|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Indirect Fluorescent Antinuclear Antibody Testing (FANA) | | | | | | |
| **Purpose** | This is an indirect fluorescent antibody test for the semi-quantitative detection of antinuclear  antibody in human serum. | | | | | |
| **Policy Statements** | • This procedure applies to all laboratory technologists performing Immunology testing, the section  supervisor, 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 | |  |  |  | | --- | --- | --- | | **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 stably  transfected with Anti–Sjogren’s Syndrome related antigen (SSA (Ro) antigens).  **SSA (Ro) Positive Control**. Ready-to-use  dropper 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 with   1. antibody specific to deoxyribonucleic acid or deoxyribonucleoprotein   (DNA and/or DNP) nuclear antigens.  **Speckled Positive Control**.  Ready-to-use dropper vial containing  0.5 ml positive human control  serum with antibodies specific to Sm  and/or Ribonucleoprotein (RNP) nuclear  antigens.  **Nucleolar Positive Control**  Ready-to-use dropper vial containing  0.5 ml positive human control  serum with antibody specific to  nucleolar antigens.  **Centromere Positive Control**.  Ready-to-use dropper vial containing  antibody specific to chromosomal  centromere (kinetochore).  **Negative Control serum**.  Ready-to-use dropper vial containing  1.0ml negative human control  serum. It demonstrates no discernable  pattern of nuclear staining.  **Titratable Control Serum**. Ready to  use vial containing 1.0 ml positive human  control serum to be treated as an undiluted  patient sample.  **Fluorescent Antibody Reagent**.  Goat anti-human IgG (heavy and light  chains) conjugated to fluorescein  isothiocyanate (FITC). Reagent comes  ready-to-use in dropper bottles with  9.0 ml for each of the 10 slides in  complete 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 of  powder in 1 liter of deionized or distilled  water, cover and store refrigerated at 2-10° C  for up to 4 weeks or until signs of  contamination or other visible changes occur.  **Semi**-**Permanent Mounting Medium**.  Ready-to use dropper vial containing 5.0 ml  glycerol-based mounting medium,  pH 9.1 + 0.2. | Pasteur pipettes  12 X 75 test tubes  Deionized or distilled  Water  1 liter containers for PBS  Bibulous paper or paper  Towels  Volumetric Pipettes  Incubator tray  Slide washing chambers  Timer  Squeeze bottle of PBS  Coverslips.( 24 X 60 mm) | Fluorescent microscope equipped with 495 nm exciter filter and 515 nm barrier filter | | | | | | |
| Sample  **Quality Control** | Serum, collected by aseptic techniques into a red top Vacutainer or other suitable tube  without 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 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 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 for  corrective action/problem resolution. Notify the Medical director of test system failure. Notify  the ordering physician if prolonged delays in test reporting are likely. Document observations  and corrective   1. 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   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. | | | | | |
|  | . | | | | | |
| **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 and  squeeze gently until drop is visible at the tip. Gently touch the drop to  the well while avoiding direct contact of the dropper tip with slide  surface. For general screening, the Homogeneous control is  recommended. 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 remaining  dilutions 1:160 through 1:1280 to determine the titer if the screening  dilution is positive. | | | |  |
|  | 5 | **Incubate Slides**: Place a small volume of water in the incubator tray  and 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 rinse  briefly with PBS. Avoid squirting the wells directly and  cross-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 the  negative control well and a clearly discernable pattern of staining can be seen in the  majority of the cells.  **Titers**: When reading titers, begin reading with the well that contains the most dilute sample  and 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 be  confused with the presence or absence of antinuclear antibodies. The key factor to  consider 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 are  considered medium titers; >1:640 high titers.  **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. | | | | | |
| **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, Solid  Nuclear Antigens: Double Stranded DNA (dsDNA), Native DNA (nDNA) ,  Deoxyribonucleoprotein (DNP), Histones  Disease Association: High titers suggestive of SLE; low titers suggestive of SLE  or 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, Membranous  Nuclear Antigens: dsDNA, Single Stranded DNA (ssDNA), nDNA, DNP, Histone  Disease Association: High titers suggestive of SLE; low titers suggestive of SLE  or 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 Antibody  Nuclear 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 Nuclear  Membrane” 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: Unknown  Nuclear 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 titer  Follow-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: None  Nuclear Antigens: Smith (Sm), Ribonucleoprotein (RNP)  Disease Association: High titers suggestive of SLE (Sm antigen) or RNP  antigen 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 speckles  are very discrete and are usually in some multiple of 46 (usually 23-46  speckles/nucleus). Because centromeres are constrictions where spindle fibers  attach on chromosomes, mitotic cells will show the same speckling  reaction 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 Sclerosis  Report As: “Centromere pattern” and titer.  Follow-up testing: None, confirmed by HEp-2000 only. Detected by  immunoblotting. | | | | | |
|  |  | | | | | |
| **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 Pattern  Test: **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.- Proceed  according 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. | | | | | |
| **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 | |