For Individual Laboratory to Complete:

Laboratory Name		
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ENA-6 Screen
Enzyme Immunoassay

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

Systemic rheumatic disease is characterized by the presence of circulating autoantibodies that are widely reactive with both nuclear and cytoplasmic antigens. Antibodies to Sm (Smith) antigen are present in 25-40% of patients with systemic lupus erythematosus (SLE) and are considered to be highly specific markers for this disease (1). Antibodies to Sm/RNP are detected in up to 40% of patients with SLE (1,2). High titers of anti-RNP, in the absence of other autoantibodies, are correlated with mixed connective tissue disease (MCTD) (3). Antibodies to SSA (Ro) are present in approximately 60 to 70% of patients with Sjogren's Syndrome and 30 to 40% of patients with SLE (4). Antibody to SSA occurs in about 60% of patients with "ANA negative" SLE, 63% of patients with subacute cutaneous erythematosus and in 75% of the homozygous C2-deficient patients with an SLE-like presentation (5,6). Antibodies to SSB (La) are found in 11-24% of patients with SLE. They are also considered a serologic marker for Sjogren's Syndrome and are detected in up to 60% of these patients (4,7). Antibodies to Scl-70 are present in approximately 20-33% of patients with scleroderma, but are rarely seen in patients with other connective tissue disorders (1,8). Antibodies to Jo-1 are present in up to 35% of patients with polymyositis and much less commonly found in dermatomyositis. Anti-Jo-1 antibodies are rare in other rheumatic diseases (1,9).

Until recently, laboratories have detected ENAs by immunodiffusion or counter-immunoelectrophoresis. These methods are time consuming and insensitive relative to newer methods. Enzyme immunoassay (EIA) has advantages over these methods in terms of sensitivity, specificity, ease of automation and testing turnaround time.

The Diamedix Immunosimplicity® Is-ENA-6 Screen Test Kit is an EIA procedure intended for the detection of IgG antibodies to six ENAs. Positive samples can be further evaluated using specific ENA test kits. This test can be performed either manually or in conjunction with one of the Diamedix Automated EIA Systems.

Principle of the Procedure

Scl-70 and Jo-1) bound to the solid surface of a microtiter well. If IgG antibodies against any of these antigens are present in the samples they will bind to the respective antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (alkaline phosphatase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. Substrate is then added and incubated. In the presence of bound enzyme the substrate is converted to a colored end product. The absorbance of this end product can be read spectrophotometrically at 405 nm (reference 600-630 nm).

Specimen Collection

Whole blood should be collected by accepted medical techniques. The serum is separated from the clot and refrigerated at 2 to 8°C. Freeze sera at -20°C if not tested within 24 hours. 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. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used.

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

Reagents

Antigen Wells	Twelve, 8-well microwell breakapart strips, color-coded gold, coated with extractable nuclear antigens(SSA, SSB, Sm/RNP, Scl-70 and Jo-1).
Cut-Off Calibrator	One vial with blue cap containing 0.25 ml human serum, weakly reactive for ENA IgG antibodies,0.1% sodium azide.
Low Positive Control	One vial with white cap containing 0.25 ml human serum reactive for ENA IgG antibodies, 0.1% sodium azide.
Negative Control	One vial ith black cap containing human serum, non-reactive for ENA IgG antibodies, 0.2% sodium azide.
Sample Diluent	One bottle with blue cap containing 60 ml Phosphate buffer with protein stabilizers. Contains 0.1% sodium azide and Proclin™ 300, 90 ppm active ingredient. Color coded blue.
Wash Concentrate	Two bottles with clear caps containing 50 ml of Phosphate buffered saline with detergent and Proclin™ 300, 15 ppm active ingredient. Each bottle is sufficient to make 1 liter of wash solution.
Conjugate	One bottle with red cap containing 25 ml goat anti-human immunoglobulin G labeled with alkaline phos-phatase. Also includes protein stabilizers and

Proclin™ 300,30 ppm active ingredient. Color-coded pink.

Substrate One amber bottle with brown cap containing 25 ml buffered p-Nitrophenyl phosphate in a buffered solution. *Substrate solution may develop a slight yellow color upon storage.*

Stop Solution One bottle with white cap containing 30 ml of Sodium phosphate, tribasic. **CAUTION:**Solution is caustic. Avoid contact with skin. If contact is made, flush area with copious amounts of water.

These reagents should be stored 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 405nm, 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 Calibrator and controls has been found negative for Hepatitis B surface antigen and HIV-I antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1, Hepatitis B virus, 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", 1988.
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™ 300, flush drains with copious amounts of water to dilute the active components below active levels. 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 build up 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 anti-ENA antibodies and samples whose absorbances are less than this value are considered negative for anti-ENA antibodies. To account for the inherent variations in enzyme immunoassays an equivocal range of $\pm$ 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 Low Positive and Negative Controls must be included in each test run.

b) The absorbance of the Blank must be < 0.2.

c) The Index Value of the Positive Control must be ≥ 1.1.

d) The Index Value of the Negative Control must be ≤ 0.8.

e) The absorbance of the Cut-Off Calibrator must be ≥ 0.10.

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

NOTE: Each lot of Is-ENA-6 Screen reagents is validated during quality control testing using all six antibodies. All six antibodies are not represented in the Cut-Off Calibrator and Low Positive Control materials. Additional controls may be tested according to guidelines or requirements of local, state, or federal regulations or accrediting organizations.

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 50 ml of Wash Concentrate(20X) to one liter of deionized or distilled H_2O.

Manual Users:

1. Prepare 1:101 dilutions of the Cut-Off Calibrator, controls and patient samples in Sample Diluent. (e.g., by addition of 2 µl sample to 200 µl Sample Diluent or 5 µl sample to 500 µl Sample Diluent)
2. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 µl of diluted Cut-Off Calibrator, controls and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.

NOTE: *Include one well which contains 100 µl of Sample Diluent only as the reagent blank. This will ultimately 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 ± 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 ~300 µl Wash Solution. Remove excess moisture from the wells after each washing. When using an automated washer, follow the manufacturer's instructions.
5. Place 100 µl 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 405 nm and zero against the reagent blank. A suitable reference wavelength (e.g.,600-630 nm) reading should be used. Read the plate within 60 minutes of adding Stop Solution.

Refer to the BP-96 Plate Reader Operation Manual for complete instructions on set-up and operating procedures.

Diamedix Automated EIA System Users:

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

Calculation of Results

Calculate the mean absorbance of the Cut-Off calibrator. **Note:** When calculating the mean absorbance value for the Cut-Off Calibrator exclude any absorbance value that deviates by more than 15% from the MEAN absorbance value. Calculate the mean from the two remaining absorbance values. Exclusion of more than one of the three absorbance values invalidates the run.

$$\frac{\textbf{Absorbance of Sample}}{\textbf{Mean Absorbance of Cut-Off Calibrator}} = \textbf{Index Value of Sample}$$

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

Index Value 1.570/0.277 = 5.66

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 anti-ENA IgG antibodies.
Index ≥ 1	Positive for anti-ENA IgG antibodies.
Index 0.80-1.0	Equivocal*

*When equivocal results are obtained, samples can be reported as equivocal, retested, tested by another method or a new sample can be tested. Equivocal samples that give positive results upon retest should be reported as positive. Equivocal samples that give negative results upon retest should be reported as negative.

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample Diluent, Wash Concentrate, Substrate and Stop 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 Is-ENA-6 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. Positive antibodies to ENAs 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-ENA-6 Screen will not identify the specific type of anti-ENA present in a positive sample. Positive samples must be tested for individual antibodies using the individual ENA antibody tests.

6. The performance characteristics of the Diamedix Is-ENA-6 Screen Test Kit with automated equipment other than the Diamedix Automated EIA Systems have not been established.

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