INTENDED USE

The VIRGO® Antinuclear Antibody (ANA KB) indirect fluorescent antibody (IFA) test is intended for the detection and titration of anti-nuclear antibodies in human sera.

SUMMARY

Systemic Lupus Erythematosus (SLE) is a syndrome with manifestations that range from a localized skin lesion to a destructive systemic disorder without any cutaneous change.[1] Yes, despite this marked variability in the clinical picture, all the forms of this disorder have may common characteristics.[2]

There are a number of other autoimmune connective tissue diseases with clinical manifestations similar to SLE. Some examples are scleroderma, dermatomyositis, rheumatoid arthritis, Sjogren's syndrome, myasthenia gravis, discoid lupus erythematosus, and drug-induced erythematosus.

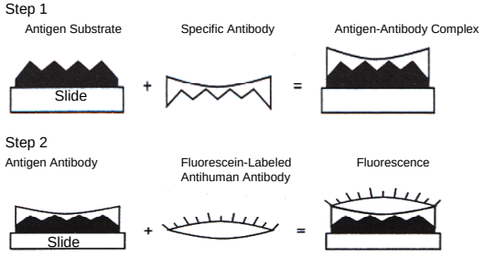
A simple, sensitive serological test for detection of SLE in patients is the indirect fluorescent antibody (IFA) test for antinuclear factor (ANF test). In this technique, first described in 1957 by Holborow, Weir and Johnson,[3] the antibody in serum of patients with autoimmunity combines with the nuclei of tissue cells. The cells are subsequently "stained" with fluorescein-labeled antihuman globulin. The positive reaction, brightly fluorescing nuclei, is visualized with the aid of a fluorescence microscope. A positive ANA test is found in most patients with active autoimmunity. The ANA titer may be lowered or absent in patients in true remission. A fall in ANA titer indicates a true remission rather than a drug-induced one, while a rise in ANA titer indicates renewed clinical activity.[4]

In general, the antibodies to SLE belong to the IgG class of immunoglobulins. Occasionally, the IgM and IgA antibodies may also be found. In rheumatoid arthritis the ANA may be predominantly IgM.

PRINCIPLE OF THE TEST

The VIRGO fluorescent antibody assays utilize the indirect method of fluorescent antibody staining, first described by Weller and Coons in 1954.[5] The procedure is carried out in two basic reaction steps. In step one, the human serum to be tested is brought into contact with the antigenic substrate. Antibody, if present in the test serum, will attach to the antigen, forming an antigen-antibody complex. If the serum being tested does not contain antibody for this particular antigen, no complex is formed and all the serum components are washed away in the rinse step. The second step involves adding a fluorescein labeled anti-human antibody to the test wells. If the specific antigen-antibody complex is formed in step one, the fluorescein labeled antibody will attach to the antibody moiety of the complex in step two. A positive reaction, bright apple-green fluorescence, can be seen with the aid of a fluorescence microscope.

Principle of Indirect Fluorescent Antibody Testing



CONTENTS OF THE KIT

902070	**902075**	**Test Kit Product Number**
48 Tests	**200 Tests**	**Number of Tests per Kit**
12	**25**	**Slides:** human KB epithelial cells
(4-well)	**(8-well)**	
1	**1**	**Vial Positive Control:** Lyophilized human serum
1	**1**	**Vial Negative Control:** Lyophilized human serum
1	**2**	**Vial(s) FITC Conjugate:** Lyophilized, inactivated goat anti-human IgG (heavy and light chains) with counterstain
3	**5**	***Packages Powdered Phosphate Buffer:** (PBS) pH 7.4 ± 0.2
1	**1**	***Vial (2 mL) Buffered Glycerol**
2	**3**	***Package(s) (5 each) Blotters**

*These components may be interchanged between different master lots. Additional supplies are available from Hemagen Diagnostics, Inc.

NOTE: A 200 test kit inclusive of all reagents **except** controls is also available. Order number is 902077.

MATERIALS REQUIRED BUT NOT SUPPLIED

Test tubes and racks for making dilutions

Pipettes for preparing dilutions

Coverslips, 22 x 50 mm., No. 1 thickness

Humidified chamber

Magnetic stir plate (optional)

Staining dish and slide-holder rack

Fluorescence microscope. Refer to manufacturer's instruction manual for the filter system that gives optimum results for FITC (Maximum excitation wavelength = 490 nm. Mean emission wavelength = 520 nm.)

PRECAUTIONS

1. HANDLE ALL ASSAY SPECIMENS, SLIDES, POSITIVE AND NEGATIVE CONTROLS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

   All human blood components of the kit have been tested by approved FDA methods and found to be negative for both hepatitis B surface antigen (HbsAg) and for antibodies to human immunodeficiency virus type 1. Because no test method can offer complete assurance that HIV, hepatitis B virus, or other infectious agents are absent, specimens and kit reagents should be handled at the Biosafety Level 2, as recommended for any potentially infectious human serum or blood specimen.[6,7]
2. The antigenic substrates are fixed in acetone.
3. Do not pipette by mouth.
4. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
5. All materials used in this assay, including reagents, samples and wiping materials should be disposed of in a manner that will inactivate infectious agents.

HANLDING PRECAUTIONS

1. **For *In Vitro* Diagnostic Use.**
2. Do not use the kit or individual reagents beyond their labeled expiration dates.
3. The components of this kit have been tested as a unit. **Do not** interchange components from other sources or from different master lots, except as noted.
4. Protect the conjugate from prolonged exposure to light.

REAGENT STORAGE AND STABILITY

1. Store kit at 2-8°C. Powdered PBS and Buffered Glycerol can be stored at 2-30°C if desired. The test kit can be used through the expiration date on the outer box label.
2. **After rehydration**, Positive Control, Negative Control and FITC Conjugate should be stored at 2-8°C or made up in aliquots and stored at -20°C or colder if not used within one week.

   NOTE: Precautions were taken in the manufacture of this product to protect the reagents from contamination. After reconstitution, care should be exercised to protect the reagents in this kit from contamination. If constant storage temperature is maintained, reagents and substrate will be stable for the dating period of the kit.

REAGENT PREPARATION

Allow reagents and slides to reach room temperature 15 to 30 minutes before use.

1. **Slides and Glycerol:** Ready to use.
2. **PBS:** Dissolve contents of one package in 1 liter of distilled or deionized water. Seal container to prevent contamination or evaporation.
3. **Controls:** Rehydrate with 1.0 mL of PBS. The controls are now at the 1:40 screening dilution. Aliquot for storage at -20°C if not used within one week.
4. **Conjugate:** Rehydrate with 2.0 mL of PBS. Aliquot for storage at -20°C if not used within one week.

SPECIMEN COLLECTION AND HANDLING

1. Serum samples may be stored at room temperature for up to 24 hours. For longer term storage, they may be stored at 2-8°C (for up to three days), or frozen at -20°C or colder. Place at 37°C only until the samples are thawed. Remove and mix thoroughly before use. Self-defrosting freezers are not recommended. Avoid multiple free-thaw cycles.[8]
2. Optimal performance of the VIRGO ANA KB IFA depends upon the use of fresh serum samples. Specimens should be collected aseptically. Early separation from the clot prevents hemolysis of serum.[9]
3. For best results, another sample should be drawn if bacteriological contamination or lipids are present. If another sample cannot be obtained, filtering (0.45µ) or centrifugation (approximately 3000 x G for 10 minutes) is required.
4. Excess lipids in the test serum may produce a "filming" reaction. The lipids "stick" nonspecifically to the glass and are extremely difficult to remove. Experience will enable the trained technician to differentiate this "film" reaction from the specific reaction.
5. Occasionally, the specimen may contain certain proteolytic enzymes which attack and digest the substrate. This is especially true of specimens contaminated with microorganisms. Such specimens may be heated to 56°C for 30 minutes. If this fails to reduce the enzymatic activity, another sample should be obtained from the patient.

TEST PROCEDURE

Specimens may contain infectious agents and should be handled accordingly.

For optimal results, DO NOT allow substrate wells to dry out while performing the test.

1. **Remove the slides and required reagents from the refrigerator** and allow them to reach room temperature (15 to 30 minutes).
2. **Remove the slides from the pouch** just before use and label.
3. **Rehydrate the controls to prepare the 1:40 screening dilution.** These are now representative of typical positive and negative fluorescence patterns.
4. **Dilute the samples** with PBS to the 1:40 screening dilultion or prepare serial two-fold dilutions for quantitative determination.
5. **It is necessary to test 1 Positive Control, 1 Negative Control, and 1 PBS Buffer Control** for each batch of slides tested.
6. **Cover each well with diluted samples or controls** (~10-20 µL per well).
7. **Incubate in a humidified chamber at 23 ± 2ºC for 30 minutes.**
8. **Rinse the slides briefly** in a light stream of PBS. Do not direct the stream into the wells.
9. **Rinse the slides thoroughly for 7 minutes** in a staining dish of PBS. **Change the buffer and wash for an additional 8 minutes.** Handle slides gently. Gentle agitation of the buffer is necessary for efficient slide washing.
10. **Blot the painted mask of the slide** with the blotters provided. **Do not allow the wells to dry before conjugate addition.**
11. **Cover each well with one drop (~10 µL) of FITC Conjugate.**
12. **Incubate in a humidified chamber at 23 ± 2ºC for 30 minutes.** Protect from intense light.
13. **Repeat steps 8 and 9.** Blot the painted mask of the slide with the blotters provided. **Do not allow wells to dry before the addition of glycerol.**
14. **Place a small drop of Buffered Glycerol in each well** and cover with a coverslip.
15. For best results, **the slides should be read immediately at a magnification of 200-500X.** Alternatively, the slides may be read within 24 hours. However, they should be stored at 2-8°C in the dark, and sealed to prevent the mounting fluid from drying.

CRITERIA FOR GRADING FLUORESCENCE INTENSITY

4+	Brilliant apple-green fluorescence
3+	Bright apple-green fluorescence
2+	Clear distinguishable apple-green fluorescence
1+	Dull apple-green fluorescence, lacking in sharpness but readable
0	No fluorescence or barely visible fluorescence

GUIDELINES FOR CHARACTERIZING FLUORESCENCE

ANA Associated Fluorescence Patterns

Homogeneous

Smooth staining of the nucleus with no apparent fluorescent staining of the nucleoli.

Peripheral, Membranous, or Shaggy

Smooth staining primarily around the outer region of the nucleus with weaker staining within the center of the nucleus.

Speckled

Fine or granular-appearing staining of the nucleus generally without fluorescent staining of the nucleoli.

Nucleolar

Large solid speckled staining within the nucleus with or without fine occasional speckles.

No Fluorescence

Absence of or less than 1+ fluorescence in the nucleus of the cells.

NOTES:

1. Samples that show a positive reaction at 1:40 should be tittered to determine the endpoint of reactivity.
2. In quantitative determinations, the endpoint titer is the highest dilution showing a 1+ fluorescence in the nucleus.
3. A number of variations is staining patterns of substrate nuclei can be detected by the ANA immunofluorescence test. These variations are attributed to antibodies reacting with different nuclear constituents. Some of the patterns observed are homogeneous, centromere, peripheral, speckled and nucleolar. The homogeneous pattern is characteristic of the insoluble DNA-histone nucleoprotein. The speckled pattern has been attributed to soluble nuclear antigens. This pattern may often be eliminated after extensive washing of the cells prior to use. Nucleolar staining may be due to staining of ribonucleic acid (RNA) located in the nucleoli. More than one pattern may exist in an individual patient's serum and patterns may change with dilution of patient's serum. The significance of the various staining patterns is not clearly defined. Provisional diagnosis should be based on all available clinical and laboratory data rather than pattern of reactivity.
4. Low titers of antinuclear antibody may be encountered in normal individuals. Because the ANA test is a screening procedure, a small percentage of the normal population may exhibit ANA activity at a 1:40 dilution. Generally, nonsignificant ANA titers are age or sex related or associated with unrelated diseases or infectious processes.

QUALITY CONTROL

1. The control sera are representative of positive and negative reactions. At the screening dilution, the Positive Control represents a strong (3-4+) reaction. If the fluorescence intensity of the Positive Control is less than the acceptable range, the test is invalid and should be repeated.
2. Each lot of Positive Control must be titrated to an endpoint dilution. The endpoint titer must be within ± one two-fold serial dilution of the Positive Control titer reported in the VIRGO ANA KB IFA Kit Notice. If the results obtained are out of range, the test is invalid and should be repeated.

3. At the screening dilution, the Negative Control should not display apple-green fluorescence. If apple-green fluorescence is observed, the test is invalid and should be repeated.
4. Quality Control results were obtained on a Nikon® microscope equipped for epiilliumination with a 50W HBO mercury ARC lamp, B filter system for FITC and a 40X dry objective (NA 0.65). Differences in endpoint reactivity and fluorescence intensity may be affected by the type and condition of fluorescence equipment used (see Microscope Specifications at the end of the package insert).

INTERPRETATION OF SAMPLE RESULTS

RESULTS	SIGNIFICANCE
Screening:	
No fluorescence or fluorescence intensity < 1+ at the 1:40 screening dilution.	No significant level of ANA detected.
≥ 1+ fluorescence at 1:40 or greater on a single serum sample.	Positive by IFA for ANA. Titer for endpoint of reactivity.

LIMITATIONS OF THE PROCEDURE

1. The VIRGO ANA KB IFA Test Procedure and Interpretation of Test Results must be followed closely to obtain reliable test results.
2. An ANA titer is rarely helpful in distinguishing between various autoimmune connective tissue disease, but titers in the range of 1:1280, particularly with speckled, nucleolar or peripheral staining patterns, are strong evidence of SLE or scleroderma[3] and mixed connective tissue disease (MCTD). However, patients with SLE or other autoimmune phenomena may show wide variation in ANA titers depending on the clinical state of the disease. Again, no single serological determination should be used as sole criterion for diagnosis; all clinical and laboratory data must be taken into account.
3. A large number of therapeutics have been reported to induce antinuclear antibodies in patients, e.g., procainamide, hydralazine, diphenylhydantoin, mephenytoin and certain antibiotics.[10,11] The clinician should be aware of this possibility.
4. A small percentage of patients with SLE may not demonstrate antinuclear antibodies by IFA testing, however, these antibodies may be detected by other techniques.[12]
5. Some autoimmune antibodies such as antimitochondrial antibody, antigolgi antibody, etc., may show fluorescence in the cytoplasm which is unrelated to an ANA reaction.
6. Results of this test should be interpreted in the light of other clinical findings and diagnostic procedures.

PERFORMANCE CHARACTERISTICS

Fluorescent antinuclear antibody titers and patterns in various connective tissue disorders (n=143) as well as a normal control population (n=149) were determined using Hemagen's human tissue culture substrate. Results are presented in Tables I and II.

Table I

Need to insert table

Table II

Need to insert table

MICROSCOPE SPECIFICATIONS

Comparable filter systems are shown below:

Transmitted Light Fluorescence:

Light Source	Exciter Filter	Barrier Filter
Mercury Vapor	**KP490 + BG38**	**KP510, K530**
200W	**(4 mm.) or**	
50W	**BG12 (4 mm.)**	
	+ BG38 (4 mm.)	
Tungsten	KP490 + BG38	KP510, K515, K530
Halogen	(4 mm.)	
50W		
100W		

Incident Light Fluorescence:

Light Source	Exciter Filter	Dichroic Beam Splitting Mirror	Barrier Filter
Mercury Vapor	**KP500 + BG38**	**TK-510**	**K510**
200W	**(4 mm.) or BG23 (4 mm.) for stronger red suppression.**		**K520**
100W			
50W	**Edgefilter 450 nm., 480 nm. For narrow band excitation, suppression of tissue auto-fluorescence**		
Tungsten	**KP500 + BG38**	**TK510**	**K510**
Halogen	**(4 mm.)**		**K515**
50W			**K530**
100W			

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SUMMARY OF VIRGO ANA KB IFA

IMPORTANT: It is recommended that one be familiar with the detailed procedure in the package insert before using this summary.

Cover wells with the 1:40 screening dilution of sample or control. For quantitative determination, prepare serial two-fold dilutions.

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Incubate slides in a humidified chamber at room temperature for 30 minutes.

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Rinse slides briefly with PBS. Wash for 15 minutes, changing buffer 1X. Blot slides.

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Cover each well with FITC Conjugate.

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Incubate slides in a humidified chamber at room temperature for 30 minutes.

↓

Repeat wash step described above.

↓

Place a small drop of Buffered Glycerol on each well and cover with a coverslip.

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Read slides immediately at 200-500X magnification on fluorescence microscope.

Antinuclear / Antibody KB/ ANA KB IFA

Immunofluorescence Test Kit for the Detection of Antinuclear Antibodies

FOR *IN VITRO* DIAGNOSTIC USE

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