

## **CLSI DISCLAIMER:**

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For Individual Laboratory to Complete:

## ANA ELISA Screen Enzyme Immunoassay

<b>Laboratory Name</b>		
<b>Adopted</b>		
<b>Reviewed</b>		
<b>Reviewed</b>		
<b>Revised</b>		
<b>Supercedes</b>		

**Method:** Diamedix Corp., Immunosimplicity®  
Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO® Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

### Clinical Significance

Antinuclear antibodies (ANAs) directed against a variety of macromolecules occur in extraordinarily high frequency in systemic rheumatic diseases(1). Although these antibodies were first associated with systemic lupus erythematosus (SLE), the list of implicated diseases has expanded and many rheumatic diseases are characterized by the presence of one or more of these ANAs. For instance, anti-SSA/Ro and anti-SSB/La antibodies are associated with SLE and Sjogren's Syndrome (SS), anti-dsDNA and anti-Sm antibodies with SLE, anti-histone antibodies with SLE and Drug Induced Lupus, anti-RNP antibodies with mixed connective tissue disease (MCTD) and SLE, anti-Scl-70 antibodies with scleroderma (progressive systemic sclerosis (PSSJ), anti-Jo1 with polymyositis and dermatomyositis and anti-centromere antibodies with CREST syndrome(2,3,4).

The Immunofluorescence assay (IFA) has been used as the standard method in the detection of ANAs (5). Although the IFA is a sensitive test, it is laborious when testing large numbers of patient samples and is subject to errors from human interpretation and from variability in fluorescent microscopes (1). The IFA HEp-2 ANA test is also subject to the following concerns: it is sometimes insensitive to certain sera containing antibodies to SS-A, SS-B, Sm, or dsDNA (6) and it tends to find sera positive in a large number of patients who do not develop systemic rheumatic disorders within a followup two year period (7). The enzyme immunoassay (EIA) test system is an excellent alternative to the IFA test system for screening patient's serum for the presence of ANAs of clinical significance. The EIA test system efficiently screens large numbers of patient samples and reduces human error.

The Diamedix Is-ANA ELISA Screen collectively detects, in one well, total ANAs against double stranded DNA (dsDNA, nDNA), Histones, SS-A/Ro, SS-B/La, Sm, Sm/RNP, Scl-70, Jo-1, and centromeric antigens, along with sera positive for IFA HEp-2 ANAs. Sera positive on the ANA Screen should be tested for the specific autoantibodies indicative of various systemic rheumatic disorders. This test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

### Principle of the Procedure

Purified antigens (dsDNA, histones, SS-A/Ro, SS-B/La, Sm, Sm/RNP, Scl-70, Jo-1, centromere and other antigens extracted from HEp-2 nucleus) are bound to microwells. Antibodies to these antigens, if present in diluted serum, will then bind to the microwells. Washing of the microwells removes unbound serum antibodies. Horseradish peroxidase (HRP) conjugated anti-human IgG immunologically binds to the bound patient

antibodies forming a "conjugate - antibody - antigen" sandwich. Washing of the microwells removes unbound conjugate. An enzyme substrate, in the presence of bound conjugate, hydrolyzes to form a blue color. The addition of an acid stops the reaction, forming a yellow end product. The intensity of the color is measured spectrophotometrically at 450nm (reference 600-630nm).

### **Specimen Collection**

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 6 hours. If assays are not complete within 8 hours, serum should be refrigerated (2 to 8° C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Avoid the use of contaminated, hemolyzed, lipemic or icteric sera.

**CAUTION:** Serum samples must not be heat-inactivated prior to use.

### **Reagents**

<b>Antigen Wells</b>	Twelve, 8-well clear microwell breakapart strips, coated with purified antigens.
<b>Cut-Off Calibrator</b>	One vial with blue cap containing 0.25 ml human serum, weakly reactive for ANA antibodies, preserved with 0.1% sodium azide.
<b>Positive Control</b>	One vial with white cap containing 0.25 ml human serum, reactive for ANA antibodies, preserved with 0.1% sodium azide.
<b>Negative Control</b>	One vial with black cap containing 0.25 ml human serum, non-reactive for ANA antibodies, preserved with 0.1% sodium azide.
<b>Sample E Diluent</b>	One bottle with blue cap containing 60 ml Phosphate buffer with Tween 20 and protein stabilizers. Contains Proclin™ 300, 15 ppm active ingredient. Color-coded blue.
<b>Wash U Concentrate</b>	Two bottles with clear caps containing 50 ml of Phosphate buffer with detergent and Proclin™ 300, 15 ppm active ingredient. Each bottle is sufficient to make 1 liter of wash solution.
<b>Conjugate</b>	One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with horseradish peroxidase. Also includes protein stabilizers and Proclin™ 300, 15 ppm active ingredient. Color-coded pink.
<b>Substrate HRP</b>	One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' Tetramethylbenzidine).
<b>Stop O Solution</b>	One bottle with white cap containing 30 ml of 1N hydrochloric acid.

**CAUTION:** Acids are corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water. See Precautions section.

**Store these reagents at 2 to 8° C.**

## **Other Materials Required**

### **Manual Users:**

1. Wash bottle or automated microplate washer.
2. Pipettors capable of dispensing appropriate volumes.
3. Timer.
4. One liter graduated cylinder.
5. One liter wash solution reservoir.
6. Deionized or distilled water.
7. Absorbent toweling.
8. Tubes or microwell plate for serum dilution.
9. Reader capable of reading absorbance at 450nm, reference at 600 or 630 nm.

### **Diamedix Automated EIA System Users:**

1. One liter graduated container.
2. Deionized or distilled water.
3. Dilution containers as appropriate to system.
4. Sample and Reagent tips required by system.
5. Reagent containers required by system.

### **Warnings:**

1. Handle samples, Calibrator, controls and the materials that contact them as potential biohazards. Each donor unit in the standards and controls has been found negative for Hepatitis B surface antigen, Hepatitis C and HIV-1 & 2 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1 & 2, Hepatitis B virus, Hepatitis C or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/ National Institutes of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. Certain of the test reagents contain Proclin™ 300 as a preservative. When disposing of reagents containing Proclin™, flush with copious amounts of water to dilute the components below active levels.
5. Serum components contain sodium azide as preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the buildup of metal azide compounds.

## **Calibration**

This test uses an in-house reference standard (or Calibrator). The Calibrator has been derived from weakly positive sera and is titrated to an absorbance value equivalent to the cut-off of the assay. Samples whose absorbances exceed this value are considered positive for ANA and samples whose absorbances are less than this value are considered negative for ANA. To account for the inherent variations in enzyme immunoassays an equivocal range of +10% has been included at the assay cut-off.

## Quality Control

- a) Each time the assay is run, the Cut-Off Calibrator must be run in triplicate. The Diluent only Blank, Positive, and Negative Controls must be included in each test run.
- b) The absorbance of the Blank must be  $< 0.250$ .
- c) The absorbance of the Cut-Off Calibrator must be  $\geq 0.150$ .
- d) The Index Value of the Positive Control must be  $\geq 1.1$ .
- e) The Index Value of the Negative Control must be  $< 0.9$ .

**If any of these criteria is not met, the run is invalid and must be repeated.**

**Note:** Each lot of Is-ANA ELISA Screen reagents is validated during quality control testing using all antibodies. All antibodies are not represented in the Cut-Off Calibrator and Positive Control materials. Additional controls may be tested according to guidelines or requirements of local, state, or federal regulations or accrediting organizations. For guidance on appropriate quality control practices, please refer to NCCLS document C24-A, Internal Quality Control Testing: Principles and Definitions.

## Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding one bottle (50 ml) of Wash Concentrate to one liter of deionized or distilled H<sub>2</sub>O.

### Manual Users:

1. Prepare 1:101 dilutions of the Cut-Off Calibrator, controls and patient samples in Sample Diluent. (e.g., by addition of 5  $\mu$ l sample to 500  $\mu$ l Sample Diluent). The Cut-Off Calibrator must be run in triplicate.
2. Mix sample dilutions gently by withdrawing and expelling in a pipette 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100  $\mu$ l of diluted Cut-Off Calibrator (in triplicate), controls and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.  
  
*NOTE: Include one well which contains 100  $\mu$ l Sample Diluent only as a reagent blank. This will be used to "zero" the photometer before reading the test results.*
3. Allow the wells to incubate at room temperature (18-30°C) for 30  $\pm$  5 minutes.
4. Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by inverting the plate and tapping firmly on paper toweling. Wash the wells by rinsing 3 times with  $\sim$  300  $\mu$ l per well of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
5. Place 100  $\mu$ l of Conjugate into each well, avoiding bubble formation.

6. Allow the wells to incubate uncovered at room temperature (18-30° C) for 30 ± 5 minutes.
7. Wash the wells as described in Step 4 above.
8. Place 100 µl of Substrate into each well, avoiding bubble formation.
9. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.
10. Place 100 µl of Stop Solution into each well, avoiding bubble formation.
11. Read the absorbance of each well at 450 nm using a reference wavelength of 600-630 nm. The plate should be read within 60 minutes after the addition of the Stop Solution.

**Diamedix Automated EIA System Users:**

If using one of Diamedix's Automated EIA System, please refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

**Calculation of Results**

Calculate the MEAN absorbance of the three Cut-Off Calibrators. **Note:** Exclude any absorbance value that deviates by more than 15% from this MEAN. Use the MEAN of the remaining two replicates in the calculations. Exclusion of more than one of the three absorbance values invalidates the run.

$$\frac{\text{Absorbance of Sample}}{\text{Mean Absorbance of Calibrator}} = \text{Index Value of Sample}$$

*Example: Absorbance values obtained for the Cut-off Calibrator: 0.289, 0.268, 0.275 (after subtraction of Blank)*

*Mean Absorbance of Cut-Off Calibrator = 0.277*

*Sample Absorbance = 1.570 (after subtraction of Blank)*

*Index Value 1.570/ 0.277 = 5.67*

The Diamedix Automated EIA Systems will calculate and print results automatically.

## Reference Ranges

The following is only a guide to interpretation. **Each laboratory can establish its own "normal" ranges based on populations encountered.**

Index < 0.90	Negative for ANA antibodies.
Index $\geq$ 1.10	Positive for ANA antibodies.
Index 0.91-1.09	Equivocal* for ANA antibodies.

\* When equivocal results are obtained, the sample should be reported as equivocal, tested by another method, or a new sample should be tested.

## Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **E** Diluent, Wash **U** Concentrate, Substrate **HRP** and Stop **O** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8°C.
4. Incubations above or below the recommended temperatures or times may give erroneous results.
5. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.
6. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
7. (*Manual Procedure Only*) The washing procedure is very important and requires special attention. (Please refer to the Procedure section)  
**NOTE:** *Improperly washed wells may give erroneous results.*

## Limitations

1. The results obtained with the Diamedix Is-ANA ELISA Screen test kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves. Test results should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
2. The test should be performed on serum. The use of whole blood or plasma has not been established.
3. ANAs may be found in apparently healthy individuals.
4. Screening tests are used for testing entire populations or subsets of such populations for the presence of a characteristic. A negative screening result implies that the individual has a high probability of being free of the characteristic, whereas a positive test may reflect only the need for further testing.
5. The Diamedix Is-ANA ELISA Screen will not identify the specific type of ANA present in a positive sample. Confirmative testing for specific antibodies should be run if a positive result is obtained.

6. The performance characteristics of the Diamedix Is-ANA ELISA Screen test kit with automated equipment other than Diamedix Automated EIA Systems have not been established.

## References

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