

CLSI DISCLAIMER:

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For the Individual Laboratory to Complete:

**anti-Scl-70
Enzyme Immunoassay**

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| Laboratory Name | | |
| Adopted | | |
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| Revised | | |
| Supercedes | | |

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. Systemic sclerosis (scleroderma) is a chronic disease of unknown etiology which affects connective tissue and small arteries. It is characterized by fibrotic and degenerative changes in the skin, blood vessels, and internal organs.^{1,3}

Two major subtypes of scleroderma have been recognized. Diffuse scleroderma is a rapidly progressive form of the disease characterized by widespread changes in the skin, the distal and proximal extremities, the trunk and the viscera. The second type is limited scleroderma, also known as CREST (*Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasis*) syndrome. In this milder form of the disease, skin involvement is limited to the fingers, hands, or face and involvement of the internal organs can be delayed for years.^{2,3}

Two autoantibodies have been identified which are present almost exclusively in scleroderma.⁴ The first of these is the anti-Scl-70 antibody, which has been identified as antibody to the nuclear enzyme, topoisomerase I. 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.^{4,5} The second autoantibody, anti-centromere, is strongly associated with limited scleroderma or CREST syndrome. In addition, from 4 to 18% of CREST have anti-topoisomerase I (anti-Scl-70) antibodies.^{4,5} The detection of either of these autoantibodies permits a high degree of certainty in the diagnosis of scleroderma and can aid in differentiating the scleroderma subtypes.⁴

Until recently, many laboratories used fluorescent antinuclear antibody (FANA) and Immunodiffusion (ID) to detect anti-Scl-70 antibodies, However, neither of these methods is quantitative and their sensitivity, reproducibility, cost-effectiveness, and ease of performance are poor and

variable. Enzyme immunoassay (EIA) has advantages over the ID method in sensitivity, specificity, ease of automation, and testing turnaround time.⁶ The Diamedix Is-anti-Scl-70 Test Kit is an EIA procedure intended for the semi-quantitation of antibodies to Scl-70 antigen. The results are reported in ELISA units (EU) per ml determined by comparison to a Calibrator.

Principle of the Procedure

Purified Scl-70 antigen from bovine spleen and/or thymus is bound to microwells. Diluted patient sera, Calibrator, and controls are placed in the microwells and incubated. Anti-Scl-70 antibodies, if present, will bind to the antigen in the microwell. After washing the microwells to remove unbound antibodies, a second incubation with anti-human IgG conjugated to alkaline phosphatase is carried out. The conjugate will bind to human anti-Scl-70 antibodies, if present, forming an immunocomplex. The microwells are then washed again to remove unbound components and the enzyme substrate, para-nitrophenylphosphate is added. The enzyme, if bound, will catalyze the hydrolysis of the substrate to para-nitrophenol and result in a yellow color formation. The reaction is then stopped and the color read with a photometer at 405 nm (reference at 600-630 nm). The intensity of the color developed is proportional to the concentration of anti-Scl-70 IgG present in the sample.

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 (up to 7 days) for short term storage, or stored frozen at -20°C for long term storage. Avoid multiple freeze-thaw cycles. 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

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| Antigen Wells | Twelve, 8-well microwell breakapart strips, color-coded silver, coated with Scl-70 antigen. |
| Calibrator | One vial containing 0.25 ml of human serum, 0.1% sodium azide. Assigned value printed on label. |
| Negative Control | One vial containing 0.25 ml of non-reactive human serum, 0.1% sodium azide. |
| Positive Control | One vial containing 0.25 ml of reactive human serum, 0.1% sodium azide. Assigned range printed on label. |
| Sample Diluent | One bottle containing 60 ml Phosphate buffer with protein stabilizers. Contains Proclin™ 300, 15 ppm active ingredient. Color coded blue. |

- Wash Concentrate** Two bottles 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.
- Conjugate** One bottle containing 25 ml goat anti-human immunoglobulin G labeled with alkaline phosphatase. Also includes protein stabilizers and Proclin™ 300, 30 ppm active ingredient. Color-coded pink.
- Substrate** One bottle containing 25 ml para-Nitrophenyl phosphate in a buffered solution. *Substrate solution may develop a slight yellow color upon storage.*
- Stop Solution** One bottle containing 25 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 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. Tubes for pre-dilution of Calibrator and controls
5. Sample and Reagent tips required by system
6. Reagent containers required by system

Warnings:

1. Handle samples, calibrators, 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). This Calibrator has been prepared from a pool of sera strongly positive for the antibody under investigation. The Calibrator functions as an internal reference preparation and is assigned a unitage in ELISA units (EU) per ml. The Calibrator is included in every test run and is diluted and run in the same way as a test sample.

These tests have been optimized to permit the use of single point calibration. This is possible because the dose response curves are sufficiently linear and pass near to, or through the origin. The linearity of the dose response has been validated by the manufacturer during quality control testing.

Patient samples which contain very high levels of antibody may produce absorbance values greater than the Calibrator absorbance. Patient sample results greater than the Calibrator value should be reported as "Greater than Calibrator value EU/ml". If numerical results are required for such samples, dilute the sample using Sample Diluent and re-assay. Several dilutions (for example 1/10, 1/50 and 1/100) of the pre-diluted sample may be re-assayed simultaneously. Select the dilution that has an absorbance reading about 50% of the absorbance reading of the Calibrator; calculate the EU/ml for this dilution and multiply by the dilution factor to obtain estimated values.

Quality Control

- a) The Positive Control must be within its assigned range.
- b) The Negative Control must be < 16 EU/ml.
- c) The absorbance of the reagent blank must be < 0.30.

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

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 H₂O.

Manual Users:

1. Prepare 1:101 dilutions of the 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 calibrator, control, or patient sample, 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 tapping on paper toweling if necessary. Wash the wells by rinsing 3 times with at least 300 µl 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 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

Determine the EU/ml (ELISA Units/ml) for each patient specimen or control using the following formula:

$$\frac{\text{EU/ml of Calibrator}}{\text{Absorbance of Calibrator}} \times \text{Absorbance of Test Sample} = \text{anti-Scl-70 EU/ml of sample}$$

Reference Ranges

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

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| Less than 16 EU/ml Index < 0.80 | Negative for antibodies to Scl-70. |
| Greater than 20 EU/ml Index > 1 | Positive for antibodies to Scl-70. |
| 16 to 20 EU/ml Index 0.80-1.0 | Equivocal for antibodies to Scl-70. |

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 mix or interchange wells, controls, or calibrators from different lots.
2. Do not use reagents beyond their expiration date.

3. Incubations above or below the recommended temperatures or times may give erroneous results.
4. The ELISA 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.
5. Antigen coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
6. (*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 analysis of a single serum sample should not be used as the sole criterion for diagnosis of an autoimmune disease.
2. The results obtained with the Is-anti-Scl-70 Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves.
3. The test should be performed on serum. The use of whole blood or plasma has not been established.

References

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4. Steen, V.D., Powell, D.L. and Medsger, T.A. 1988. Clinical Correlations and Prodnosis Based on Serum Autoantibodies in Patients with Systemic Sclerosis. Arthritis Rheum. 31:(2) 196-203.
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