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MICROWELL ELISA
ENA IgG,A,M
Catalog No: 2552

INTENDED USE
The Diagnostic Automation, Inc. (DAI) ENA Enzyme-Linked Immunosorbent Assay (ELISA) is intended for the detection of antibodies to Sm, RNP, Ro, La, Scl-70, and Jo-1 in human sera. The assay is to be used to detect antibodies in a single serum specimen. The results of the assay are to be used as an aid to the diagnosis of systemic lupus erythematosis, mixed connective tissue disease, Sjogren syndrome, scleroderma, polymyositis, and dermatomyositis. This assay does not differentiate these conditions. For *in vitro* diagnostic use.

INTRODUCTION
Systematic autoimmune disease is characterized by the presence of circulating auto-antibodies directed to a wide variety of cellular antigens (1,2,3). Systemic lupus erythematosis (SLE), commonly referred to as Lupus, is the best known of these diseases. Other possible connective tissue diseases include mixed connective tissue disease (MCTD), Sjogren syndrome, sclerodema, and polymyositis/ dermatomyositis. The majority can be diagnosed by clinical presentation and their antibody profiles to the various antigens involved, which include dsDNA, SM, RNP, Ro, La, Scl-70, Jo-1, and Histones. Therefore, immunoassays for autoantibodies are useful for diagnostic and prognostic evaluations of autoimmune disease (1,2,3).

The SM (Smith) antigen is composed of nuclear RNA and several polypeptides. Antibodies to SM are present in approximately 30% of patients with SLE (systemic lupus erythematosis). SM is a very specific marker for SLE. SM antibodies are very rare in other autoimmune diseases and normals (1,2,3).

RNP antigen is composed of RNA and several protein components. The antigen is closely associated with SM antigen. Approximately 45% of patients with SLE have antibodies to RNP. RNP antibodies are also found in patients with discoid lupus, Sjogren syndrome, scleroderma, rheumatoid arthritis and MCTD (mixed connective tissue disease). Very high titers to RNP is a characteristic of MCTD (1,2,3).

Ro (SS-A) antigen consists of protein complexed with RNA. Approximately 60% of patients with Sjogren syndrome and 35% of SLE patients have antibodies to Ro. SLE patients with Ro antibodies alone (negative La/SSB) have a greater chance of developing nephritis (1,2,3).
La (SS-B) antigen consists of a protein complexed to RNAs. Approximately 60% of patients with Sjogren syndrome and 15% of SLE patients have antibody to La. Ro (SSA) antibody occurs in virtually all sera with La antibody. However, some SLE patients have Ro only antibody profiles (1,2,3).

Scl-70 antigen is a nuclear enzyme topoisomerase 1. Approximately 20-30% of patients with scleroderma have antibodies to Scl-70. The antibody is rarely seen in other conditions. Scl-70 antibodies are usually present in the diffuse subtype of scleroderma while antibodies to centromere proteins are dominant in the limited subtype of scleroderma or CREST syndrome (1,2,3).

Jo-1 antigen is the cellular enzyme histidy1-tRNA synthetase. Approximately 30% of patients with polymyositis (PM) and 10% of patients with dermatomyositis (DM) have antibodies to Jo-1. Antibodies to Jo-1 are seen rarely in normals and other diseases except for those which overlap with PM-DM (1,2,3).

Classically, antibodies to autoimmune antigens are detected by double immunodiffusion. However, the test is lengthy and suffers weak sensitivity. Enzyme-Linked Immunosorbent Assays (ELISAs) combine greater sensitivity with ease of use. Many ELISAs have been developed and validated for detecting autoantibodies to various antigens (4,5,6,7).

PRINCIPLE OF THE TEST
The DAI ENA test is an Enzyme-Linked Immunosorbent Assay to detect IgG, IgM, and IgA antibodies to Sm, RNP, Ro, La, Scl-70, Jo-1 antigens. Purified SM, RNP, Ro, La, Scl-70, Jo-1 antigens are attached to a solid phase microassay well. Diluted test sera are added to each well. If the antibodies are present that recognize the antigen, antigen-antibody complexes are formed. After incubation the wells are washed to remove unbound antibody. An enzyme labeled anti-human IgG, M, A, is added to each well. If antibody is present the conjugate will bind to the antigen-antibody complexes. After incubation the wells are washed to remove unbound conjugate. A substrate solution is added to each well. If enzyme is present the substrate will undergo a color change. After an incubation period the reaction is stopped and the color intensity is measured photometrically, producing an indirect measurement of specific antibody in the patient specimen.

KIT COMPONENTS

1. SM, RNP, Ro, La, Scl-70, Jo-1 antigen coated microassay plate: 96 wells, provided with a strip holder and stored in a foil bag with desiccant and humidity indicator card.
2. Wash Buffer (20x concentrate): One bottle, 50 mL. Contains buffer and Tween 80.
3. Serum Diluent: One bottle, 30 mL. Contains buffer, BSA and Tween 80.
4. Conjugate: One bottle, 15 mL. Contains horseradish peroxidase conjugated anti-human IgG, IgM and IgA in a buffer.
5. Substrate: One bottle, 15 mL. Contains 3, 3', 5, 5' - tetramethylbenzidine (TMB).
6. Stop Solution: One bottle,15 mL. Contains a H_2SO_4 solution.
7. High Positive Control: One vial, 0.2 mL. Contains human serum with antibodies that react strongly with the antigen. Established range printed on vial label.
8. Negative Control: One vial, 0.2 mL. Contains human sera with antibodies that do not react with the antigen. Established range printed on vial label.
9. Low Positive Control: One vial, 0.2 mL. Contains human serum with antibodies that react weakly with the antigen. Established range printed on vial label.
10. Calibrator: One vial, 0.2 mL. Contains human serum with antibodies that react with the antigen used to calibrate the assay. Kit specific Correction Factor printed on vial label.

REAGENT STORAGE CONDITION

1. All kit components that are stored at their recommended storage conditions are stable until the expiration date on their label. Do not use past their expiration date.
2. Antigen coated wells. Unused strips should be immediately resealed in the foil bags with desiccant and humidity indicator card and returned to storage at 2-8° C. If the bag is resealed with tape the wells are stable for 30 days. If the bag is resealed with a heat sealer the wells are stable until their expiration.
3. All other reagents are stored at 2-8° C in their original containers.
4. Store 1X (diluted) Wash Buffer at room temperature (21° to 25° C) for up to 5 days, or 1 week between 2° and 8° C.

PRECAUTIONS

1. Each donor unit used in the preparation of the Calibrator and Controls was tested by an FDA approved method for the presence of the antibody to HIV-1 as well as for hepatitis B surface antigen and found to be negative. Because no test method can offer complete assurance that human immunodeficiency virus (HIV-1), hepatitis B virus, or other infectious agents are absent, these specimen/reagents as well as patient samples, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control / National Institute of Health manual "Biosafety in Microbiology and Biomedical Laboratories", 1984 and FDA labeling guidelines for *in vitro* diagnostic reagent manufactures, sec. 1985. (8).
2. Certain reagents in this kit contain sodium azide for use as a preservative. Azides may react with lead and copper plumbing to form explosive azide compounds. When disposing of reagents, flush with copious quantities of water to minimize azide build up.
3. This product is for IN VITRO DIAGNOSTIC USE only.
4. Reagents contain preservatives which may be toxic if ingested.
5. Do not pipette by mouth. Avoid contact of reagents and patient specimens with skin or mucous membranes.
6. Do not allow the stop solution to contact skin or eyes. If contact occurs, immediately flush with copious quantities of water.
7. Avoid splashing or generation of aerosols.
8. Do not use heat inactivated sera.
9. Do not mix or interchange reagents between lots of kits or from other manufacturer.
10. Do not dilute or adulterate kit reagents.
11. Do not cross contaminate reagents or specimens.
12. Do not use TMB Substrate solution if it has begun to turn blue.
13. Reusable glassware must be washed out and thoroughly rinsed free of all detergents.
14. Do not vary reagent and incubation temperatures above or below room temperature (21 - 25° C).
15. Washing is important. Improperly washed wells will give erroneous results. Do not allow the well to dry out between incubations.

SPECIMEN COLLECTION

1. Aseptically collect blood samples by venipuncture and prepare serum using accepted technique (9).
2. Serum containing visible particulate matter can be spun down utilizing slow speed centrifugation.
3. Sera may be stored up to five days at 2-8° C. If a further delay in testing is needed store frozen at -20 to -70° C in a non-defrosting freezer. Avoid multiple freeze/thaw of patient samples.
4. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
5. Do not heat inactivate sera.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Wash bottle, automated or semi-automated microwell plate washing system.
2. Micropipettes, including multichannel, capable of accurately delivering 10-200 mL volumes (less than 3% CV).
3. One liter graduated cylinder.
4. Paper towels.
5. Test tube for serum dilution.
6. Reagent reservoirs for multichannel pipettes.

7. Pipette tips.
8. Distilled or deionized water, CAP Type 1 or equivalent.
9. Timer capable of measuring to an accuracy of +/- 1 second.
10. Disposal basins and 0.5% sodium hypochlorite (50 mL bleach in 950 mL H_20).
11. Single or dual wavelength microplate reader with 450 nm filter. If dual wavelength is used, set the reference filter to 600-650 nm. Read the operators' manual or contact the instrument manufacturer to establish linearity performance specifications of the reader.

PREPARATION OF REAGENTS

1. All reagents must be removed from refrigeration and allowed to come to room temperature (21 - 25° C) before use. Return all reagents to refrigerator promptly after use.
2. All samples and controls should be vortexed before use.
3. Dilute 50 mL of the 20X Wash Buffer to 1 L with distilled and/or deionized H_20. Mix well.

TEST PROCEDURE

1. Determine the number of patients to be assayed. For each assay the Calibrator should be run in duplicate. Also the High Positive Control, Low Positive Control, Negative Control, and a reagent blank (RB) should be run on each assay. Check software and reader requirements for the correct Calibrator/Control configurations.

   Example Configuration:

1A	RB	2A	Patient #3
1B	NC	2B	Patient #4
1C	Cal	2C	Patient #5
1D	Cal	2D	Patient #6
1E	HPC	2E	Patient #7
1F	LPC(run as 1st Pt)	2F	Patient #8
1G	Patient #1	2G	Patient #9
1H	Patient #2	2H	Patient #10

2. For each test serum, Calibrator and Control to be assayed prepare a 1:21 serum dilution. Add 10 µL of each serum sample to 200 µL of Serum Diluent . Mix well.
3. Remove the number of wells needed from the plate bag and arrange in a strip holder. The remaining strips should be resealed in the plate bag with desiccant and humidity card. The bag should be reheat sealed or rolled over and the end taped. If the color of the humidity card changes from blue to pink the strips should not be used.
4. Transfer 100 µL of the prediluted samples to the reaction wells, using a multichannel pipette. Withdraw and expel each sample at least three times to ensure proper mixing of the sample before transferring to the reaction plate. Use new fresh pipettes tips for each sample. Add 100 µL of Serum Diluent to the reagent blank.
5. Incubate the plate at room temperature (21-25° C) for 30 minutes $\pm$1 minute.
6. Wash the reaction plate three times with 1x Wash Buffer. Shake all of the liquid out of the wells. With a wash bottle, automated or semi-automated wash system fill each well with 250-300 µL Wash Buffer making sure no air bubbles are trapped in the wells. Shake all of the Wash Buffer out of the wells. Repeat the wash two more times. A total of up to 5 washes may be necessary with automated equipment. After the last wash shake out the Wash Buffer and remove residual Wash Buffer by tapping the plate firmly on a paper towel. The Wash Buffer can be collected in a basin and treated with 0.5% sodium hypochlorite (bleach) at the end of the days run.
7. Add 100 µL of the Conjugate to each well of the reaction plate, including reagent blank.
8. Incubate each well of the reaction plate at room temperature (21-25°C) for 30 minutes $\pm$ 1 minute.
9. Repeat wash as described in Step 6.
10. Add 100 µL of the TMB Substrate to each well of the reaction plate, including reagent blank.
11. Incubate each well of the reaction plate at room temperature (21-25° C) for 15 minutes $\pm$ 1 minute.
12. Add 100 µL of the Stop Solution to each well, including reagent blank, at the same rate as the TMB Substrate was added. Positive samples will turn from blue to yellow. Tap plate to ensure mixing.
13. Read the plate using a spectrophotometer at a wavelength of 450 nm. If dual wavelength is used, set the reference filter to 600-650 nm. Measure each optical density (OD) against the reagent blank. The plate should be read within 30 minutes of assay completion.

CALCULATIONS

1. Calibrator Value - Calculate the mean value for the Calibrator from the two Calibrator determinations.
2. Correction Factor - To account for day to day fluctuations in assay activity due to room temperature and timing, a correction factor is determined by DAI for each lot of kits. The correction factor is printed on the Calibrator vial.
3. Cutoff O.D. Value - The cutoff O.D. value for each assay is determined by multiplying the correction factor by the mean Calibrator value determined in step 1.
4. Index Value - Calculate an index value for each specimen by dividing the specimen O.D. value by the cutoff O.D. determined in step 3.

Example :
- O.D.s obtained for Calibrator = 0.38, 0.42
- Mean O.D. for Calibrator = 0.40
- O.D. obtained for patient sera = 0.60
- Correction factor = 0.50

Cutoff value	= 0.50 x 0.40 = 0.20
Index Value	= 0.60/0.20 = 3.00

INTERPRETATION OF RESULTS

Index Value	Interpretation
≤ 0.90	Negative
0.91 - 1.09	Equivocal
≥ 1.10	Positive

Specimens with index values in the equivocal range should be retested. If still equivocal retest by an alternate method or test a new sample.

QUALITY CONTROL

1. Calibrator and Controls must be run with each test run.
2. Reagent blank must be <0.15 O.D. at 450 nm.
3. The mean O.D. value for the Calibrator should be ≥ 0.30 at 450 nm.
4. The index values for the High, Low, and Negative Control should be in their respective ranges printed on the vials. If the control values are not within their respective ranges, the test should be considered invalid and the test should be repeated.
5. If above criteria are not met on repeat, contact DAI Technical Service.

LIMITATIONS

1. The result of the assay should not be interpreted as being diagnostic. The results should only be used as an aid to diagnosis. The results should be interpreted in conjunction with the clinical evaluation of the patient.
2. Sera from patients with other autoimmune diseases and from normal individuals may contain autoantibodies.
3. The assay should be used only with serum. Icteric, lipemic, hemolyzed and heat inactivated serum should be avoided.
4. Index Values of ≥ 10.00 should be reported as greater than 10.

EXPECTED VALUES

1. From 1-5% of apparently normal individuals may contain autoantibodies (1,2,3).
2. Antibodies to the various ENA's vary depending on the disease state. The following are the reported incidences of ENA antibodies in various diseases.

	DISEASE					
Antibody	**SLE**	**SS**	**PSS**	**MCTD**	**PM**	**DM**
SM	~ 30%	-	-	~ 2 %	-	-
RNP	~ 45%	~ 5 %	~ 25%	~ 95%	-	-
Ro	~ 30%	~ 60%	-	-	-	-
La	~ 15%	~ 60%	-	-	-	-
Scl-70	-	-	~ 25%	-	-	-
Jo-1	-	-	-	-	~ 30%	~ 10%

SLE - systemic lupus erythematosis
SS - Sjogren syndrome
PSS - scleroderma
MCTD - mixed connective tissue disease
PM - polymyositis
DM - dermatomyositis

PERFORMANCE CHARACTERISTICS

Sensitivity and Specificity

The DAI ENA ELISA kit was evaluated relative to a commercially available ELISA test kit Table 1 summarizes the data.

Table 1
Sensitivity and Specificity of the DAI ENA ELISA Kit
DAI ENA ELISA Kit

		Positive* ≥ 1.10	Equivocal 0.91-1.09	Negative < 0.90	Total
Alternate	Positive > 25	70	-	-	70
ELISA	Equivocal 20-25	1	-	-	1
Kit	Negative < 25	3	-	128	131

Total	74	0	128	202

Sera falling in the equivocal range were excluded from the following calculations

Relative Sensitivity	= 70/73	= 96 %
Relative Specificity	= 128/128	= 100 %
Relative Agreement	= 198/201	= 98.5 %

* The positive sera consisted of the following specificities SM-18, RNP-17, Ro-11, La-6, Scl-70-10, Jo-1-12

Precision

The precision of the DAI ENA ELISA kit was determined by testing seven different sera eight times each on three different assays. The data is summarized in Table 2. With proper technique the user should obtain C.V's of less than 15%.

Table 2
Precision Data

	Assay 1 (n=8)			Assay 2 (n=8)			Assay 3 (n=8)			Inter Assay (n=24)		
Serum #	X	S.D.	C.V.	X	S.D.	C.V.	X	S.D.	C.V.	X	S.D.	C.V.
1	6.89	0.319	4.63%	7.29	0.351	4.81%	7.46	.247	3.31 %	7.21	.383	5.31 %
2	3.24	0.416	12.8%	3.40	0.251	7.38%	3.67	.196	5.34 %	3.44	.342	9.94 %
3	6.24	0.253	4.05%	6.83	0.249	3.65%	6.94	.307	4.42 %	6.67	.407	6.10 %
4	5.67	0.186	3.28%	5.89	0.161	2.74%	6.24	.271	4.34 %	5.93	.312	5.26 %
5	5.95	0.382	6.42%	6.04	0.321	5.31%	6.36	.202	3.18 %	6.12	.348	5.69 %
6	0.17	0.065	38.2%	.273	0.061	22.3%	.197	.098	33.7 %	.261	.071	27.2 %
7	0.22	0.031	14.2%	.260	0.195	75.0%	.291	.170	86.3 %	.206	.149	72.3 %

X = Mean ENA Value
S.D. = Standard Deviation
C.V. = Coefficient of Variation

Linearity

The DAI ENA index values were determined for serial twofold dilutions of five positive sera. The index values were compared to $\log_2$ of dilution by standard linear regression. The data in Table 3 indicates that the assay is linear.

Table 3
Linearity

Serum #	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	r^2
1	6.53	5.59	4.61	3.78	3.10	2.25	1.38	0.78	0.997
2	6.40	4.83	3.45	2.38	1.57	1.07	0.74		0.944
3	5.21	4.23	3.53	2.94	2.40	1.93	1.43	1.01	0.982
4	8.97	8.22	7.39	5.98	4.61	3.14	1.87	1.14	0.991
5	8.05	7.44	6.53	5.24	4.17	2.52	1.68	1.09	0.988

r^2 = coefficient of determination. Linear regression compared ENA Index Value to $\log_2$ of dilution.

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