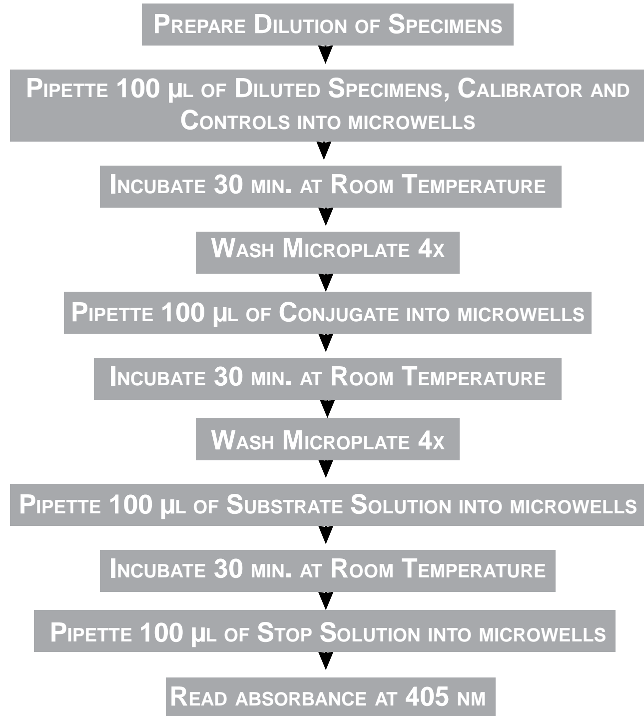
ImmuLisa™ Procedure at a Glance



For technical assistance please contact:

IMMCO Diagnostics, Inc.
60 Pineview Drive
Buffalo, NY 14228-2120
Telephone: (716) 691-0091
Fax: (716) 691-0466
Toll Free USA/Canada: 1-800-537-TEST
E-Mail: info@immcodiagnostics.com

or your local product distributor

ImmuLisa™ Anti-Extractable Nuclear Antigen (ENA) Antibodies ELISA

For *in vitro* Diagnostic Use

PRODUCT INSERT

Product Code: 1126	**anti-RNP/Sm**	96 Determinations
Product Code: 1127	**anti-Sm**	96 Determinations
Product Code: 1128	**anti-SS-A (Ro)**	96 Determinations
Product Code: 1129	**anti-SS-B (La)**	96 Determinations

INTENDED USE

Enzyme-linked immunosorbent assays for the detection and quantitation of IgG antibodies to extractable nuclear antigens (ENA) [RNP, Sm, SS-A (Ro), or SS-B (La)] in human serum. Antibodies to Sm aid in the diagnosis of systemic lupus erythematosus (SLE). RNP antibodies aid in the diagnosis of mixed connective tissue disease (MCTD) and SLE. SS-A (Ro) and SS-B (La) antibodies aid in the diagnosis of SLE, subacute cutaneous lupus erythematosus (SCLE) and Sjögren's syndrome.

SUMMARY AND EXPLANATION

ENA are soluble ribonucleoprotein (snurps) complexes. Autoantibodies 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].

These antibodies can be detected by various methods. Because of the difficulties in obtaining ENA antigens in a purified form, the gel immunodiffusion method has been widely used. Immunodiffusion exhibits sufficient specificity but is a qualitative method and lacks sensitivity. IMMCO Diagnostics has available ENA antigens in a highly purified form and has developed an ELISA method for the detection of ENA antibodies. ELISA methodology has many advantages over immunodiffusion: assay performance times are reduced, individual subjectivity in reading results is eliminated, quantitation is achieved without serum titration, there is potential for automation and greater sensitivity is achieved[14].

PRINCIPLES OF PROCEDURES

The test is performed as a solid phase enzyme linked immunosorbent assay (ELISA). Microwells are coated with purified RNP/Sm, Sm, SS-A (Ro) or SS-B (La) 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.

In the RNP test an Sm/RNP antigen is used to coat the wells since the RNP antigen is found associated with the Sm antigen. Therefore in order to calculate the EU/ml for RNP, the EU/ml of Sm for that same serum sample must be determined *(Cat. No. 1127)* and this value subtracted from the EU/ml obtained with the RNP test kit *(Cat. No. 1126)*.

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.

When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date. Reconstitute the wash buffer with distilled or deionized water.

REFERENCES

1. Tan EM. Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine. Adv Immunol 33: 167-240, 1982.
2. Tan EM. Special antibodies for the study of systemic lupus erythematosus. Arth Rheum 25: 753-756, 1982.
3. Tan EM, Chan E, Sullivan KF and Rubin RL. Antinuclear anti- bodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. Clin Immunol Immunopathol 47: 121-141, 1988.
4. Reichlin M. Current perspectives on serological reactions in SLE patients. Clin Exp Immunol 44: 1-10, 1981.
5. Reichlin M and Harley JB. Antibodies to extractable nuclear antigens: clinical significance. In “Immunopathology of the Skin”, Beutner EH, Chorzelski TP and Kumar V, Eds, John Wiley and Sons, New York, 3rd Ed, 555-563, 1987.
6. Kumar V, Beutner EH and Chorzelski TP. Autoimmunity and the skin. In “Concepts in Immunopathology”, Vol 1, Cruse JM and Lewis RE, Eds, Karger, Basel, 318-353, 1985.
7. McCarty GA. Autoantibodies and their relation to rheumatic diseases. Medical Clinics of North America 70: 237-261, 1986.
8. Hardin JA. The lupus autoantigens and the pathogenesis of systemic lupus erythematosus. Arth Rheum 29: 457-460, 1986.
9. Williamson GG and Boyle JA. Antigenic relatedness of small ribonucleoprotein particles. Biochem Biophys Acta 798149-155, 1984.
10. Clark G, Reichlin M and Tomasi TB. Characterization of a soluble cytoplasmic antigen reactive with sera from patients with systemic lupus erythematosus. J Immunol1l02:117-124, 1969.
11. Alspaugh MA and Maddison P. Resolution of the identity of certain antigen antibody systems in systemic lupus erythematosus and Sjogren’s syndrome: an interlaboratory collaboration. Arth Rheum 22: 796-798, 1979.
12. Sontheimer RD, Thomas JR and Gilliam JN. Subacute cutaneous lupus erythematosus subset. Arch Dermatol ll5: 1409-1415, 1979.
13. Sontheimer RD, Maddison PJ and Reichlin M. Serologic and HLA associations in subacute cutaneous lupus erythematosus, a clinical subset of lupus erythematosus. Ann Intern Med 97:664-671, 1982.
14. Maddison PJ, Skinner RP, Vlachoyiannopoukos P, Brennard DM and Hough D. Antibodies to nRNP, Ro (SS-A) and La (SS-B) detected by ELISA: their specificity and inter-relations in connective tissue disease sera. Clin Exp Immunol 62: 337- 245, 1985.
15. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control, National Institutes of Health, (HHS Pub No [CDC] 88-8395), 1988.

ImmuLisa™ SS-B (La)

Other ELISA	Positive	Negative	Total
Positive	14	1	15
Negative	5	42	47
Total	19	43	62

Agreement: 90%
Sensitivity: 93%
Specificity: 89%

Depending on the concentration of the analyte the intra-assay Coefficient of Variation (CV) for the ImmuLisa™ anti-ENA antibody test is 4-11%. The inter-assay CV is 4-10%.

Precautions

For *in vitro* Diagnostic Use. 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 practices 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 Test (RNP) *Product Code: 1126*
ImmuLisa™ Anti-ENA Antibody Test (Sm) *Product Code: 1127*
ImmuLisa™ Anti-ENA Antibody Test SS-A (Ro) *Product Code: 1128*
ImmuLisa™ Anti-ENA Antibody Test SS-B (La) *Product Code: 1129*

Kits contain sufficient reagents to perform 96 determinations each.

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

1 x 1.5 ml *Ready to use **Positive Control**. Contains human serum. 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.

4 x 1.5 ml *Ready to use **set of 4 Calibrators**; Calibrator A *(green cap),* Calibrator B *(violet cap),* Calibrator C *(blue cap) and* Calibrator D *(yellow cap)*. Contains human serum. Expected concentration in EU/ml is printed on the label.

1 x 12 ml *Ready to use **anti-human IgG 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

**CAUTION - Contains <0.1%* NaN_3

** Anti-ENA Antibody Test (RNP). *Product Code 1126* contains ready to use Positive Control, Calibrators, and anti-human IgG Conjugate.

** Anti-ENA Antibody Test (Sm). *Product Code* 1127 contains ready to use Positive Control, Calibrators, and anti-human IgG Conjugate.

** Anti-ENA Antibody Test [SS-A(Ro)]. *Product Code 1128* contains ready to use Positive Control, Calibrators, and anti-human IgG Conjugate.

** Anti-ENA Antibody Test [SS-B (La)]. *Product Code 1129* contains ready to use Positive Control, Calibrators, and anti-human IgG Conjugate.

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 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.**

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

PERFORMANCE CHARACTERISTICS

The ImmuLisa™ anti-ENA antibody tests [Sm, RNP, SS-A (Ro) or SS-B (La)] were compared with other commercially available ELISA test kits. A total of 67 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) antibodies.
These sera were tested according to the procedure recommended by the manufacturer. The results are summarized in the following tables:

Comparison of ELISA Methods
for the Detection of Antibodies to ENA

ImmuLisa RNP

Other ELISA		Positive	Negative	Total
	Positive	11	0	11
	Negative	5	46	51
	Total	16	46	62

Agreement: 92%
Sensitivity: 100%
Specificity: 90%

ImmuLisa™ Sm

Other ELISA		Positive	Negative	Total
	Positive	3	1	4
	Negative	5	53	58
	Total	8	54	62

Agreement: 90%
Sensitivity: 75%
Specificity: 91%

ImmuLisa™ SS-A (Ro)

Other ELISA		Positive	Negative	Total
	Positive	25	1	26
	Negative	9	27	36
	Total	34	28	62

Agreement: 84%
Sensitivity: 96%
Specificity: 75%

Calibrator

The Ready to Use Calibrators are included to provide semi-quantitation and must be used with each run. Patient samples containing higher antibody levels may give absorbance values greater than that of the Calibrator A. For determining accurate semi-quantitative values such serum samples should be further diluted so they fall within the range of the calibrator curve when retested. For determining EU/ml, multiply the units obtained by the dilution factor.

Interpretation

The following serves only as a guide in the interpretation of the results. Each laboratory must determine its own normal values. These may vary with the population examined.

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

LIMITATIONS OF THE PROCEDURE

The ImmuLisa™ anti-ENA Test 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.

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%

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

- 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.

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 For a **qualitative determination** use only the Ready to Use Low Calibrator D (*vial with yellow cap*)

or For a **semi-quantitative determination** use the Ready to Use Calibrators A through D as depicted in the sample layout below.

QUALITATIVE DETERMINATION

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

SEMI-QUANTITATIVE DETERMINATION

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

Step 4 Prepare a **1:101** dilution of patient specimen by pipetting **5µl** of serum into **0.5 ml** of Serum Diluent. **Mix well**.

Step 5 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 6 Pipette **100 µl** of Ready to Use Calibrator, Positive and Negative controls and patient samples to the appropriate microwells as per above sample layout.

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

Step 8 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.

Step 9 Pipette **100 µl** of Conjugate into microwells.

Step 10 Incubate **30 minutes** (± 5 min) at room temperature.

Step 11 Wash all microwells as in Step 7.

Step 12 Pipette **100 µl** of Enzyme Substrate to each well in the same order and timing as for the conjugate.

Step 13 Incubate **30 minutes** (± 5 min) at room temperature.

Step 14 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 15 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

Calibrators, 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 A should have an absorbance reading of not less than 1.0, otherwise the test must be repeated. The negative control must be <20 EU/ml. If the test is run in duplicate, the mean of the two readings should be taken for determining EU/ml. While performing Qualitative determinations, the absorbance of the Calibrator D must be greater than that of the negative control and lesser than the absorbance of the positive control. For semi-quantitative determinations the positive control must give values in the range stated on the vial.

RESULTS

Calculations

The concentrations of the patient samples can be determined by either of two methods:

1. QUALITATIVE DETERMINATION

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

2. SEMI-QUANTITATIVE DETERMINATION

Plot absorbance of Calibrator A through D against their respective concentration on a linear-linear graph paper. Plot the concentration in EU/ml on the X-axis against the absorbance of the Y-axis and draw the best fit curve. Determine the concentrations of the patient samples from the curve against its corresponding absorbance value.