greater at a serum dilution of 1:40 or greater.

.... Multiple antinuclear antibodies may be present in a given specimen; one masking the other. Serially diluting the specimen will aid in distinguishing these patterns.

.... Report all titers and patterns seen.

TITRATION:

If a semi-quantitative titration is performed, the result should be reported as the reciprocal of the last dilution in which 1+ apple-green fluorescent intensity with a clearly discernible staining pattern is detected. When reading fourfold serial dilutions, endpoints can be extrapolated where necessary.

EXAMPLE OF ENDPOINT EXTRAPOLATION:

1:40 = 3+
1:160 = 2+
1:640 = +/-
1:2560 = Neg

The extrapolated endpoint is reported as 320.

Report all titers and patterns seen, extrapolating the titer where necessary.

EXAMPLE:

1:40 = 4+ Peripheral and 3+ Homogeneous
1:160 = 3+ Homogeneous
1:640 = 2+ Homogeneous and 3+ Speckled
1:2560 = 1+ Homogeneous and 2+ Speckled
1:10,240 = +/- Speckled

Report: 40 Peripheral, 2560 Homogeneous and 5120 Speckled.

TROUBLESHOOTING

Possible solutions to problems that may occur in immunofluorescent assays are discussed in an accompanying brochure entitled "TROUBLESHOOTING IN IMMUNOFLUORESCENCE".

INTERPRETATION OF RESULTS

Four major staining patterns which may occur singly or in combinations have been described:

1. Peripheral (shaggy, rim, membranous)
2. Homogeneous (diffuse, solid)
3. Speckled (including ACA)
4. Nucleolar

Other patterns less frequently seen include Spindle and Ribosomal RNP.

1. PERIPHERAL - smooth staining primarily around the outer region of the nucleus with weaker staining in the center. Not all cells within a well may appear peripheral; some may appear homogeneous. The chromosome region of mitotic cells will exhibit a bright positive staining pattern.

   Note: A very thin distinct line around the nucleus is not a peripheral ANA, but a nuclear membrane antibody. It differs in that the chromosome region within the mitotic cells is negative.

   NUCLEAR ANTIGENS: nDNA or Histones

   DISEASE ASSOCIATION: High titers to nDNA are suggestive of active SLE. Lower titers are suggestive of SLE or other connective tissue diseases.[22]

2. HOMOGENEOUS - diffuse staining of the entire nucleus, with or without apparent masking of the nucleoli. Some specimens may exhibit moderately large irregular areas of more intense staining; or the pattern may appear granular, especially as the antibody reaches its endpoint. The chromosome region of mitotic cells will exhibit a bright positive staining pattern.

   NUCLEAR ANTIGENS: nDNA, DNP or Histones.

   DISEASE ASSOCIATION: High titers are suggestive of SLE, while low titers may be found in SLE, and Rheumatoid Arthritis.[5]

   a. Antibodies to DNP have the same specificity as the LE Cell Factor.[11]
   b. Antibodies to Histone alone have a high association with drug-induced lupus.[11]

3. SPECKLED - fluorescent aggregates throughout the nucleus which can be very fine to very coarse depending on the type of antibody present. More than one type of speckle may be seen in any one specimen. The chromosome region of mitotic cells is usually negative.

   a. Sm and nRNP antibodies usually present as a coarse speckle with the chromosome region of mitotic cells negative.
   b. SS-A/Ro and SS-B/La antibodies present as small uniform speckles in a uniform distribution with the chromosome region of mitotic cells negative.
   c. Fine dense speckles with positive staining of the nucleoli and chromosome region of mitotic cells may indicate an Scl-70 antibody.
   d. Varying fluorescence of fine to coarse speckles, in approximately 30-60% of cells with positive or negative staining of the mitotic cells, may represent a PCNA (Proliferating Cell Nuclear Antigen) antibody.

   NUCLEAR ANTIGENS: Sm, nRNP, SS-A/Ro, SS-B/La, Scl-70 (DNA-Topoisomerase 1) or PCNA (Cyclin).

   DISEASE ASSOCIATION:

   a. Sm antibodies are highly specific for SLE and appear to be a "marker" for this disease.[23]
   b. nRNP along with other types of ANA's have been found in SLE, Rheumatoid Arthritis (RA), and Progressive Systemic Sclerosis (Scleroderma). High levels of nRNP antibodies alone are characteristic of Mixed Connective Tissue Disease (MCTD).[23]
   c. SS-A/Ro and SS-B/La antibodies are frequently present in patients with Sjogren's Syndrome without associated RA. Both may be found less frequently in patients with SLE. SS-A/Ro antibodies are found in a high percentage of infants with congenital heart block, neonatal lupus or both.[24]
   d. Scl-70 antibodies appear to be a "marker" for Progressive Systemic Sclerosis (Scleroderma).[23]
   e. Anti-PCNA has been found in a small percentage of patients with SLE.[15]

4. ANTI-CENTROMERE ANTIBODY (ACA) - discrete uniform speckles throughout the nucleus, the number of which corresponds to a multiple of the normal chromosome number. The staining pattern of the mitotic cells will follow that of the chromosomes, with pairs of dots arranging themselves in an equatorial plane during metaphase and then moving towards their respective centrosomes during anaphase.

   Note: An antibody closely resembling the ACA is the Pseudo Centromere (or NSp-1) Antibody. This antibody can be differentiated from the ACA in that the chromosome region of mitotic cells does not stain.[25]

   NUCLEAR ANTIGEN: Centromere/kinetochore portion of the chromosome.

   DISEASE ASSOCIATION: This antibody is considered a "marker" for the CREST variant of Progressive Systemic Sclerosis.[26] It is infrequently found in diffuse Scleroderma and Raynaud's disease.[27]

5. NUCLEOLAR - fluorescent staining of the nucleoli within the nucleus, sharply separated from the unstained nucleoplasm. The nucleolar fluorescence may be homogeneous, speckled or clumpy. Frequently accompanied by a speckled pattern.

   NUCLEAR ANTIGENS: PM/Scl (PM-1), RNA Polymerase 1 or Fibrillarin (U3).[28]

   DISEASE ASSOCIATION: High titers are highly specific for Progressive Systemic Sclerosis (PSS), with lower titers found in PSS, SLE, Sjogren's Syndrome and Raynaud's disease.[29,30]

6. ANTI-SPINDLE ANTIBODY - a network of threads connecting the centrosomes to each other in the mitotic cells.

   NUCLEAR ANTIGEN: Spindle apparatus in cells undergoing mitosis.

   DISEASE ASSOCIATION: There may be some association with Carpal Tunnel Syndrome.[14]

INTERPRETATION OF RESULTS (continued)

7. CYTOPLASMIC FLUORESCENCE:
   a. Ribosomal RNP (rRNP) - diffuse granular fluorescence throughout the cytoplasm. May be confirmed by placing the specimen on a stomach section, where it will stain the chief cells. Frequently accompanied by a nucleolar pattern.

   NUCLEAR ANTIGEN: Ribosomal or cytoplasmic RNP.

   DISEASE ASSOCIATION: This antibody has been found in a small percentage of cases of SLE.[31]

   b. Non-ANA Cytoplasmic Fluorescence:
   The two most common autoantibodies are:
   1) Anti-Mitochondrial Antibody (AMA) - discrete speckles throughout the cytoplasm in a fibrous network, with denser speckling in the perinuclear region. May be confirmed by testing on appropriate tissue substrate.
   2) Anti-Smooth Muscle Antibody (ASMA) - fluorescent strands in the cytoplasm in a spidery network, with fibrils extending from the cell membrane. May be confirmed by testing on appropriate tissue substrate.

CORRELATION OF ANA's WITH IFA STAINING PATTERNS

Antibody Against:	Staining Pattern:	Mitotic Cells:
nDNA	Peripheral & Homogeneous	Positive
Histones	Peripheral & Homogeneous	Positive
DNP	Homogeneous	Positive
Sm	Coarse Speckle	Negative
nRNP	Coarse Speckle	Negative
SS-A	Small Uniform Speckle	Negative
SS-B	Small Uniform Speckle	Negative
Scl-70	Fine Dense Speckle & Nucleolar	Positive
PCNA	Variable Speckle	Negative or Positive
ACA	Discrete Uniform Speckle	Positive Centromeres
PM/Scl	Nucleolar (homogeneous)	Negative
RNA Polymerase 1	Nucleolar (speckled)	Few Discrete Speckles
Fibrillarin	Nucleolar (clumpy)	Positive fibers
Spindle	Spindle Apparatus	
rRNP	Cytoplasmic	Negative

LIMITATIONS OF THE PROCEDURE

1. ANA serological test results should be used in conjunction with information available from the clinical evaluation and other diagnostic information.
2. Two to ten percent of a normal adult population have antinuclear antibodies.[32]
3. Antinuclear antibodies are known to be age and sex related. With increasing age there is an increased incidence of ANA's; therefore, a positive low titer result may be normal for certain individuals in the absence of other clinical signs and symptoms. Antinuclear antibodies are not usually found, however, in normal young individuals.
4. Some positive reactions have been reported in relatives of patients suffering from a connective tissue disease who may develop such a disease at a later time.[33]
5. Positive test results from cord blood or neonates should be interpreted with caution. The presence of antinuclear antibodies in cord blood is usually the result of passive transfer from mother to the fetus. A negative test, however, may be useful in excluding a possible autoimmune process.
6. Positive ANA results may be seen in a small percentage of patients with Infectious and/or Neoplastic diseases, and also in diseases of drug etiology.[22,34]
7. SLE patients undergoing steroid therapy or in remission may have a negative ANA.[11]
8. Positive test results may not be valid in persons who have received blood transfusions or various blood products within the past several months.
9. Test results on specimens from immunosuppressed patients and pregnant women may be difficult to interpret.
10. Endpoint reactions may vary between laboratories due to differences in type or condition of fluorescence microscope employed or assay procedure used.[35]
11. If both the positive and negative control substrate cells are not visible when viewed using the fluorescence microscope, it may be necessary to replace or realign the light source and check the specific filters.
12. Cell culture substrate slides may exhibit nonspecific fluorescence due to contamination of antibodies or PBS rinse-wash solutions with bacteria or fungi. It is very important that personnel reading the staining results have experience in fluorescence microscopy.
13. In general titers of 1:40 and 1:80 are considered low titers, 1:160 and 1:320 are considered medium titers, and 1:640 and greater are considered high titers. It is recommended that each laboratory establish it's own reference ranges.

EXPECTED VALUES

The following chart presents the incidence of antinuclear antibodies utilizing a HEp-2 cell ANA substrate in patient population studies performed at the Duke University Medical Center Division of Rheumatic and Genetic Disease laboratories over a two-year period. This represents a study of over 9,000 control sera and over 4,500 abnormal sera.

Clinical Diagnosis	% Positive
Controls:	
20-60 years	2.0%
70-80 years	3.5%
SLE	95.0%
RA	40.0%
MCTD	99.0%
PSS (Diffuse)	85.0%
PSS (CREST variant)	93.0%
PM/DM	25.0%

Clinical Diagnosis	% Positive
Vasculitides	20.0%
Childhood SLE	64.0%
JRA	
Systemic	14.0%
Polyarticular	6.0%
Pauciarticular	
HLA B27 pos.	0.0%
HLA B27 neg.	26.0%

SPECIFIC PERFORMANCE CHARACTERISTICS

To investigate the relative specificity and sensitivity of the Bion ANA TEST SYSTEM, one hundred twenty serum specimens were compared qualitatively and semi-quantitatively with another commercially available indirect fluorescent HEp-2 ANA test system. All Tables represent averaged results from two independent readers.[36]

The relative sensitivity and specificity are summarized in TABLE 1. The one specimen in which the two kits did not agree was a CDC Reference Serum having high levels of antibodies to SS-A.

TABLE 1 - SUMMARY OF RELATIVE COMPARISON TESTING

		BION KIT		BION Relative Sensitivity	BION Relative Specificity
		Positive	Negative		
OTHER KIT	Positive	71	0	100%	100%
	Negative	1	48		

SPECIFIC PERFORMANCE CHARACTERISTICS (continued)

In the following tables H = Homogeneous pattern, S = Speckled pattern, C = Centromere antibody, N = Nucleolar pattern, cR = cRNP antibody (cytoplasmic pattern). TABLE 2 represents the titers and patterns obtained with the 72 positive specimens in TABLE 1.

TABLE 2 - Summary of Relative Sensitivity Testing

Spec #	BION	Other	Spec #	BION	Other	Spec #	BION	Other
1	80 H	80 H	25	40 S	40 S	49	320 S	160 S
2	40 H	40 H	26	80 S	80 S	50	640 S	640 S
3	80 H	40 H	27	80 S	40 S	51	640 S	160 S
4	40 H	40 H	28	40 S	40 S	52	160 S	40 S
5	160 H	80 H	29	40 S	40 S	53	2560 S	320 S
6	320 H	160 H	30	80 S	40 S	54	1280 S	640 S
7	1280 H	640 H	31	2560 S	2560 S	55	640 S	640 S
8	5120 H	2560 H	32	10240 S	5120 S	56	2560 S	2560 S
9	5120 H	5120 H	33	2560 S	40 S	57	160 S	<40
10	640 H	320 H	34	640 S	640 S	58	640 S	320 S
11	10240 H	10240 H	35	2560 S	640 S	59	80 S	40 S
12	2560 H	1280 H	36	320 S	320 S	60	40 S	40 S
13	640 H	320 H	37	1280 S	1280 S	61	640 S	320 S
14	1280 H	640 H	38	10240 S	5120 S	62	640 C	640 C
15	640 H	320 H	39	5120 S	40 S	63	10240 C	5120 C
16	640 H	320 H	40	5120 S	640 S	64	320 C	320 C
17	1280 H	1280 H	41	10240 S	5120 S	65	640 C	320 C
18	1280 H	640 H	42	640 S	1280 S	66	1280 N	1280 N
19	640 H	320 H	43	10240 S	5120 S	67	640 N	320 N
20	80 H	40 H	44	160 S	160 S	68	320 N	160 N
21	1280 H	640 H	45	10240 S	10240 S	69	160 N	160 N
22	80 S	80 S	46	5120 S	5120 S	70	80 N	80 N
23	40 S	40 S	47	160 S	160 S	71	320 cR	640 cR
24	320 S	320 S	48	160 S	80 S	72	640S/160H	160 H

All but nine of the positive specimens agreed within a twofold dilution. The nine serum specimens were characterized by additional testing as follows:

#'s 33, 39, 57 (CDC Reference Serum) and 72 were characterized as having antibodies against SS-A.
#35 was characterized as having antibodies against Scl-70.
#40 was characterized as having antibodies to both SS-A and SS-B.
#53 (CDC Reference Serum) was characterized as having antibodies against SS-B.
#'s 51 and 52 were unable to be characterized by additional testing.

Interlot precision of the Bion ANA TEST SYSTEM, represented in TABLE 3, was evaluated by testing eleven serum specimens (2 negative and 9 positive over a range of titers) on three successive days using three different lot numbers. There was no more than a twofold difference (+/-) in titer between any of the comparison testings, which is within the confidence limits of this methodology.[35]

Intralot precision of the Bion ANA TEST SYSTEM, represented in TABLE 4, was evaluated by running nine different types of antinuclear antibodies three times within one run using three different slides from the same lot. Again, there was no more than a twofold difference in titer between any of the comparison testings.[35]

TABLE 3 - Summary of Interlot Precision

Spec #	Lot 1	Lot 2	Lot 3
1	160 N	160 N	160 N
2	160 S	160 S	80 S
3	320 S	640 S	320 S
4	2560 S	2560 S	1280 S
5	640 H	1280 H	1280 H
6	<40	<40	<40
7	320 C	640 C	640 C
8	40 S	80 S	40 S
9	<40	<40	<40
10	2560 S	5120 S	2560 S
11	2560 S	5120 S	2560 S

TABLE 4 - Summary of Intralot Precision

Spec #	Test #1	Test #2	Test #3
1	1280 N	1280 N	1280 N
2	320 H	320 H	320 H
3	1280 S	1280 S	1280 S
4	1280 C	1280 C	1280 C
5	2560 S	5120 S	5120 S
6	320 S	320 S	320 S
7	320 S	320 S	320 S
8	80 cR	80 cR	80 cR
9	320 S	320 S	320 S

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