



HEp-2000[®] IgG FLUORESCENT ANA-Ro TEST SYSTEM

***For in vitro Diagnostic Use
For Professional Use***

INTENDED USE: This is an indirect fluorescent antibody test for the semi-quantitative detection of IgG antinuclear antibody (ANA) in human serum by manual fluorescent microscopy or with the Image Navigator[®] Fluorescence Semi-Automated Microscope. This test system uses transfected[†] HEp-2 cells, which allow specific identification of autoantibodies to the SSA/Ro antigen. Autoantibodies to SSA/Ro may show a distinctive staining pattern on the transfected cells. When this pattern is present, it is considered to be confirmatory evidence that anti-SSA/Ro antibodies are present.

Absence of this distinctive pattern does not rule out the possible presence of anti-SSA/Ro antibodies.

This test system is to be used as an aid in the detection of antibodies associated with systemic rheumatic disease in conjunction with other laboratory and clinical findings. A trained operator must confirm results generated with the Image Navigator[®] semi-automated device and software.

SUMMARY AND EXPLANATION OF THE TEST

Antinuclear antibody (ANA) is a general term used to describe autoantibodies against various cell nuclear proteins. Early studies of these autoantibodies, using immunofluorescent techniques, revealed a select few nuclear protein specificities (1). Because of the high correlation of positive ANA with systemic lupus erythematosus (SLE), a negative ANA essentially ruled out the disease (2).

Although antibodies specific to DNA continue to show a high disease correlation with SLE (3), a number of nuclear (4) and cytoplasmic (5-7) macromolecules have also been detected and associated with other connective tissue diseases (8-10). Some of these antibodies appear to have diagnostic and/or prognostic significance in progressive systemic sclerosis (11-12), mixed connective tissue disease (13-15), Sjögren's syndrome (16-17), polymyositis (18), and/or rheumatoid arthritis (19). Because of these disease associations, ANA testing is now recognized as a general screening tool for connective tissue disease (20).

Sensitivity of the ANA test varies with the type of substrate used, fixative procedure, and types of ANA present in sera. Cell culture substrates generally show greater sensitivity than tissue sections (21-24). Detection of autoantibodies to the SSA/Ro antigen is especially variable. Rodent tissues do not contain detectable levels of SSA/Ro antigen (25), and reports of detection of anti-SSA/Ro antibodies in cell culture substrates vary in sensitivity from 50 to 90% (26-27). The Immuno Concepts HEp-2000[®] ANA Test System with transfected mitotic* human epithelioid cells (HEp-2), represents an advanced immunofluorescent system for detection of ANA.

[†]The transfected cells and their application are protected by U.S. Patent 5,518,881 and other U.S and foreign patents pending.

*Mitosis is a term used to describe the cell division process. It is generally divided into six phases including interphase, prophase, metaphase, anaphase, telophase, and cytokinesis.



HEp-2 cells with mitotic figures have been shown to have greater sensitivity and yield sharper pattern recognition than classical mouse kidney substrate in detecting antibodies in progressive systemic sclerosis (PSS) (28). Mitotic figures aid in differential pattern recognition as well as in detecting previously unreported nuclear antigens present in higher concentrations in mitotically active cells (29-31). The HEp-2 cells in this test system have been transfected with multiple copies of the specific DNA sequence that carries the information for the SSA/Ro autoantigen. Approximately 10-20% of the transfected cells over-express this antigen, so detection of autoantibodies to SSA/Ro is more consistent than it is on HEp-2 cells that have not been transfected. Autoantibodies to SSA/Ro may show a distinctive staining pattern on the transfected cells. When this pattern is present, it is considered to be confirmatory evidence that anti-SSA/Ro antibodies are present.

Absence of this distinctive pattern does not rule out the possible presence of anti-SSA/Ro antibodies.

PRINCIPLE OF THE TEST

The Immuno Concepts Fluorescent ANA Test System uses the indirect fluorescent antibody technique first described by Weller and Coons (32). Patient samples are incubated with antigen substrate to allow specific binding of autoantibodies to cell nuclei. If ANA's are present, a stable antigen-antibody complex is formed. After washing to remove non-specifically bound antibodies, the substrate is incubated with an anti-human antibody conjugated to fluorescein. When results are positive, there is the formation of a stable three-part complex consisting of fluorescent antibody bound to human antinuclear antibody, which is bound to nuclear antigen. This complex can be visualized with the aid of a fluorescent microscope. In positive samples, the cell nuclei will show an apple-green fluorescence with a staining pattern characteristic of the particular nuclear antigen distribution within the cells. If the sample is negative for ANA, the nucleus will not show a clearly discernible pattern of nuclear fluorescence.

SYSTEM COMPONENTS - MATERIALS PROVIDED

Use: All components come ready to use with no aliquoting or reconstitution required (except the PBS buffer which must be dissolved in deionized or distilled water before use).

Storage: All components can be stored under refrigeration at 2-10°C. After reconstitution, PBS buffer should be stored in screw-cap containers and store between 2-25°C.

Stability: All components remain stable, both in the closed kit and after opening, at least 12 months from date of manufacture. Do not use any component beyond its expiration date.

REACTIVE REAGENTS

Substrate Slides [SLIDE]: ANA substrate slides using HEp-2000[®] cells (with mitotic figures) grown and stabilized directly on the test wells. These are HEp-2 cells that have been stably transfected with the SSA/Ro autoantigen. Unique moat slide design minimizes cross contamination of wells during testing. The slide pouch is filled with an inert non-toxic gas that contributes to the stability of the cells. Slides are available with 7 wells, 13 wells, 14 wells, 18 wells, or 21 wells, depending on the needs of the laboratory.

SSA/Ro Positive Control [CONTROL] +: Catalog No. 2035-Ro. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to SSA/Ro antigens. This serum demonstrates the fine speckled staining reaction which is typical of anti-SSA/Ro seen on Immuno Concepts' HEp-2000[®] cell substrate. The expression is predominantly nuclear in localization, with prominent nucleolar staining. Weak cytoplasmic staining may also be seen in the over-expressing cells. The chromosome region of mitotic cells demonstrates a negative staining reaction.

Homogeneous Positive Control [CONTROL] +: Catalog No. 2021. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to DNA and/or DNP nuclear antigens. This serum demonstrates a homogeneous staining reaction on Immuno Concepts' HEp-2000[®] cell substrate. The chromosome region of mitotic cells demonstrates the same homogeneous staining reaction.

Speckled Positive Control [CONTROL] +: Catalog No. 2022. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to Sm and/or RNP nuclear antigens. This serum demonstrates one of the most common speckled staining reactions seen on Immuno Concepts' HEp-2000[®] cell substrate. The chromosome region of mitotic cells demonstrates a negative staining reaction.

Nucleolar Positive Control [CONTROL] +: Catalog No. 2023. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to nucleolar antigens. This serum demonstrates a nucleolar staining reaction on Immuno Concepts' HEp-2000[®] cell substrate.

Centromere Positive Control [CONTROL] +: Catalog No. 2025. Ready-to-use dropper vial containing 1.0 ml positive human control serum with IgG antibody specific to chromosomal centromeres (kinetochore). This serum demonstrates a discrete speckled staining reaction on Immuno Concepts' HEp-2000[®] cell substrate. The chromosome region of mitotic cells demonstrates the same discrete speckled staining reaction.

Titrateable Control Serum [TC]: Catalog No. 2026. Ready-to-use vial containing 0.5 ml positive human control serum with IgG antibodies to be treated as an undiluted patient sample.

Negative Control Serum [CONTROL] -: Catalog No. 2031. Ready-to-use dropper vial containing 1.0 ml negative human control serum. Although the negative control serum may demonstrate weak fluorescence of the cytoplasm with brighter staining of the nonchromosome region of the mitotic cell, it demonstrates no discernible pattern of nuclear staining.

Fluorescent Antibody Reagent [CONJ|FITC]: Catalog No. 2009CS (9.0 ml), 2009G-Ro (9.0 ml), 2009GCS-Ro (9.0 ml), 2075CS (23 ml), 2075G-Ro (23 ml), 2075GCS-Ro (23 ml), 2009B (9.0 ml), 2075B (23 ml). Anti-human IgG (gamma chain specific) conjugated to fluorescein isothiocyanate (FITC). Reagent comes ready-to-use in precision dropper bottles with 9.0 ml for each 10 slides in complete test kits.

NON-REACTIVE COMPONENTS

PBS Buffer Powder [PWDR|PBS]: Catalog No. 1011. Phosphate-buffered saline powder (0.01 M, pH 7.4 ± 0.2). Each pouch contains sufficient buffer powder to make 1 liter. (One pouch of buffer powder is supplied for each five slides in complete test kits.)

Preparation: Dissolve one pouch of buffer powder in 1 liter of deionized or distilled water, cover, and store between 2-25°C for up to four weeks or until signs of contamination or other visible changes occur.

Semi-Permanent Mounting Medium [SOLN|MM]: Catalog No. 1111. Ready-to-use dropper vial containing 5.0 ml glycerol-based mounting medium.

Coverslips [CVSLP]: Catalog No. 1042. Each packet contains ten 24 x 64 mm No. 1 glass coverslips.

ADDITIONAL MATERIALS REQUIRED - BUT NOT PROVIDED

Volumetric pipettes to deliver 20-25 µl volumes
Coplin jars or staining dishes
Squeeze bottle or Pasteur pipettes
Serological pipettes
One-liter containers (for PBS buffer)
Deionized or distilled water
Test tubes to prepare serum dilutions
Petri dish or other chamber for incubation
Bibulous paper or paper towels
Disposable gloves
Lab timer
Fluorescent microscope equipped with 495 nm exciter filter and 515 nm barrier filter

PRECAUTIONS

1. All human source materials used for this product have been tested and found to be negative (not repeatedly reactive) for antibodies to Human Immunodeficiency Virus-1 (HIV-1), Human Immunodeficiency Virus-2 (HIV-2), hepatitis C virus (HCV), and for hepatitis B surface antigen (HBsAg) by FDA approved methods. However, no test method can offer complete assurance that HIV-1, HIV-2, hepatitis C, hepatitis B, or other infectious agents are absent. Thus, all kit materials should be handled in the same manner as potentially infectious materials.

2. All patient samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual: *Biosafety in Microbiological and Biomedical Laboratories, 1999 Edition*.
3. Dilution of the components or substitution of components other than those provided in this system may yield inconsistent results.
4. Sodium azide (0.09%) is used as a preservative. Sodium azide may react with lead or copper plumbing and form explosive metal azide salts. When disposing of reagents, flush with ample volumes of tap water to prevent potential residues in plumbing. Sodium azide is a poison and may be toxic if ingested.
5. This kit is for *in vitro* diagnostic use.
6. In the event hemolyzed or lipemic sera must be used, heat inactivate sera 30 minutes at 56°C for optimal results. Microbially contaminated sera should not be used.
7. The titratable control serum is intended for use in monitoring lot-to-lot and run-to-run reproducibility. It is not intended as a measurement of overall sensitivity or specificity of the assay.
8. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
9. Avoid splashing or generation of aerosols at all times.
10. Incubation times and temperatures other than those specified may give erroneous results.
11. Cross contamination of reagents or samples may give false results.
12. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
13. Bring all reagents, slides, and specimens to room temperature (18-25°C) prior to use.
14. Wear disposable gloves when handling specimens and reagents, and wash hands thoroughly afterwards.
15. Microbial contamination of reagents or samples may give false results.
16. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidal soap and copious amounts of water.

SPECIMEN COLLECTION

Collection: Serum is the preferred specimen. Approximately 5 ml of whole blood should be collected aseptically by venipuncture using a sterile vacuum collection tube or other suitable collection system. Allow blood to clot at room temperature (18-25°C). Serum should be separated from the clot by centrifugation as soon as possible to minimize hemolysis.

Interfering Substances: Sera exhibiting a high degree of hemolysis, icterus, lipemia, or microbial growth should not be used because these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Storage: Sera may be stored at 2-10°C up to one week. If testing is further delayed, sera should be stored frozen at -20°C or lower. Serum should not be stored in a self-defrosting refrigerator or freezer.

CAUTION: Repeated freeze/thawing of patient samples may yield false positive or false negative results.

INTERPRETATION OF RESULTS

QUALITY CONTROL

Positive, negative, and PBS controls should be tested one time per run. The positive control should show bright apple-green fluorescence in the nuclei of the cells, with a clearly discernible pattern characteristic of the control serum that was used. The negative control should show low intensity, nonspecific dull green fluorescence in both the cytoplasm and nucleus, but with no discernible pattern of nuclear staining. The PBS control is used to observe non-specific staining by the antibody reagent, and should not exhibit any green fluorescence. If the controls do not appear as described, the test is invalid and should be repeated. The five pattern controls should be run at least once for each lot of kits to demonstrate the expected appearance of the ANA patterns. If the HEp-2000[®] ANA test is to be used for confirmation of the presence of anti-SSA/Ro antibodies, the SSA/Ro Positive Control, catalog number 2035-Ro, must be run on at least one slide in that day's run.

OPTIONAL TITRATABLE CONTROL

When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read "backwards" to the 1:40 dilution. The first well in which a clearly discernible nuclear staining pattern is visible is the titer end-point. We recommend this technique for determining titer end-points.

The mean titer and titer range (\pm one dilution on either side of the mean) determined for this lot number were established in our laboratory and are stated as a guide. This control is provided to allow each laboratory to assess the reproducibility (precision) of its ANA testing. Since this control is not intended to be an indicator of titer accuracy, each laboratory should establish its own mean titer end-point for this sample, and should use this information to assess run-to-run reproducibility (precision).

Through multiple testing of this titratable control, using the Immuno Concepts Fluorescent ANA Test System, a mean titer value has been established for each lot number. The lot number, mean titer and titer range (\pm one twofold dilution on either side of the mean) are stated on the vial label and should be used as a guide for the test system performance.

It is important that the intensity of fluorescence not be confused with the presence or absence of antinuclear antibodies. The key factor to consider in determining whether a given dilution of serum is positive is the appearance of a clearly discernible pattern, irrespective of the intensity of the fluorescent staining.

This titratable control will show the typical speckled pattern associated with the RNP antibody. Also present may be a second pattern of MND (several discrete speckles in the nucleus of interphase cells), however, it is the typical RNP speckled pattern that is to be used for the purpose of reading end-point.

The values obtained in our laboratory may differ from your values. Some of the many factors that can affect your results may include, but are not limited to:

1. The type of light source used. Mercury light sources will produce greater excitation energy at 495 nm than Quartz/Halogen. The 50-watt, 100-watt, and 200-watt mercury light sources differ little in excitation energy at 495 nm. The 100-watt Quartz/Halogen light sources will produce greater excitation energy at 495 nm than 50-watt Quartz/Halogen.
2. The condition and age of the light source. This is particularly true for mercury light sources, which generally exhibit a gradual reduction in excitation energy at 495 nm prior to burning out. This gradual reduction in excitation energy can result in a significant loss in sensitivity over several weeks. This problem can be avoided by keeping a time log. For best results, replace 50-watt mercury bulbs at 100 hours, and 100 or 200-watt mercury bulbs at 200 hours. Quartz/Halogen light sources generally do not exhibit a gradual reduction in excitation energy prior to burning out.
3. The type of exciter filter used. Interference exciter filters provide greater sensitivity over a much narrower wavelength than absorption exciter filters. Refer to your fluorescent microscope manual or sales representative for more information.
4. Proper alignment of the microscope light path. Refer to your fluorescent microscope manual for instructions.
5. The numerical aperture of the objective. With incident light fluorescence (Epi), fluorescence is increased exponentially as the numerical aperture (NA) of the objective is increased additively. This may cause a 40X objective with a NA of 0.65 to read one or more dilutions lower than a 40X objective with a NA of 0.85. The numerical aperture is printed on the side of the objective. Sensitivity of transmitted light fluorescent microscopy is not affected by NA.
6. Suppression filters. Suppression filters reduce specific wavelengths of excitation and may be used in reducing sensitivity. Refer to your fluorescent microscope manual or sales representative for more information.
7. Precision and accuracy of dilution technique, equipment, and performance of the test procedures.

INTERPRETATION OF PATIENT RESULTS

200X total magnification is recommended for screening positive/negative and titer end-point determination, while 400X total magnification is recommended for pattern recognition and viewing mitotic cells.

Negative: A serum is considered negative for antinuclear antibodies if nuclear staining is less than or equal to the negative control well with no clearly discernible pattern. The cytoplasm may demonstrate weak staining, with brighter staining of the nonchromosome region of mitotic cells, but with no clearly discernible nuclear pattern.

Positive: A serum is considered positive if the nucleus shows a clearly discernible pattern of staining in a majority of the interphase cells.

SSA/Ro: A serum is considered positive for SSA/Ro antibodies if 10-20% of the interphase nuclei show the distinctive SSA/Ro staining pattern, which appears as a distinct bright speckled pattern with prominent staining of the nucleoli. These are the over-expressing transfected cells. The remaining 80-90% of the interphase nuclei may or may not demonstrate a fine speckled staining of the nucleus with or without fluorescent staining of the nucleoli.

Titers: When reading titers, many laboratories begin reading with the well that contains the most dilute sample and read “backwards” to the 1:40 dilution. The first well in which a clearly discernible pattern is visible is the titer end-point. We recommend this technique for determining titer end-points. It is important that the intensity of staining not be confused with the presence or absence of antinuclear antibodies. The key factor to consider in determining whether a given dilution of serum is positive is the appearance of a clearly discernible nuclear pattern, irrespective of the staining intensity.

Due to the increased concentration of SSA/Ro antigen in the over-expressing cells, it is not unusual to see staining of these cells in very high titers. The clinical significance of these high titers is unknown.

CAUTION: Some sera may demonstrate nuclear and cytoplasmic staining with no apparent nuclear pattern. This phenomenon is generally due to heterophile antibodies and should be reported as negative (33).

FLUORESCENT INTENSITY

Fluorescent intensity may be semi-quantitated by following the guidelines for fluorescent antibody reagents established by the Centers for Disease Control and Prevention, Atlanta, Georgia (CDC).

- 4+ Brilliant yellow-green (maximal fluorescence): clear-cut cell outline; sharply defined cell center.
- 3+ Less brilliant yellow-green fluorescence: clear-cut cell outline; sharply defined cell center.
- 2+ Definite cell pattern but dim fluorescence: cell outline less well defined.
- 1+ Very subdued fluorescence: cell outline almost indistinguishable from cell center in most instances.

A standard slide for the determination of these fluorescent intensities, FITC QC Slide™, catalog number 1900, is available from Immuno Concepts, N.A. Ltd.

REPORTING OF RESULTS

Screening: Results should be reported as positive or negative at the 1:40 dilution, and the nuclear staining pattern should be reported for positive samples.

Titering: Results should be reported as the last serial dilution in which clearly discernible staining is seen. Results with a strong reaction at the highest dilution should be reported as greater than that dilution. Titers of 1:40 to 1:80 are considered low titers; 1:160 to 1:320 are considered medium titers; and 1:640 and greater are considered high titers. It is not necessary to determine the titer endpoint. Any ANA titer greater than or equal to 1:640 is considered a high titer, and will alert the clinician to do additional testing. Each laboratory should establish its own titering scheme, based on the antibodies that are detected in the patient population.

PATTERN DETECTION

Homogeneous: A solid staining of the nucleus with or without apparent masking of the nucleoli. The chromosome region of metaphase mitotic cells is clearly positive with a smooth or peripheral staining intensity greater than, or equal to, interphase nuclei.

Synonyms: Diffuse; solid.

Nuclear antigens: dsDNA; nDNA; DNP; histone.

Disease association: High titers are suggestive of SLE. Lower titers are suggestive of SLE or other connective tissue diseases (34).

Peripheral: A solid staining, primarily around the outer region of the nucleus, with weaker staining toward the center of the nucleus. The chromosome region of metaphase mitotic cells is clearly positive with a smooth or peripheral staining intensity greater than, or equal to, interphase nuclei.

Synonyms: Rim, shaggy, membranous.

Nuclear antigens: dsDNA, ssDNA, nDNA, DNP, histone.

Disease association: High titers suggestive of SLE; lower titers suggestive of SLE or other connective tissue diseases (34).

Speckled: A coarse or fine granular staining of the nucleus generally without fluorescent staining of the nucleoli. The non-chromosome region of metaphase mitotic cells demonstrates staining, while the chromosome region is negative.

Nuclear antigens: Sm; RNP; Scl-70; SSA/Ro; SSB/La; and other antigen/antibody systems not yet characterized.

Disease association: High titers are suggestive of SLE (Sm antigen), mixed connective tissue disease (RNP antigen), scleroderma (Scl-70 antigen), or Sjögren's syndrome-sicca complex (SSA/Ro or SSB/La antigen). Lower titers may be suggestive of other connective tissue disease (35).

Nucleolar: Large coarse speckled staining within the nucleus, generally less than 6 in number per cell, with or without occasional fine speckles, 5-10 in number. The nonchromosome region of metaphase mitotic cells demonstrates strong staining, while the chromosome region may demonstrate faint staining. Anaphase and telophase cells may demonstrate similar staining to interphase nuclei.

Nuclear antigens: Generally referred to as 4-6s RNA's and other nuclear antigens such as fibrillarin, RNA Polymerase I, NOR 90, and PM/Scl.

Disease association: High titers prevalent in scleroderma and Sjögren's syndrome (36).

Centromere: A discrete speckled staining pattern highly suggestive of the CREST[§] syndrome variant of progressive systemic sclerosis (28). The nuclear speckles are very discrete and usually some multiple of 46 (usually 23-46 speckles per nucleus). Because centromeres are constrictions where spindle fibers attach on chromosomes, mitotic cells will show the same speckling reaction in the chromosome region (12).

Synonyms: ACA; discrete speckled.

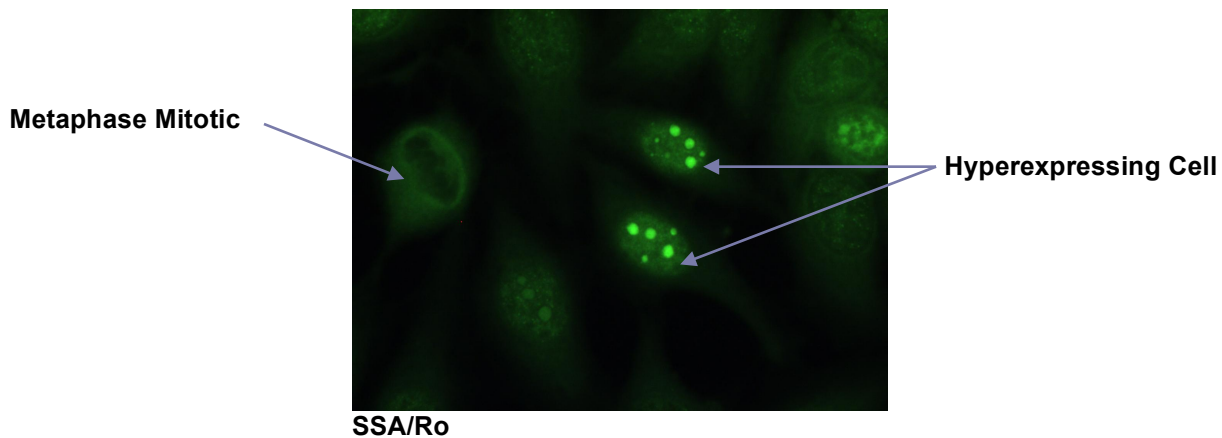
Nuclear antigens: Chromosomal centromere (Kinetochore).

Disease association: Highly suggestive of the CREST syndrome variant of progressive systemic sclerosis (28)

SSA/Ro: A distinct bright speckled pattern with prominent staining of the nucleoli in 10-20% of the interphase nuclei. These are the over-expressing transfected cells. The remaining 80-90% of the interphase nuclei may or may not demonstrate a fine speckled staining of the nucleus with or without fluorescent staining of the nucleoli. The nonchromosome region of metaphase mitotic cells demonstrates staining, while the chromosome region is negative.

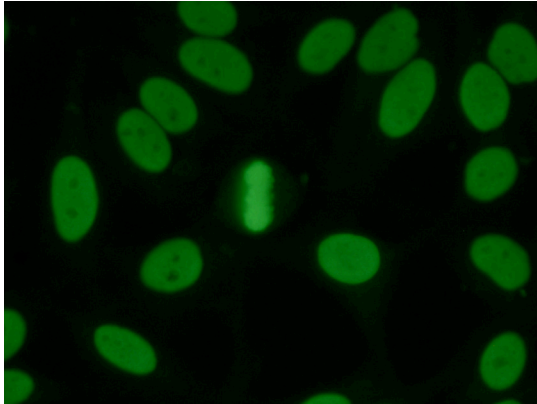
Nuclear antigens: SSA/Ro (60kD).

Disease association: Seen in 60-70% of patients with primary Sjögren's Syndrome, 30-40% of patients with SLE, and greater than 95% of patients with subacute cutaneous lupus (37).

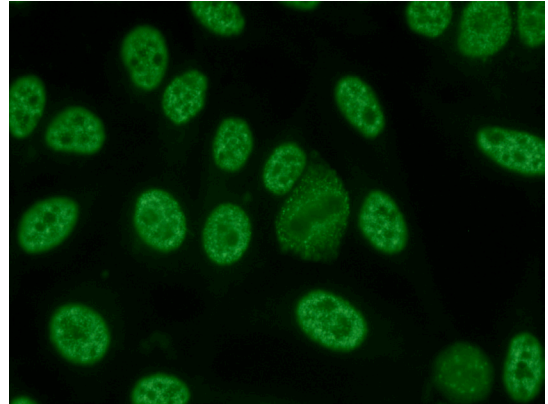


[§]CREST is a form of PSS with prominent calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, and telangiectasia.

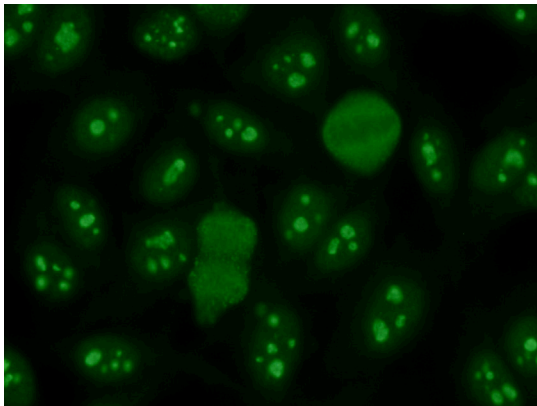
BASIC STAINING PATTERNS



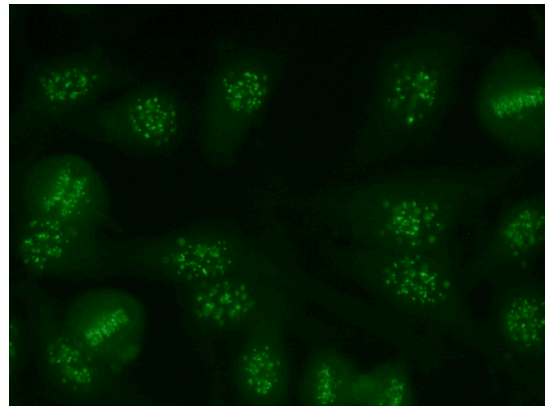
Homogeneous



Speckled



Nucleolar



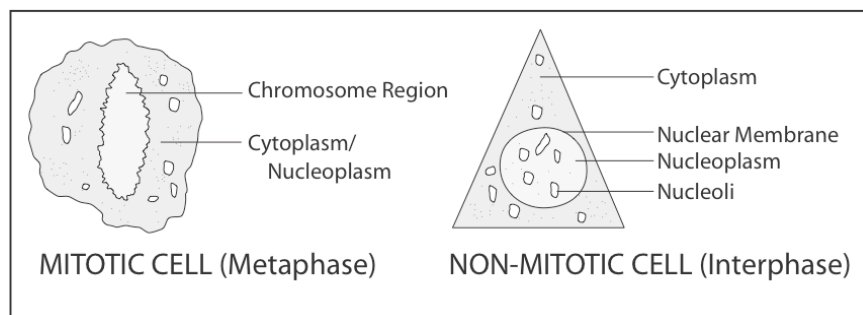
Centromere

MITOTIC CELLS

DETECTION

Mitotic cells should be visible on every field when viewed at 200X magnification or lower. To verify whether a cell is in mitosis, view at 400X magnification. Mitotic cells show a characteristic round cell shape with no detectable nuclear membrane. The chromosome region of mitotic cells will generally show an irregular shape within the cell, due to the lack of nuclear membrane, and extreme constriction of the chromosomes.

Sera positive for DNA and/or DNP and/or histone (such as the Immuno Concepts homogeneous positive control) will show bright staining of the chromosome region of these cells. In samples negative for DNA and/or DNP and/or histone (such as the Immuno Concepts speckled positive control), the mitotic cells will not show chromosome staining and may be difficult to see.



USE OF MITOTIC CELLS

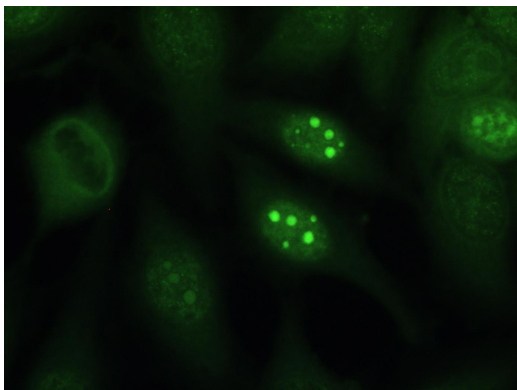
Discerning Speckled vs. Homogeneous Antibody: A fine speckled pattern of staining is sometimes difficult to differentiate from homogeneous staining. If the pattern is homogeneous, there will be solid staining of the chromosomes of the mitotic cells. If the pattern is strictly speckled, the region outside of the chromosomes will show a fine speckled reaction.

NOTE: If fine speckling of the entire mitotic cell occurs along with solid staining of the chromosome region, it is highly probable that two or more antibodies are present. Report the screening dilution as speckled/ homogeneous and titer each antibody to endpoint.

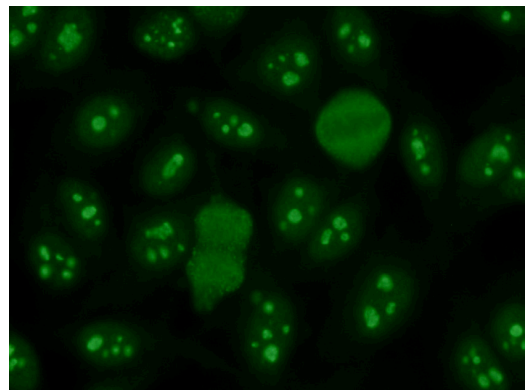
Peripheral vs. Nuclear Membrane Antibody: Antibody that shows a peripheral pattern is generally associated with DNA/DNP nuclear antigens. High titers of these antibodies are suggestive of SLE. In substrates that do not include mitotic cells, the peripheral pattern can be difficult to distinguish from nuclear membrane antibody. By using Immuno Concepts' mitotic cells, these patterns can be differentiated, because the chromosome region of the mitotic cells will be stained intensely in a peripheral pattern, but will not be stained by nuclear membrane antibody. This distinction is clinically important because nuclear membrane antibody does not have DNA/DNP specificity and is not associated with SLE (38).

Anti-Centromere Antibody (ACA) vs. Atypical speckled antibody resembling Centromere: In order to verify anti-centromere antibody, the chromosome region of the mitotic cells should stain brightly with discrete speckles. If the chromosome region does not stain, the antibody is not anti-centromere, and should be reported as "atypical speckled" (39).

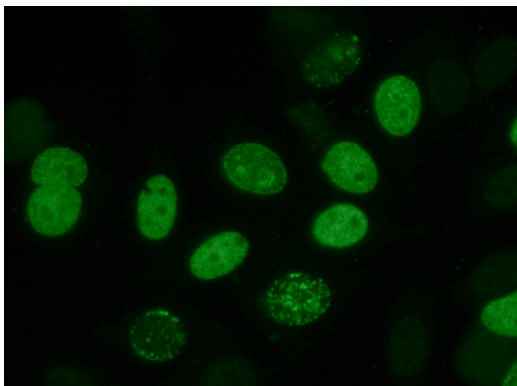
SSA/Ro vs. Patterns which may resemble SSA/Ro staining: The distinctive SSA/Ro staining is seen as a distinct bright speckled pattern with prominent staining of the nucleoli in 10-20% of the interphase nuclei. The remaining 80-90% of the interphase nuclei may or may not demonstrate a fine speckled staining of the nucleus with or without fluorescent staining of the nucleoli. The chromosome region of metaphase mitotic cells does not demonstrate staining. The nucleolar pattern can be differentiated by large coarse speckled staining in all of the nuclei, generally fewer than 6 per cell. The Scl-70 pattern shows fine speckled staining and nucleolar staining in all of the interphase nuclei and staining of the chromosomal region of the metaphase mitotic cells. Antibodies to Proliferating Cell Nuclear Antigen (PCNA) show variable coarse and fine speckling in 30-50% of the interphase nuclei.



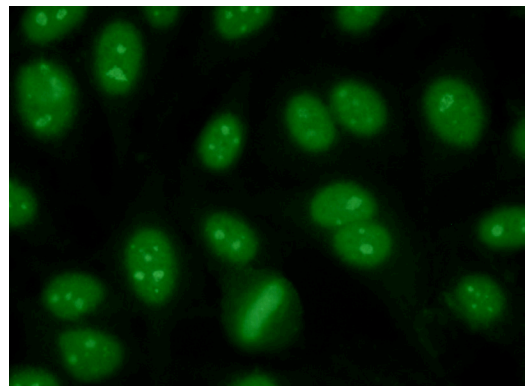
SSA/Ro



Nucleolar



PCNA



Scl-70

CYTOPLASMIC FLUORESCENCE

Although autoantibodies to cytoplasmic antigens are not commonly associated with connective tissue disease, these antibodies may be detected using epithelial cell culture substrates (40). Mitochondrial and smooth muscle antibodies are the two most commonly detected antibodies and are generally associated with mononucleosis, chronic active hepatitis, and liver disease (41-42). Using the HEp-2 cell substrate, smooth muscle antibody has also been demonstrated in patients with warts (43).

Anti-Mitochondrial Antibody (AMA): Discrete speckles concentrated in the perinuclear region of the cell and extended in lower density to the outer regions of the cytoplasm. This should be distinguished from anti-Golgi antibody, which generally stains only one side of the perinuclear region, and from anti-ribosomal antibody, which demonstrates finer speckles with a strand like appearance consistent with the location of the endoplasmic reticulum within the cell (50).

NOTE: Perinuclear speckles can most easily be distinguished from peripheral nuclear staining by noting that the mitochondrial speckles form an interrupted speckled staining around the outside of the nuclear membrane, while peripheral sera form a solid smooth staining inside the nuclear membrane.

REPORT SERA AS NEGATIVE FOR ANTINUCLEAR ANTIBODIES AND VERIFY POSITIVE FOR ANTIMITOCHONDRIAL ANTIBODY ON AMA SPECIFIC SUBSTRATE.

Anti-Smooth Muscle Antibody (ASMA): Very fine fibrous staining over the entire cytoplasm of cells with a "spiderweb" appearance. Unlike mitochondrial antibody, smooth muscle antibody staining is uniform over the entire cytoplasm and may also extend over the nucleus.

Mitotic cells generally show large discrete speckles outside the chromosome region (50). Smooth muscle antibody has been shown to have a high specificity to actin (44-45).

REPORT SERA AS NEGATIVE FOR ANTINUCLEAR ANTIBODY AND VERIFY POSITIVE FOR ANTI-SMOOTH MUSCLE ANTIBODY ON ASMA SPECIFIC SUBSTRATE.

LIMITATIONS OF THE TEST

1. Diagnosis cannot be made on the basis of antinuclear antibody detection alone. The physician must interpret these results in conjunction with the patient's history and symptoms, the physical findings, and other diagnostic procedures.
2. Treatment should not be initiated on the sole basis of a positive test for antinuclear antibodies. Clinical indications, other laboratory findings, and the physician's clinical impression must be considered before any treatment is initiated.
3. Certain drugs, including procainamide and hydralazine, may induce a lupus erythematosus-like disease (46). Patients with drug-induced LE may demonstrate positive homogeneous or homogeneous/peripheral ANAs commonly directed against nuclear histones (47).
4. A small percentage of patients with SLE may not demonstrate ANAs by indirect immunofluorescence but may have ANAs by other techniques (48).
5. It is not necessary to determine the titer endpoint. Any ANA titer greater than or equal to 1:640 is considered a high titer, and will alert the clinician to do additional testing. Each laboratory should establish its own titration scheme, based on the antibodies that are detected in the patient population. Although a high-titered ANA may be highly suggestive of connective tissue disease, it should not be considered diagnostic but rather viewed as a part of the overall clinical history of a patient.
6. Staining patterns often change with progressive titration of sera. This phenomenon is generally due to the presence of more than one nuclear antibody.
7. Because of the many options available in fluorescent microscopes, it is recommended that light sources, filters, and optics be standardized when comparing patient titers between laboratories.
8. Positive ANAs are also seen in a small percentage of patients with infectious and/or neoplastic diseases (9).
9. Autoantibodies to SSA/Ro show a distinctive staining pattern on the transfected cells. When this pattern is present, it is considered to be confirmatory evidence that anti-SSA/Ro antibodies are present. Absence of this pattern does not rule out the possible presence of anti-SSA/Ro antibodies.

10. Because of the overexpression of the SSA/Ro autoantigen in the HEp-2000[®] cells, samples that contain anti-SSA/Ro antibodies show higher titer values on these cells than the values obtained on non-transfected HEp-2 cells. Since none of the other autoantigens in the HEp-2000[®] cells are affected by the transfection process, sera with other autoantibody specificities do not show significant titer differences between the transfected HEp-2000[®] cell line and non-transfected HEp-2 cells.

EXPECTED VALUES

In a large university medical center, using HEp-2 cell ANA substrate, the following data were generated over a two-year period (49). Table 1.

TABLE 1

Diagnosis	Pattern Segregation	% Positive
Abnormal Population (Over 4,500 Sera Tested):		
Systemic lupus erythematosus	S, P+H, H, P	93
Rheumatoid arthritis	S, H	40
Mixed connective tissue disease	S	99
Progressive systemic sclerosis-diffuse	S, N	85
Progressive systemic sclerosis-CREST	ACA	93
Juvenile rheumatoid arthritis		
Systemic	S	14
Polyarticular	S	13
Pauciarticular-B27+	-	0
DM/PM	S	25
Vasculitis	S	20
Normal Population (Over 9,000 Sera Tested):		
20-60 Years	S	2
70-80 Years	S	3.5

Abbreviations: S=Speckled, H=Homogeneous, P=Peripheral, N=Nucleolar, ACA=anti-Centromere

REFERENCE RANGE

The reference range for detection of antinuclear antibodies in the normal population is "Negative". As shown in the Table above, a small percentage of normal individuals will exhibit a positive ANA. Each laboratory should establish and maintain its own reference (normal) range values, based on the patient population and other local factors.

PERFORMANCE CHARACTERISTICS

The Immuno Concepts IgG Fluorescent ANA-Ro Test System was compared to another ANA-Ro fluorescent test system that is in commercial distribution. The population studied consisted of 113 samples which were submitted to clinical laboratories for ANA testing. All samples were tested in parallel on the predicate device and the subject device. Based on this comparison, the following data were obtained:

		Predicate ANA-Ro Test	
		Positive	Negative
Immuno Concepts IgG ANA-Ro Test	Positive	41	3
	Negative	0	69

These data yield the following statistics: relative sensitivity, 100%; relative specificity, 95.8%; positive predictive value, 93.2%; negative predictive value, 100%; and overall agreement, 97.3%

USE WITH THE IMAGE NAVIGATOR[®]

The Image Navigator[®] is Immuno Concepts' semi-automated microscope system for reading the HEp-2000[®] Fluorescent ANA slides. In a comparison of the Image Navigator[®] with conventional reading of fluorescent ANA slides, the following data were obtained:

Positive agreement, 99.8%; negative agreement, 98.9%; overall agreement, 99.4% (n=739 samples).

Clinical sensitivity for detection of ANA in patients known to have Systemic Lupus Erythematosus was 93.8% for conventional reading and 94.1% for the Image Navigator[®]. In 301 patients with known connective tissue diseases, the clinical sensitivity was 82.7% for conventional reading and 83.1% for the Image Navigator[®].

In a group of 296 patients with diseases other than connective tissue diseases and 142 healthy control subjects (total n=438), clinical specificity was shown to be 87.3% for conventional reading and 87.4% for the Image Navigator[®].

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Cat E2000G-Ro-I, 4.11.02.003.110-En

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HEP-2000® IGG FLUORESCENT ANA-Ro TEST PROCEDURE

NOTE: If the laboratory is using an automated sample processing system, the procedure and recommendations of the manufacturer of the processor should be followed. The slide processing system should be programmed for the appropriate sample dilutions, dispense volumes, and incubation times as outlined below.

- 1. RECONSTITUTE BUFFER (PBS)**
Dissolve contents of one buffer pouch in one liter of deionized or distilled water. The PBS buffer may be covered and stored at 2-25°C up to four weeks.
- 2. DILUTE PATIENT SAMPLES**
Screening: Dilute patient samples to 1:40 by adding 0.05 ml (50 µl) serum to 1.95 ml reconstituted PBS. *
Semi-Quantitative Titering: Make serial dilutions of screening sample(s) (e.g. 1:80, 1:160, 1:320...etc.) using PBS.
- 3. PREPARE SUBSTRATE SLIDES (20-25 µl/well)**
Remove slide(s) from pouch(es) and place control sera on control wells as follows: Invert control dropper bottle and squeeze gently until drop is visible at the tip. Gently touch the drop to appropriate control well while avoiding direct contact of dropper tip with slide surface. Add 1 drop (20-25 µl) patient sample to the numbered wells.
NOTE: For general screening, the homogeneous positive control is recommended. For semi-quantitative titering, the Titratable Control, catalog number 2026, should be run with each batch of patient samples. All pattern controls should be run with each lot number of kits to demonstrate the expected appearance of the ANA patterns. If the HEP-2000® ANA test is to be used for confirmation of the presence of anti-SSA/Ro antibodies, the SSA/Ro Positive Control, catalog number 2035-Ro, must be run on at least one slide in that day's run.
CAUTION: DIRECT CONTACT OF DROPPER TIP WITH SLIDE SURFACE MAY RESULT IN DAMAGE TO THE ANTIGEN SUBSTRATE.
- 4. INCUBATE SLIDES (30 ± 5 minutes at room temperature, i.e. 18-25°C)**
Place slide(s) into a moist covered chamber (a petri dish with moistened paper toweling will be adequate). Incubate, with lid in place, for 30 minutes (± 5 minutes) at room temperature (18-25°C).
- 5. PBS RINSE**
Remove slide(s) from incubator tray and rinse briefly with PBS using a squirt bottle, Pasteur, or serological pipette. Do not squirt buffer directly on the wells.
- 6. PBS WASH (10 minutes)**
Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended 10-30 minutes with no variability in final test results. Discard PBS wash solution after use.
- 7. FLUORESCENT ANTIBODY REAGENT (Cover the wells with 12-14 drops)**
Remove one slide at a time from PBS and dip 3-5 times in deionized or distilled water. Tap slide on its side against bibulous paper or paper toweling to remove excess water. Immediately return slide to the incubation chamber and cover the wells completely using fluorescent antibody reagent; begin by placing a drop over each well. Repeat for each slide. Fluorescent antibody reagent has been titered to compensate for residual deionized or distilled water remaining on the slide after rinsing.
NOTE: It is important that slide wells do not dry during this procedure or damage to the substrate may occur.
- 8. INCUBATE SLIDES (30 ± 5 minutes at room temperature, i.e. 18-25°C)**
Place lid on incubation chamber and cover with a paper towel to prevent exposure to light if the chamber is not opaque. Allow slide(s) to incubate 30 minutes (± 5 minutes) at room temperature (18-25°C).
- 9. PBS RINSE**
Remove slide(s) from incubator tray and rinse briefly with PBS. Do not squirt buffer directly on the wells.
- 10. PBS WASH (10 minutes)**
Wash slide(s) 10 minutes with PBS in a slide staining dish or Coplin jar. This wash may be extended 10-30 minutes with no variability in final test results when counterstain is not used.
Optional Counterstain: Add 5-10 drops counterstain (0.5% Evans blue) per 100 ml PBS prior to slide immersion. Because the degree of counterstaining desired may vary among individuals, counterstaining intensity may be increased or decreased by simply adjusting the number of drops added to the PBS in this wash.
- 11. MOUNT COVERSLIP**
Remove one slide at a time from PBS and dip 3-5 times in deionized or distilled water (Optional). Tap slide on its side against bibulous paper or paper toweling to remove excess water.
DO NOT BLOT OR DRY THE SLIDE IN ANY MANNER OR ALLOW TO SIT WITHOUT COVERSLIP FOR LONGER THAN 15 SECONDS. Add 4-5 drops of semi-permanent mounting medium along midline of each slide. Carefully place coverslip in position, avoiding air pockets, by gently lowering coverslip from one end of the slide to the other.
NOTE: Excess mounting medium on slide may result in high background fluorescence, due to light scattering, or lack of clear resolution of cells (blurred image). Excess mounting medium may be removed from slide by gently blotting coverslip with bibulous or lens paper while avoiding any direct movement of the coverslip.
- 12. READ SLIDE**
If the slide is to be read using conventional fluorescent microscopy, it can be read immediately, using the standard procedure of the laboratory. Slides must always be read by a trained user who is familiar with fluorescent ANA interpretation and patterns. Consult the supplier of the fluorescent microscope for instructions for use of the microscope.

If the slide is to be read using the Image Navigator®, Immuno Concepts' automated microscopy and image capture system, consult the Operator's Manual for the Image Navigator® system. The Image Navigator® must only be operated by a trained user.

FOR TECHNICAL ASSISTANCE:
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Email: technicalsupport@immunoconcepts.com

* Laboratories that want to screen at a dilution other than the recommended dilution must validate the use of the alternative screening dilution in their patient population.