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| Indirect Fluorescent Antinuclear Antibody Testing (FANA) |
| **Purpose** | This is an indirect fluorescent antibody test for the semi-quantitative detection of antinuclearantibody in human serum. |
| **Policy Statements** | • This procedure applies to all laboratory technologists performing Immunology testing, the sectionsupervisor, and section pathologist. |
| **Principle and Clinical Significance** | The Anti Nuclear Antibody (ANA) test is based on indirect immunofluorescence. Serum is incubated on a slide containing a monolayer of human epithelial cells ( Hep-2 cell line ). If the antibody is present, it binds to the cell nuclei. Bound antibody is then detected by adding fluorescent antihuman IgG. Positive cells demonstrate a bright green fluorescence with a distinct staining pattern. Positive samples are then diluted and both the fluorescent pattern and titer are reported. The titer is the highest dilution of serum that still shows immunofluorescent nuclear staining.Antinuclear antibodies (ANA) are a central feature of Systemic Lupus Erythematosis (SLE) and related rheumatic diseases, presently called the connective tissue diseases. ANA’s are thought to be directly involved in the pathogenesis of these disorders. The antinuclear antibody (ANA) test provides good sensitivity for SLE, and therefore is often used to screen for autoimmune rheumatic diseases. Nearly all patients with active, untreated SLE have a positive ANA, and a negative ANA effectively rules out SLE.  |
| **Test Code** | FANA (Orderable by Laboratory only)  |
| Materials |

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| **Reagents** | **Supplies** | **Equipment** |
| **HEp-2000 Substrate** **slides** – ImmunoConcepts Catalog # 2007-Ro(7-well) or 2013-Ro(13-well) ANA substrate slides. These are HEp-2 cells that have been stablytransfected with Anti–Sjogren’s Syndrome related antigen (SSA (Ro) antigens).**SSA (Ro) Positive Control**. Ready-to-usedropper vial containing 1.0 ml positive human control with antibody specific to SSA antigens.**Homogeneous Positive control**.Ready-to-use dropper vial containing 1.0 ml positive human control serum with1. antibody specific to deoxyribonucleic acid or deoxyribonucleoprotein

(DNA and/or DNP) nuclear antigens.**Speckled Positive Control**.Ready-to-use dropper vial containing0.5 ml positive human controlserum with antibodies specific to Smand/or Ribonucleoprotein (RNP) nuclear antigens.**Nucleolar Positive Control**Ready-to-use dropper vial containing0.5 ml positive human controlserum with antibody specific tonucleolar antigens.**Centromere Positive Control**.Ready-to-use dropper vial containingantibody specific to chromosomalcentromere (kinetochore).**Negative Control serum**.Ready-to-use dropper vial containing1.0ml negative human control serum. It demonstrates no discernablepattern of nuclear staining.**Titratable Control Serum**. Ready touse vial containing 1.0 ml positive humancontrol serum to be treated as an undilutedpatient sample.**Fluorescent Antibody Reagent**. Goat anti-human IgG (heavy and lightchains) conjugated to fluoresceinisothiocyanate (FITC). Reagent comesready-to-use in dropper bottles with9.0 ml for each of the 10 slides incomplete test kits.**Phosphate-buffered saline powder (PBS)*** 1. M, pH 7.4 + 0.2). Each pouch
	2. contains sufficient powder to make
	3. 1 liter.

**Preparation**: Dissolve one pouch ofpowder in 1 liter of deionized or distilledwater, cover and store refrigerated at 2-10° Cfor up to 4 weeks or until signs ofcontamination or other visible changes occur.**Semi**-**Permanent Mounting Medium**.Ready-to use dropper vial containing 5.0 mlglycerol-based mounting medium, pH 9.1 + 0.2. | Pasteur pipettes12 X 75 test tubesDeionized or distilledWater1 liter containers for PBSBibulous paper or paperTowelsVolumetric PipettesIncubator traySlide washing chambersTimerSqueeze bottle of PBSCoverslips.( 24 X 60 mm)  | Fluorescent microscope equipped with 495 nm exciter filter and 515 nm barrier filter |

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| Sample**Quality Control** |  Serum, collected by aseptic techniques into a red top Vacutainer or other suitable tubewithout anticoagulant. Allow blood to clot at room temperature. Separate serum from clot as soon as possible to minimize hemolysis.Interfering Substances: Sera exhibiting a high degree of hemolysis, icterus, or microbial growthshould not be used because these conditions may cause aberrant results. Specimenscontaining visible particulate matter should be clarified by centrifugation before testing.Storage: Sera may be stored at 2-10° C up to 1 week. If testing is further delayed, sera should be frozen at -20° C or lower. Serum should not be stored in a self-defrosting refrigerator or freezer.Positive, negative and PBS controls should be run on the wells provided for them on each slide.If the controls do not appear as described, the test should be repeated and further investigated forcorrective action/problem resolution. Notify the Medical director of test system failure. Notifythe ordering physician if prolonged delays in test reporting are likely. Document observationsand corrective1. Positive Control : Should show bright apple-green fluorescence in the nuclei of the cells,

 with a clearly discernable pattern characteristic of the control that was used.1. Negative Control: Although the negative control may demonstrate weak fluorescence of the

 cytoplasm with brighter staining of the nonchromosomal region of the mitotic cell, it should show no discernable pattern of nuclear staining.1. PBS Control: Used to observe nonspecific staining by the antibody reagent and should

 not exhibit any green fluorescence.1. Microscope Quality Control: Refer to Microscope Maintenance Log sheet. Lamp alignment,

 Number of hours of use and ability to detect 1+ fluorescence from ImmunoConcepts FITC Quality Control slide will be documented each day of use.1. Lot to Lot ANA Kit QC: A titratable serum control is included in each kit and is to be used to

 verify the reproducibility of this assay. Treat as an undiluted patient serum. Expected results should be within +/- one dilution of the mean titer reported by the kit manufacturer. Run the titratable control serum each time a new kit lot number is opened. Document the result on the log sheet specified for that purpose.1. PBS Control: Used to observe nonspecific staining by the antibody reagent and should

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| **Procedure** | **Step** | Action | **Related Document** |
|  | 1 | **Prepare Patient Samples**: Mix sera well and remove any fibrin strands. |  |
|  | 2 | **Prepare Screening dilution**:**1;40** - 0.025 ml serum + 0.975 ml PBS**1:80** -Transfer 0.2 ml of well-mixed 1:40 dilution into 0.2 ml PBS. This is the initial ANA screening dilution. |  |
|  | 3 | **Prepare serial dilutions for semi-quantitative titering**: This step is necessary only if the screening dilution is positive.**1:160** - Transfer 0.2 ml of well-mixed 1:80 dilution into 0.2 ml PBS**1:320** -Transfer 0.2 ml of well-mixed 1:160 dilution into 0.2 ml of PBS**1:640** - Transfer 0.2 ml of well-mixed 1:320 dilution into 0.2 ml PBS**1:1280** - Transfer 0.2 ml of well-mixed 1:640 dilution into 0.2 ml of PBS |  |
|  | 4 | **Prepare Substrate Slides**: Remove slide(s) from pouches and place control sera on control wells as indicated. Invert control dropper bottle andsqueeze gently until drop is visible at the tip. Gently touch the drop tothe well while avoiding direct contact of the dropper tip with slidesurface. For general screening, the Homogeneous control isrecommended. For semi-quantitative titering, select the positive control illustrating the most similar pattern to the positive patient screen. Add 1 drop of patient 1:80 dilution for screening slides. Run the remainingdilutions 1:160 through 1:1280 to determine the titer if the screeningdilution is positive. |  |
|  | 5 | **Incubate Slides**: Place a small volume of water in the incubator trayand place slide(s) on the slide ridges. Incubate, with lid in place,for 30 + 5 minutes at room temperature. |  |
|  | 6 | **PBS Rinse and Wash**: Remove slides from incubator tray and rinsebriefly with PBS. Avoid squirting the wells directly andcross-contaminating wells. Wash slide(s) 10 minutes with PBS with gentle agitation. The wash may be extended 10-30 minutes with no variability in final test results. Discard PBS wash solution after use. |  |
|  | 7 | **Fluorescent Antibody Reagent**: Remove one slide at a time from PBS and dip 3-5 times in distilled water. Tap slide on its side against paper toweling to remove excess water. Immediately return slide to incubator tray and flood wells completely with fluorescent antibody reagent. Repeat for each slide. Note: Fluorescent Antibody reagent has been titered to compensate for residual distilled water remaining on the slide after rinsing. **\*\*Do not blot or dry slides in any manner or** **allow slides to sit without fluorescent antibody reagent for longer** **than 15 seconds.** |  |
|  | 8 | **Incubate slides**: Incubate slides 30 + 5 minutes at room temperature. |  |
|  | 9 | **PBS Rinse/Wash**: Rinse and wash slides as in step 6. |  |
|  | 10 | **Mount Coverslip**: Remove one slide at a time from PBS and dip 3-5 times in distilled water. Tap excess water from slide on paper towels. **\*\*Do not blot or dry slides in any manner or allow** **slides to sit without coverslip for longer than 15 seconds**. Add 2-3 drops of semipermanent mounting media 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. **Excess mounting media on slides may result in high** **background fluorescence due to light scattering, and lack** **of clear resolution of cells (blurred image**). Excess mounting medium may be removed from slides by gently blotting coverslip with paper towels while avoiding any direct movement of the coverslip. |  |
| **Interpretation/ Results** | 1. Expected Result: Normal is negative or no fluorescence observed.
2. Interpretation of patient results:

**Magnification**: 200X total magnification is recommended for screening positive vs. negative, while 400X total magnification is recommended for pattern recognition, viewing mitotic cells and titer end-point determination.**Negative**: A serum is considered negative for antinuclear antibodies if nuclear staining is < the negative control well. The cytoplasm may demonstrate weak staining, with brighter staining of the nonchromosomal region of the mitotic cells, but no clearly discernable nuclear pattern.**Positive**: A serum is considered positive if the nucleus shows staining greater than thenegative control well and a clearly discernable pattern of staining can be seen in themajority of the cells.**Titers**: When reading titers, begin reading with the well that contains the most dilute sampleand read “backwards” to the lowest dilution**. The first well in which a clearly discernable****pattern is visible is the titer end-point.** It is important that the intensity of staining not beconfused with the presence or absence of antinuclear antibodies. The key factor toconsider in determining whether a given dilution is positive is the appearance of a clearly discernable pattern, irrespective of the staining intensity. Titers of 1:160 and 1:320 areconsidered medium titers; >1:640 high titers.**Caution:** Some sera may demonstrate nuclear and cytoplasmic staining with no apparentnuclear pattern. This phenomenon is generally due to Heterophile Antibodies and should bereported as negative. |
| **Result Reporting** |  See ANA test Algorithm for additional testing strategy.1. **Screening:** Report as Positive or Negative. The titer should be ordered and charged if the

 screen is positive.1. **Titering:** Results should be reported to the last serial dilution in which a clearly discernable

 pattern is seen. Results with a positive staining at 1:1280 should be reported as > 1:1280.1. **Pattern Recognition:**
2. **Use of Mitotic Cells**:
3. Mitotic cells are used to identify positive chromosomes/homogeneous patterns.
4. Used to discern fine speckling vs. homogeneous staining.
5. Used to discern cases wherein two or more antibodies are present i.e. mixed patterns.

Used to discern peripheral staining vs. nuclear membrane antibodies i.e. Nuclear Lamins.1. **Homogeneous**: A solid staining of the nucleus with or without apparent masking of the

 nucleoli. The chromosome region of the metaphase mitotic cells is clearly positive with a smooth or peripheral staining intensity greater than or equal to the interphase nuclei.Synonyms: Diffuse, SolidNuclear Antigens: Double Stranded DNA (dsDNA), Native DNA (nDNA) , Deoxyribonucleoprotein (DNP), HistonesDisease Association: High titers suggestive of SLE; low titers suggestive of SLEor other connective tissue diseases.Report as: “Homogeneous pattern” and titer.Follow-up testing: Extractable Nuclear Antigens, Anti-dsDNA, Anti-DNP or Anti-Histone.1. **Peripheral**: A solid staining around the outer region of the nucleus with weaker staining

 toward the center. The chromosome region of the metaphase mitotic cells is clearly positive with a smooth or peripheral staining intensity greater than or equal to interphase nuclei.Synonyms: Rim, Shaggy, MembranousNuclear Antigens: dsDNA, Single Stranded DNA (ssDNA), nDNA, DNP, HistoneDisease Association: High titers suggestive of SLE; low titers suggestive of SLEor other connective tissue diseases.Report As: “Peripheral pattern” and titer.Follow-up testing: Anti-dsDNA, Anti-DNP or anti-Histones in some cases.1. **Atypical Homogenous**: Antinuclear membrane antibody is characterized by

 homogeneous and peripheral nuclear staining of the nuclei of the interphase cells. The chromosomes of the mitotic cells are negative, indicating that anti-nDNA antibodies are absent. This antibody may be reacting with nuclear pore complexes. It is clinically important to make the distinction between the Peripheral pattern and the Nuclear Membrane (Lamins) because the nuclear membrane antibody does not have DNA/DNP specificity and is not associated with SLE.Synonyms: Nuclear Lamins, Antinuclear Membrane AntibodyNuclear Antigens: Nuclear membrane – Nuclear envelope or Nuclear Lamin proteins (A,B,C).Disease Association: Autoimmune Hepatitis (Lupoid)Report As: “Atypical Homogeneous pattern suggestive of antibodies to NuclearMembrane” and titer.Follow-up testing: None, confirmed on HEp-2000 only.1. **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 nonchromosomal region of metaphase mitotic cells demonstrates strong staining, while the chromosome region may demonstrate faint staining. Anaphase and telophase sells may demonstrate similar staining to interphase nuclei.Synonyms: UnknownNuclear Antigens: Generally referred to as 4-6 Ribonucleic Acid (RNA)s and other unknown nuclear antigens.Disease Association: High titers prevalent in scleroderma and Sjogren’s syndrome.Report As: “Nucleolar pattern” and titerFollow-up testing: None, confirmed on HEp-2000 only.1. **Speckled**: A fine of grainy appearing staining of the nucleus generally without

 fluorescent staining of the nucleoli. The nonchromosomal region of the metaphase mitotic cells demonstrates a very strong staining, while the chromosome region is weakly stained with occasional patchy staining at the periphery. Interphase cells take on a characteristic “Swiss cheese” appearance, while mitotic cells are negative and appear to have a “coin slot” in them.Synonyms: NoneNuclear Antigens: Smith (Sm), Ribonucleoprotein (RNP)Disease Association: High titers suggestive of SLE (Sm antigen) or RNPantigen is associated with Mixed Connective Tissue Disease (MCTD).Report As: “Speckled pattern” and titer Follow-up testing: Extractable Nuclear Antigens, Anti-dsDNA. 1. **Centromere**: A discrete speckled staining pattern highly suggestive of five main features;

**C**alcinosis, **R**aynaud’s phenomenon, **E**sophageal **D**ysmotility, **S**clerodactyly, and **T**elangiectasia(**CREST** syndrome) variant of Progressive Systemic Sclerosis (PSS). The nuclear specklesare very discrete and are usually in some multiple of 46 (usually 23-46speckles/nucleus). Because centromeres are constrictions where spindle fibersattach on chromosomes, mitotic cells will show the same specklingreaction in the chromosome region.Synonyms: CREST variant of PSS, Anti Centromere antibody(ACA), Discrete Speckled.Nuclear Antigens: Chromosomal centromere (kinetochore).Disease Association: Highly suggestive of the CREST syndrome variant of Progressive Systemic SclerosisReport As: “Centromere pattern” and titer.Follow-up testing: None, confirmed by HEp-2000 only. Detected byimmunoblotting.  |
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| **Result Reporting (Computer)** | Test: **FANA Screen** – **Negative at screening titer of 1:80:** Report as Neg-TL80 (Negative-; Titer less than 1:80)**Positive at 1:80** - Report as **POSF -**Proceed with titer. Report titer and PatternTest: **Reflexive ANA Profile** –**Negative at 1:80** Report as Neg-TL80 (Negative-; Titer less than 1:80)**Positive at 1:80** - Report as **POSF -**Proceed with titer. Report titer and Pattern.- Proceedaccording to ANA Algorithm.Titers of <1:160 are not considered clinically significant and no further testing is necessary.Refer to Autoimmune Antibody testing algorithm: [Document A - Autoimmune Testing Algorithm](http://khan.childrensmn.org/Manuals/Lab/SOP/Imm/Res/204862.pdf) [Document B - Reflexive ANA Resulting Scenarios](http://khan.childrensmn.org/Manuals/Lab/SOP/Imm/Res/208947.pdf)All ANA results are appended with the following comment:“ANA testing is performed by Indirect Immunofluorescence used to detect antibodies with affinity of HEp-2 CELLS, if present the pattern of ANA Immunofluorescence is also reported.” (comment code ANAC1). |
| **References** | 1. Antinuclear Antibodies, Contemporary Techniques and Clinical Application to Connective Tissue
2. Diseases. McCarty GA, Valencia DW, Fritzler MJ. Oxford University Press. 1994.
3. Manual of Clinical Laboratory Immunology, 4th ed. Chapters 105-107. 1992.
4. ImmunoConcepts HEp-2000® Product insert. ImmunoConcepts NA Ltd. Sacramento, CA. 1999.
5. Visions II and III Technical Training Program. ImmunoConcepts NA Ltd. Sacramento, CA. 2000.
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| **Historical Record** |  |  |  |  |
|  | **Version** | **Written/Revised by:** | **Effective Date:** | **Summary of Revisions** |
| 1 | Shirley Kruchten | 07/25/97 | Initial Version |
| 2 | Shirley Kruchten | 09/09/98 | Revised initial procedure |
| 3 | Colleen Berglund  | 06/01/2001 |  |
|  | 4 | Colleen Berglund | 06/15/2006 |  |
|  | 5 | Colleen Berglund | 12/16/2006 |  |
|  | 6 | Jim Berger | 10/29/2009 |  |
|  | 7 | Al Quigley | 03/31/2013 | Reformatted for CMS Web |
|  | 8 | Al Quigley | 2/14/20 | Added comment code per CAP checklist requirement, IMM.39700  |