The ZEUS ANA HeP-2 Test System is a prestandardized kit designed for the qualitative and semi-quantitative detection of antinuclear antibodies. The test is intended to aid in determining SLE and differentiating clinically similar connective tissue disorders, and is for in vitro diagnostic use.

**SIGNIFICANCE AND BACKGROUND**

The indirect fluorescent antibody (IFA) technique method has been used extensively for detecting the presence of ANA in the sera of patients with systemic lupus erythematosus (SLE), and other clinically similar connective tissue disorders (1 - 5). In addition, ANA may be associated with numerous drug-induced lupus syndromes (6 - 7) which clinically mimic the spontaneous form of SLE. The IFA technique was adapted to ANA testing by several investigators (8 - 9) following the basic methods originally described by Coons (10). ANA are primarily composed of IgG antibodies; however, IgA and IgM ANA may also be detected (11). It is now recognized that many sources of nuclear material may be employed as a substrate for ANA testing. Although most of the original ANA research was performed using rat or mouse liver or kidney tissue section substrate, the use of human or animal embryonic tissue cell culture substrates has provided a reliable and easy to interpret alternative substrate for ANA testing. The HeP-2 cell line is a recommended substrate for detecting centromere antibody, which is highly indicative of the CREST variant of progressive systemic sclerosis (27). There are several different patterns of nuclear and cytoplasmic immunofluorescence and the basis for them are as follows:

**Homogeneous** - Homogeneous or diffuse staining patterns of the nucleus is consistent with autoantibodies to native DNA (nDNA) histones and/or deoxyribonucleoprotein (DNP) (12, 13). The chromosomes of the mitotic cells (dividing cells) are important indicators of a homogeneous pattern because they will stain as irregularly shaped masses with more intensely stained outer edges.

**Speckled Patterns** - The speckled pattern is the most commonly observed ANA pattern. A uniform "true speckled" pattern may be seen with centromere antibodies in cells not in division. A clumpy speckled pattern may be seen with antibodies to n-RNP, Sm, and SSA/La.

1. **Fine speckled pattern, chromosome-negative**: Numerous small and uniform points of fluorescence uniformly scattered throughout the nucleus. The nucleoli will generally appear unstained. The mitotic cells may demonstrate a few speckles in their cytoplasm, but the chromosomes will be negative.

2. **Course speckled pattern, chromosome-negative**: Medium-sized points of fluorescence will be scattered throughout the nuclei with distinct nuclear margins. Larger-sized points of fluorescence may also be observed; however, they are too numerous and variable in size to be identified as a nucleolar pattern. The chromosomes in the mitotic cells will be negative.

3. **Discrete speckled, chromosome (centromere specificity) positive**: The chromosomes will be positive in mitotic cells; in fact, the discrete speckles will only be clustered in the chromosome mass clearly demonstrating the various stages of mitosis. The centromere pattern has been recognized to be associated with the CREST syndrome, which is a milder variant of progressive systemic sclerosis (PSS). The centromere pattern will demonstrate discrete and uniform points of fluorescent speckles scattered throughout the nucleus. Mitotic cells will be positive, demonstrating a clustering of the centromeres in the chromosomes in different arrangements according to the mitotic stage. Harmovic et al. (27) demonstrated that samples containing highly monospecific anti-SSA/Ro gave an IF-ANA test pattern of discrete nuclear speckles on a wide variety of human cells and tumor nuclei. Such serum samples with monospecific anti-SSA/Ro produced very little cytoplasmic staining of substrate cells. A distinct, large, variable speckled pattern of 3 to 10 large speckles in the nucleus has been described. These patients with large, variable speckles have undifferentiated rheumatic disease syndromes with IgM antihistone H-3 antibody (18).

**Nucleolar Pattern** - The nucleolar pattern demonstrates a homogeneous or speckled staining of the nucleolus. This pattern is often associated with a dull, homogenous fluorescence in the rest of the nucleus. The chromosomes in the mitotic cells will be negative. The nucleolar pattern suggests autoantibodies to 4-6S RNA. The nucleolar fluorescence will appear as homogenous, clumped, or speckled, depending on the antigen to which the autoantibody reacts. Antinucleolar antibodies occur primarily in the sera of patients with scleroderma, systemic lupus erythematosus, Sjögren’s syndrome, or Raynaud’s phenomenon (19).

**Peripheral (Rim)** - The nuclei stain predominantly at their periphery. The chromosomes of the mitotic cells stain as irregularly shaped masses with more intensely stained outer edges. This pattern is often seen with autoantibodies to nDNA (3, 14 - 16). If the chromosomes of the mitotic cells are negative, then the pattern would be suggestive of autoantibodies to the nuclear membrane and not to nDNA, and not reported as a peripheral pattern. (See nuclear membrane interpretation below).

**Additional Patterns**

1. **Spindle fiber pattern, chromosome-positive**: The spindle fiber pattern is unique to cells undergoing mitosis where only the spindle apparatus fluoresces. This pattern has a “spider web” appearance extending from the centriole to the centromeres. The pattern is suggestive of autoantibodies to the microtubules and its significance is unclear; however, an association between the spindle fiber pattern and carpal tunnel syndrome has been suggested.

2. **Midbody pattern**: The midbody pattern is a densely staining region near the cleavage furrow of telophase cells, that is, in the area where the two daughter cells separate. The clinical significance of the pattern is unknown; however, the pattern has been recognized in selected patients with systemic sclerosis.

3. **Centriole pattern**: The centriole pattern is characterized by two distinct points of fluorescence in the nucleus of the mitotic cells or one distinct point of fluorescence in the resting cell. The significance of this pattern is not known; however, it has been observed in PSS.

4. **Proliferating cell nuclear antigen (PCNA) pattern**: The proliferating cell nuclear antigen pattern is observed as a fine to coarse nuclear speckling in 30 - 60% of the cells in interphase, and a negative staining of the chromosome region of mitotic cells. The PCNA is very specific for patients with SLE but not detected in other connective tissue disease disorders. It has been reported that SLE patients with the PCNA pattern have a higher incidence of diffuse glomerulonephritis.

5. **Antinuclear membrane (nuclear laminae)**: The antinuclear membrane pattern appears as a rim around the nucleus and resembles a rim pattern; however, it is distinguished from the rim pattern by the fact that the metaphase chromosome stage is negative. This autoantibody is important to report because it was recently recognized to be associated with autoimmune liver disease.

**Cytoplasmic Patterns**

1. **Mitochondrial (AMA) pattern**: The pattern will characteristically have numerous cytoplasmic speckles with the highest concentration in the peri-nuclear area. The pattern can be observed in interphase and mitotic cells. The clinical significance of AMA is most frequently an association with primary biliary cirrhosis, especially when the AMA is a high titer.

2. **Golgi apparatus pattern**: The golgi apparatus pattern is characterized by positive cytoplasmic staining that is concentrated on only one side of the perinuclear region. The clinical significance is uncertain, but reports in the literature have suggested an association with SLE and Sjögren’s Syndrome.

3. **Lysosomal pattern**: The lysosomal pattern is observed as a few discrete speckles sparsely spaced throughout the cytoplasm. The pattern is observed in the cytoplasm of interphase and mitotic cells. The clinical significance is unknown.

4. **Ribosomal pattern**: The ribosomal pattern is characterized by numerous cytoplasmic speckles with the highest concentration around the nucleus. It is distinguished from the mitochondrial pattern because of the smaller specks and higher density. The significance of the pattern is unknown.

5. **Cytoskeletal pattern**: The cytoskeletal pattern is characterized by a distinct “spider web” or fibrous appearance throughout the cell. It has been reported to be associated with autoimmune liver disease (anti-smoothmuscle).

**ANA Negative**

Autoantibody to SSA/Ro is present in high frequency in a clinical subset of lupus called subacute cutaneous lupus erythematosus (SCLE). Many patients with SCLE have been falsely labeled as having “ANA-negative” lupus. We now know that many of these so-called “ANA-negative” LE patients will demonstrate a positive IF-ANA on substrate of HeP-2 cells containing the SSA/Ro antigen (20). Anti-SSA/Ro antibodies may be present in the absence of traditional ANAs, with SLE seen in persons genetically deficient in C4 and occasionally other genetic deficiencies (21, 22). This combination may be more common in black persons (23, 24). In addition, C4 deficiency may be associated with increased susceptibility to development of SLE upon treatment with hydralazine (25). These patients, if female, are likely to deliver infants with congenital heart block or lupus dermatitis (26). Although the level of ANA may not correlate with the clinical course of a particular autoimmune disease state (6), the various patterns of nuclear staining may be associated with specific disease states (3, 16, 28 - 31). The following table summarizes the various autoantibodies noted above with respect to disease association:

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**Quest ANA HeP-2 Package Insert (R0201)**

(Rev. Date 9/20/2016)
The ZEUS ANA Hep-2 Test System is designed to detect the presence of circulating ANA in human sera. The system employs tissue cell culture substrate and goat anti-human immunoglobulin adjusted for optimum use and free of nonspecific background staining. The reaction occurs in two steps:

1. During the first (sample) incubation, any ANA present in the patient sample may bind to the cell substrate, forming an antigen-antibody complex. Other serum components are subsequently washed away.

2. During the second (conjugate) incubation, anti-human immunoglobulin labeled with FITC is allowed to react with any human immunoglobulin that bound to the substrate during the sample incubation. This will form a stable antigen-antibody-conjugate complex at the location where the initial patient antibody bound to the cell substrate. Excess conjugate is subsequently washed away.

3. The results of the assay can be visualized using a properly equipped fluorescent microscope. Any positive reactions will appear as apple-green fluorescent staining within the cell. If the sample had no specific ANA, there will be no distinct nuclear staining of the cell.

### KIT COMPONENTS

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONI</td>
<td>Conjugal: Dripper-tipped bottle containing goat anti-human immunoglobulin labeled with fluorescein isothiocyanate (FITC) and counterstain. Ready to use, 7 mL.</td>
</tr>
<tr>
<td>CONTROL +</td>
<td>Homogenous Positive Control: Dripper-tipped bottle containing human ANA-positive control serum producing 4+ homogeneous nuclear staining with the substrate. Ready to use, 1.0mL.</td>
</tr>
<tr>
<td>CONTROL -</td>
<td>Negative Control: Dripper-tipped bottle containing human ANA-negative control serum. Ready to use, 1.0mL.</td>
</tr>
<tr>
<td>MRC</td>
<td>Minimally Reactive Control: Dripper-tipped bottle containing human ANA-positive control serum producing a 1+ homogeneous nuclear staining with the substrate. Ready to use, 1.0mL.</td>
</tr>
<tr>
<td>BUF PBS</td>
<td>PBS: Phosphate-buffered-saline, pH 7.2 ± 0.2.</td>
</tr>
<tr>
<td>MNTMED</td>
<td>Mounting Media: Phosphate buffered glycerol, 3mL.</td>
</tr>
</tbody>
</table>

NOTES: Mounting Media (Product Number: FA0009S), and PBS (Product Number: 0008S) are not Test System Lot Number dependent and may be used interchangeably with the ZEUS IFA Test Systems. Test System also contains a Component Label containing lot specific information inside the Test System box.

### PRECAUTIONS

1. For in vitro diagnostic use.
2. Reagents may contain a preservative that may be toxic if ingested.
3. Non-nuclear staining of the cell substrate may be observed with some human sera.
4. Do not apply pressure to slide envelope. This may damage the substrate.
5. Reagents from other sources or manufacturers should not be used. Follow test procedure carefully.
6. Reconstitute reagents gently but thoroughly. Reagents should be free of particulate matter. If reagents become cloudy, suspect bacterial contamination.
7. DO NOT freeze and thaw reagents more than once. Repeated freezing and thawing destroys antibody activity.
8. Controls are POTENTIALLY BIOHAZARDOUS MATERIALS. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg, and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, handle these products at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": current edition; and OSHA’s Standard for Bloodborne Pathogens (39).
9. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.

### MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipets capable of delivering 10 to 200μL.
2. Disposable reagent reservoirs.
3. Cover slips, thickness No. 1.
4. Moist chamber.
5. Distilled or deionized water.

The following filter systems or their equivalent have been found to be satisfactory for routine use with transmitted or incident light dark-field assemblies:

### TRANSMITTED LIGHT

<table>
<thead>
<tr>
<th>Excitation Filter</th>
<th>Barrier Filter</th>
<th>Red Suppression Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP490</td>
<td>K510 or K530</td>
<td>BG38</td>
</tr>
<tr>
<td>BG12</td>
<td>K510 or K530</td>
<td>BG38</td>
</tr>
</tbody>
</table>

### INCIDENT LIGHT

<table>
<thead>
<tr>
<th>Excitation Filter</th>
<th>Dichroic Mirror</th>
<th>Barrier Filter</th>
<th>Red Suppression Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP500</td>
<td>TK510</td>
<td>K510 or K530</td>
<td>BG38</td>
</tr>
</tbody>
</table>

QUEST ANA Hep-2 Package Insert (R2021) 2 (Rev. Date 9/20/2016)
1. ZEUS Scientific recommends that the user carry out specimen collection in accordance with CLSI document M29: Protection of Laboratory Workers from Occupationally Acquired Infectious Diseases. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.
2. Only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures with this assay (32, 33). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
3. Store sample at room temperature for up to 8 hours. Store sera between 2 - 8°C if testing is not performed within 8 hours. If a delay in testing (48 hours or more) is anticipated, store test sera at ~20°C or lower. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and give erroneous results. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine stability criteria for its laboratory (40).

<table>
<thead>
<tr>
<th>STORAGE CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2°C – 8°C</td>
</tr>
<tr>
<td>Mounting Media, Conjugate, Slides, Positive Control and Negative Control.</td>
</tr>
<tr>
<td>Rehydrated PBS (Stable for 30 days).</td>
</tr>
</tbody>
</table>

**ASSAY PROCEDURE**

Preparation of Reagents:
1. Phosphate-buffered-saline (PBS). Empty contents of one buffer packet into one liter of distilled or deionized water. Mix until all salts are thoroughly dissolved.
2. Human ANA positive controls. Use as packaged. Do not dilute.
3. Human ANA minimally reactive positive control. Use as packaged. Do not dilute.
4. Human ANA negative control. Use as packaged. Do not dilute.
5. Goat anti-human immunoglobulin FITC labeled conjugate. Use as packaged. Do not dilute.

**NOTE: The Controls are intended to be used undiluted.** As an option, users may titrate the Positive Control(s) to endpoint. In such cases, the Control(s) should be diluted two-fold in PBS. When evaluated at ZEUS, an endpoint dilution is established and printed on the Homogeneous Positive Control vial (~ one dilution). It should be noted that due to variations within the laboratory (equipment, etc.), each laboratory should establish its own mean titer for each lot of Control.

**Test Procedure:**
1. Remove slides from storage and allow them to warm to room temperature (20 - 25°C). Tear open the protective envelope and remove slides containing the HEp-2 cell culture substrate. DO NOT APPLY PRESSURE TO FLAT SIDES OF PROTECTIVE ENVELOPE.
2. Prepare patient sera at 1:40 dilution in reconstituted PBS. When evaluated at ZEUS, an endpoint dilution is established and printed on the Homogeneous Positive Control vial (~ one dilution). It should be noted that due to variations within the laboratory (equipment, etc.), each laboratory should establish its own mean titer for each lot of Control.
3. Identify each well with the appropriate patient sera and controls.
4. If test samples are to be titered, serial dilutions should be made in reconstituted PBS.
5. Using suitable dispenser (capillary, Pasteur, or automatic pipette), dispense one drop or approximately 20µL of patient sera and control sera on the cells in the respective wells. Spread sera over entire area of the wells being careful not to touch substrate with pipette tip.
6. Incubate slides in a moist chamber at room temperature (20 - 25°C) for 20 - 30 minutes.
7. Remove slides from the moist chamber one at a time and gently rinse with a stream of PBS. DO NOT DIRECT THE STREAM OF PBS INTO THE TEST WELLS. NOTE: To avoid cross-contamination when using the 96-well test system, place slide in palm of hand and grasp edges with fingertips. Quickly invert the slide and, using a “snap” motion, expel excess sera.
8. Place slides in a staining dish and wash in PBS for 2, 5-minute intervals with one change of PBS. Use a magnetic mixing setup or other means of gentle agitation.
9. Remove slides from PBS one at a time. Invert slide and key wells to holes in blotters provided. Blot slide by wiping the bottom side with an absorbent wipe. CAUTION: Position the blotter and slide on a hard, flat surface. Do not invert the slide on top of paper towels. Blotting on paper towels may destroy the substrate. DO NOT ALLOW THE SLIDES TO DRY DURING THE TEST PROCEDURE.
10. Add one drop of mounting media (according to the number of wells per slide) to each slide (between the wells) and coverslip.
11. Repeat steps 6 through 9.
12. Apply a suitable number of drops of mounting media (according to the number of wells per slide) to each slide (between the wells) and coverslip. NOTE: Be sure each well has mounting media coverage.
13. Examine slides immediately with an appropriate fluorescence microscope assembly. NOTE: If delay in examining slides is anticipated, seal coverslip with nail polish and store in refrigerator. It is recommended that slides be examined on the same day of testing.

**QUALITY CONTROL**

1. A Positive Control (4+ homogeneous), a Minimally Positive Control (1+ homogeneous), a Negative Control, and a Buffer Control must be included with each run.
2. It is recommended that the Controls be read prior to evaluating the test samples. If the controls do not appear as described, results may be invalid.
   a. Negative Control - characterized by the absence of specific fluorescence and a red background staining of all cells due to counterstain.
   b. Homogeneous Positive Control - characterized by apple-green fluorescence. The staining pattern is a diffused uniform staining of the entire nucleus.
3. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

**NOTES: Non-specific reagent trapping may exist.** It is important to adequately wash slides to eliminate false positive results. The intensity of the observed fluorescence may vary with the microscope and filter system used. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

**INTERPRETATION OF RESULTS**

1. The interpretation of the results depends on the pattern observed, the titer of the autoantibody, and the age of the patient. The elderly, especially women, are prone to develop low-titered autoantibodies (<1:80) in the absence of clinical autoimmune disease. In contrast, a 1:20 titer of a significant pattern of autoantibody(s) in a young person may suggest that overt disease may occur later. Experience suggests that a 1:40 dilution is a good dilution to screen for ANA. Low-titer positive results may occur in apparently healthy persons; therefore, always interpret ANA results in light of the patient’s total clinical presentation.
2. Titers less than 1:40 are considered negative.
3. Positive test: A positive reaction is the presence of any pattern of nuclear apple-green staining observed at a 1:40 dilution based on a 1+ to 4+ scale of staining intensity. 1+ is considered a weak reaction and 4+ a strong reaction. All sera positive at 1:40 should be titered to endpoint dilution. This is accomplished by making 1:40, 1:80, 1:160, etc. serial dilutions of all positives. The endpoint titer is the highest dilution that produces a 1+ positive reaction.
4. Homogeneous patterns with peripheral accentuation are frequently found in sera from patients with SLE.

**Pattern** | **Disease Most Frequently Found In** | **Reference** |
---|---|---|
Homogeneous High Titer | Rheumatoid Arthritis and other diseases | (3, 8, 9, 16) |
Homogeneous Low Titer | SLE | (1) |
Centromere |CREST Syndrome variant of PSS | (27) |
Speckled | Sjögren’s Syndrome | (34-36) |
Nucleolar | Scleroderma | (37) |
Peripheral | SLE | (2, 8, 9, 16) |

Quest ANA HEp-2 Package Insert (R2021) 3 (Rev. Date 9/20/2016)
LIMITATIONS

1. The ANA test is a laboratory diagnostic aid and by itself is not diagnostic. Positive ANA may be found in apparently healthy individuals. It is therefore imperative that ANA results be interpreted in light of the patient's clinical condition by a medical authority.

2. SLE patients undergoing steroid therapy may have negative test results.

3. Many commonly prescribed drugs may induce ANA (6, 7).

4. One autoantibody pattern may partially or completely obscure the diagnostic features of other. In such instances, it is necessary to titrate the serum.

5. No definitive association between the pattern of nuclear fluorescence and any specific disease state is intended with this product.

EXPECTED RESULTS

The expected value in the normal population is negative. However, apparently healthy individuals may contain ANA in their sera (38). This percentage increases with aging, particularly in the 7th decade of life.

PERFORMANCE CHARACTERISTICS

1. The ZEUS ANA Hep-2 Test System was tested in parallel with a reference procedure employing rat liver substrate. Routine ANA testing was performed by both procedures on 434 patient specimens. Of these 434 sera, 116 were positive by both procedures. The ZEUS ANA Hep-2 Test System showed 97% agreement with respect to positive and negative results, and 100% with respect to staining pattern. Of the 21 discrepancies in titer, the ZEUS ANA Hep-2 test was one dilution lower in 18 specimens. Five of these 18 specimens that were negative using the ANA Hep-2 test were positive at 1:20 by the rat liver reference procedure.

2. A study was performed using 206 samples obtained from a plasma donor center and a reference laboratory. The ZEUS ANA Hep-2 Test System with ZORBA-NS was compared to a commercial ANA Hep-2 test system (PBS sample diluent) and the ZEUS ANA Hep-2 Test System (PBS sample diluent). With respect to positive and negative results, there was 96% agreement (198/206) between the ZEUS ANA Hep-2 with ZORBA-NS and the ZEUS ANA Hep-2 with PBS. In addition, there was 96% agreement (198/206) between the ZEUS ANA Hep-2 with ZORBA-NS and the commercial ANA Hep-2 test system with PBS. The discrepant results involved 15 different samples. All 15 were borderline positive when screened using PBS as the sample diluent and negative when screened using ZORBA-NS. One sample was positive on both tests that used PBS as the sample diluent. Seven samples were positive using the ZEUS ANA Hep-2 with PBS diluent. The other seven samples were positive on the commercial ANA/Hep-2 with PBS diluent. The discrepant samples were retested at 1:40 and titered in the ZEUS ANA Hep-2 (PBS) test system and the commercial ANA Hep-2 (PBS) test system. All 15 samples were again borderline reactive at 1:40, but negative at 1:80. Seventy-nine samples were positive by all three assays with no discrepancies in staining patterns. Twenty-five of the positive samples were used for an endpoint titer analysis. The endpoint titer results are as follows:

<table>
<thead>
<tr>
<th>ZEUS ANA Hep-2 with ZORBA-NS</th>
<th>Commercial ANA Hep-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Idenital Titer</strong></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>± one, two-fold dilution</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>± two, two-fold dilutions</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

REFERENCES


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