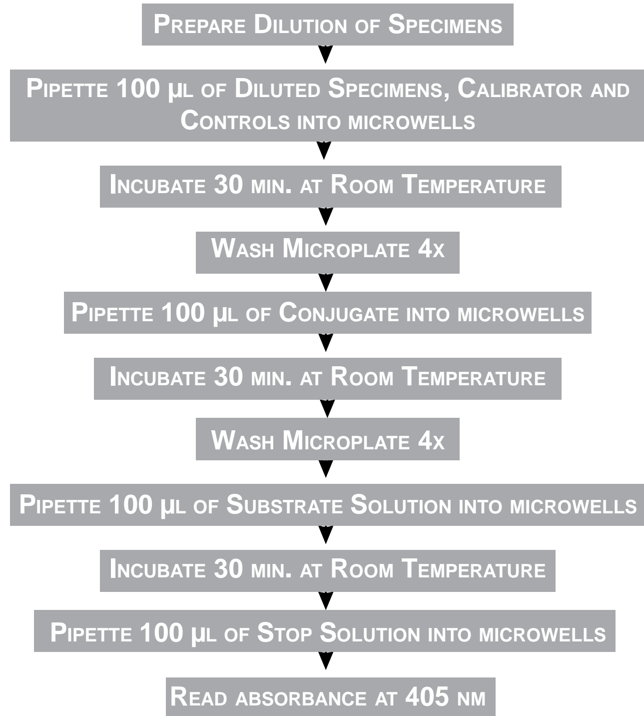
ImmuLisa™ Procedure at a Glance



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ImmuLisa™

Anti-Extractable Nuclear Antigen (ENA) Screen ELISA

For Research Use Only

PRODUCT INSERT

Catalog No. 1149 96 Determinations

INTENDED USE

Enzyme-linked immunosorbent assay for the detection and semi-quantitation of IgG antibodies to extractable nuclear antigens RNP, Sm, SS-A (Ro), SS-B (La), Jo-1 and Scl-70 in human serum of patients suspected with collagen vascular disorders.

SUMMARY AND EXPLANATION

Extractable nuclear antigens (ENA) are soluble ribonucleoprotein (snurps) complexes. Auto-antibodies directed against various ENA have proven to be of value in the diagnosis and monitoring of various systemic connective tissue diseases. Anti-Sm antibodies are disease specific and thus are a marker for *systemic lupus erythematosus* (SLE). Antibodies to Sm occur in approximately 30-40% of SLE patients. They are rare in other systemic connective tissue diseases and if present, indicate either overlap of disease or patients that have not yet fulfilled the ARA criteria for SLE[1-9]. Other antibodies such as those directed against SS-A (Ro), SS-B (La) and RNP are not disease specific. Antibodies to RNP occur in 35-45% of SLE patients and in over 95% of patients with *mixed connective tissue disease* (MCTD). Occasionally they are also found in *scleroderma*, *rheumatoid arthritis* and *drug induced LE*[1-6]. Patients with anti-RNP antibodies have a lower incidence of renal disease as compared to patients with anti-Sm antibodies. Antibodies to SS-A (Ro) and SS-B (La) occur in approximately 30-40% and 10-15% of SLE patients and 60-70% and 40-60% of patients with *Sjögren's syndrome* respectively. Antibodies to SS-B (La) occur frequently in association with SS-A (Ro) antibodies. Antibodies to SS-A (Ro) also occur in 60% of patients with *subacute cutaneous LE*, in almost all cases of neonatal LE and in two thirds of SLE patients with C2 deficiency[10-13]. Antibodies to Scl-70 are a specific serological marker for systemic scleroderma. Varying frequencies of Scl-70 antibodies have been reported. In early studies this antibody was detected in approximately 20% of patients with scleroderma but later studies reported an incidence of 75% in patients with diffuse scleroderma and 44% in patients with acrosclerosis[5].

Myositis specific antibodies are present in 25-40% of adult patients with idiopathic inflammatory myopathies and are mostly specific for cytoplasmic t-RNA synthetases[12]. Of the five anti-synthetase antibodies described, anti-histidyl-tRNA synthetase (Jo-1) antibodies in patients with myositis are associated with clinical features like interstitial lung disease, pulmonary fibrosis, Raynaud's phenomenon, fever and non-erosive symmetric small joint arthritis[12].
Antibodies against extractable nuclear antigens can be detected by various methods. Due to complexities in obtaining highly purified ENA antigens, gel immunodiffusion methods have traditionally been used. However, the ELISA methodology has many advantages over gel immunodiffusion:greater sensitivity[14], reduced assay times, no subjectivity in reading results, quantitation without serum titration and potential for automation. The ImmuLisa™ ENA Screen ELISA utilizes highly purified antigens to detect ENA antibodies.

PRINCIPLES OF PROCEDURES

The test is performed as a solid phase enzyme linked immunosorbent assay (ELISA). Microwells are coated with purified Sm, RNP, SS-A (Ro) or SS-B (La), Scl-70 and Jo-1 antigens followed by blocking the unreacted sites to reduce non-specific binding. Controls, calibrator and patient serum samples are incubated in the antigen coated wells which allows specific anti-ENA antibodies present in the serum to bind. Unbound-antibody and other serum proteins are removed by washing the microwells. Bound antibodies are detected by an enzyme labeled anti-human IgG conjugate added to the wells. Unbound conjugate is removed by washing. Specific enzyme substrate (pNPP) is then added to the wells and the presence of antibodies is detected by a color change produced by the conversion of pNPP substrate. The reaction is stopped and the intensity of the color change, which is proportional to the concentration of antibody, is read by a spectrophotometer. Results are expressed in ELISA units (EU)/ml and the results are reported as positive, borderline or negative.

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.**

Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (20-25°C) prior to use.

Coated microwell strips are for one time use only.

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Precision:

Two samples with known concentrations of ENA were assayed in 10 replicates over a period of two weeks. Intra- and inter-assay coefficient of variation (CV) were as follows:

	inter-assay % CV IgG	intra-assay %CV IgG
Sample 1	3.6	4.5
Sample 2	5.4	3.7

Recovery:

Samples with known anti-ENA concentrations were mixed with appropriate dilutions of another sample with known amounts of anti-ENA. Anti-ENA levels of the mixed samples were determined and from the values obtained the percent recovery were calculated. These results are as follows:

	Anti-ENA Ab. conc. added (EU/ml)	Anti- ENA Ab. conc. obtained (EU/ml)	% Recovery
Sample 1	85	81	95
Sample 2	34	37	107
Sample 3	51	57	112

Precautions

All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. However human blood derivatives and patient specimens should be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials[15].

WARNING - Sodium azide (NaN_3)may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources other than the same catalog number from IMMCO DIAGNOSTICS. Follow good laboratory pratices to minimize microbial and cross contamination of reagents when handling. Do not use beyond expiration date on the label.

Materials provided

ImmuLisa™ Anti-ENA Antibody Screen Test *Catalog No. 1149*

Kit contains sufficient reagents to perform 96 determinations.

12 x 8 Ready to use **Microplate** with individual breakaway microwells. Coated with ENA antigens.

1 x 1.5 ml *Ready to use **Positive Control** (*red cap*). Contains human serum. The expected concentration range is printed on the label in EU/ml.

1 x 1.5 ml *Ready to use **Negative Control** *(white cap)*. Contains human serum.

1 x 1.5 ml *Ready to use **Calibrator** *(green cap)*. Contains human serum The expected concentration in EU/ml is printed on the label.

1 x 12 ml *Ready to use **anti-human Alk. Phos. Conjugate**. Color coded pink.

1 x 60 ml *Ready to use **Serum Diluent**. Color coded blue.

1 x 12 ml *Ready to use **Enzyme substrate**. Contains pNPP. **Protect from light.**

1 x 12 ml Ready to use **Stop Solution**.

2 x vials **Powder Wash Buffer**. Reconstitute to one liter each.

1 extra Frame Holder

2 Protocol Sheets

*Contains <0.1% NaN_3

Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer or Automatic microplate washer capable of dispensing 200 µl
- Pipettes capable of delivering 5 µl to 1000 µl
- Disposable pipette tips
- Clean test tubes 12 x 75 mm and test tube rack
- Timer
- Absorbent paper
- Microplate reader capable of reading absorbance values at 405 nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm.

SPECIMEN COLLECTION AND HANDLING

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples.

PROCEDURE

Procedural Notes

- Before starting with the assay read carefully the product insert.
- Let serum specimens and test reagents equilibrate at room temperature for at least 30 minutes before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
- All dilutions of the patient samples should be prepared prior to starting with the assay.
- Good washing technique is critical. If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. **An automated microplate washer is recommended.**
- Use a multichannel pipette capable of delivering 8 wells simultaneously. This speeds the process and provides for a more uniform incubation time.
- For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
- Addition of all samples and reagents should be performed at the same rate and in the same sequence.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.

PERFORMANCE CHARACTERISTICS

The ImmuLisa™ Anti-ENA Antibody Screen ELISA Test was compared with the ENA ImmunoBlot. A total of 125 sera from a clinical reference laboratory were identified by immunodiffusion as being positive or negative for anti-RNP, anti-Sm, anti-SS-A (Ro) or anti-SS-B (La), anti-Scl-70 and anti-Jo-1. These sera were tested according to the procedure recommended by the manufacturer. The results are summarized in the following table:

Comparison of ELISA Methods for the Detection of Antibodies to ENA

ImmuLisa ENA Screen

ENA ImmuBlot	Positive	Negative	Total
Positive	50	2	52
Negative	0	73	73
Total	50	75	125

Agreement: 98%
Sensitivity: 96%
Specificity: 100%

EXPECTED VALUES

The incidence of ENA antibodies in various systemic connective tissue diseases is summarized in the following table:

Diagnostic Significance of Antibodies to Various Soluble Nuclear Antigens

Antibody Isotype	Disease Association
Sm	SLE - 10-40%
RNP	SLE - 20-30% MCTD - 95-100%
SS-A (Ro)	SLE - 15-33% SCLE - 60% Neonatal LE - 100% Sjögren's syndrome - 40-70%
SS-B (La)	SLE - 10-15% SCLE - 25% Sjögren's syndrome - 15-60%
Jo-1	Polymyositis - 32% Polymyositis overlap Dermatomyositis - 20%
Scl-70	Scleroderma - 20-40% Acrosclerosis - 44%

SLE = systemic lupus erythematosus
MCTD = mixed connective tissue disease
SCLE = subacute cutaneous lupus erythematosus

Note: The frequency of each antibody specificity in a disease represent compilation from the literature[3]. The incidence varies depending on the patient population.

Test Method

Step 1 Let all reagents equilibrate at room temperature.

Step 2 Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.

Step 3 Prepare a **1:101** dilution of patient specimen by pipetting **5µl** of serum into **0.5 ml** of Serum Diluent. **Mix well**.

Step 4 Remove the required microwells from pouch and return unused strips in the sealed pouch to refrigerator. Securely place the microwells into the extra provided holder.

Step 5 Pipette **100 µl** of Ready to Use Calibrator, Positive and Negative controls and patient samples to the appropriate microwells as per the sample layout below.

Note: Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank. The absorbance of the reagent blank should not be more than 0.3 when read against air.

Step 6 Incubate **30 minutes** (± 5 min) at room temperature.

Step 7 Wash **4x** with the wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper. For automatic washers, program the washer as per manufacturer's instructions.

	1	2	3	4
A	BLANK	S5		
B	NEG	S6		
C	POS	S7		
D	CAL	S8		
E	S1	S9		
F	S2	S10		
G	S3	S11		
H	S4	S12		

Step 8 Pipette **100 µl** of Conjugate into microwells.

Step 9 Incubate **30 minutes** (± 5 min) at room temperature.

Step 10 Wash all microwells as in Step 7.

Step 11 Pipette **100 µl** of Enzyme Substrate to each well in the same order and timing as for the conjugate.

Step 12 Incubate **30 minutes** (± 5 min) at room temperature.

Step 13 Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the enzyme substrate. Read absorbance within 1 hour of adding Stop Solution.

Step 14 Read absorbance of each microwell at **405 nm** using a single or 405/630nm dual wavelength microplate reader against the reagent blank set at zero absorbance.

Quality Control

Calibrator, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.3. The calibrator should have an absorbance reading of not less than 1.0, otherwise the test must be repeated. The positive control must give EU/ml in the range stated on the vial. If the test is run in duplicate the mean of the two readings should be taken for determining EU/ml.

RESULTS

Calculations

QUALITATIVE DETERMINATION

$$\frac{\text{Abs. of Test Sample}}{\text{Abs. of Calibrator}} \text{ X EU/ml of Calibrator = EU/ml Test Sample}$$

This method of calculation should be used only to determine whether a specimen is negative or positive.

Calibrator

The Calibrator must be included with each run. Patient samples containing higher antibody levels may give absorbance values greater than that of the Calibrator. In such an instance, these serum samples should be diluted further with Serum Diluent and retested. For determining EU/ml, multiply the units obtained by this dilution factor.

Interpretation

The following serves only as a guide in the interpretation of the results. Each laboratory must determine its own normal values.

Anti-ENA EU value	Interpretation
<20 EU/ml	Negative
20-25 EU/ml	Indeterminate (Borderline)
>25 EU/ml	Positive

LIMITATIONS OF THE PROCEDURE

The ImmuLisa™ Anti-ENA Screen ELISA should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples. This method should be used for testing human serum samples only. A diagnosis should not be made solely on the basis of ELISA test results alone.