INTENDED USE

The RhiGene MESACUP-2 TEST Sm is a semi-quantitative enzyme-linked immunosorbent assays for the detection of antibodies to Sm in human serum. The RhiGene MESACUP-2 TEST Sm is intended for *in vitro* diagnostic use as an aid in the determination of certain autoimmune diseases.

The RhiGene MESACUP-2 TEST RNP is a semi-quantitative enzyme-linked immunosorbent assays for the detection of antibodies to RNP in human serum. The RhiGene MESACUP-2 TEST RNP is intended for *in vitro* diagnostic use as an aid in the determination of certain autoimmune diseases.

The RhiGene MESACUP-2 TEST SSA (Ro) is a semi-quantitative enzyme-linked immunosorbent assays for the detection of antibodies to SSA in human serum. The RhiGene MESACUP-2 TEST SSA is intended for *in vitro* diagnostic use as an aid in the determination of certain autoimmune diseases.

The RhiGene MESACUP-2 TEST SSB (La) is a semi-quantitative enzyme-linked immunosorbent assays for the detection of antibodies to SSB in human serum. The RhiGene MESACUP-2 TEST SSB is intended for *in vitro* diagnostic use as an aid in the determination of certain autoimmune diseases.

The RhiGene MESACUP-2 TEST Scl-70 is a semi-quantitative enzyme-linked immunosorbent assays for the detection of antibodies to Scl-70 in human serum. The RhiGene MESACUP-2 TEST Scl-70 is intended for *in vitro* diagnostic use as an aid in the determination of certain autoimmune diseases.

SUMMARY AND EXPLANATION

Antinuclear antibodies (ANA) are a group of autoantibodies against various cell nuclear antigens, some which are considered to be quite useful as disease markers in autoimmune disorders. (1,2,3) Although antibodies specific to DNA have a high correlation with SLE (4), antibodies to a number of other nuclear antigens appear to be of diagnostic and/or prognostic significance in SLE and other diseases such as Progressive Systemic Sclerosis (5,6), Mixed Connective Tissue Disease (7), Sjögren's Syndrome (8), and Polymyositis (9), making ENA testing useful not only for SLE, but also as a general diagnostic tool for connective tissue diseases. (10)

RNP antibody is an ENA (extractable nuclear antigen) antibody, present with high frequency in the sera of patients with collagen diseases such as SLE and mixed connective tissue disease (MCTD). (11,12) Mixed connective tissue disease is a clinical disease combining features of SLE, Progressive Systemic Sclerosis (PSS) and Polymyositis/Dermatomyositis (PM/DM). The presence of RNP antibody in the sera of patients is essential for diagnosis of MCTD. RNP antibody, when present alone at high levels, is diagnostic of MCTD. Lower levels of anti-RNP, in conjunction with other autoantibodies may be observed in PSS, Sjögren's Syndrome (SS) and Rheumatoid Arthritis (RA). (7)

Sm antibody is an ENA antibody named after "Smith", a patient who suffered from Systemic Lupus Erythematosus (SLE). Sm antibody is frequently found with RNP antibody. It is detected in 10-30% of SLE patients. At high levels, Sm antibody is indicative of SLE and is rarely detected in other diseases. It has been adopted as a marker for diagnosis of SLE by the American Rheumatism Association (ARA). (13) Anti-Sm is observed at a high titer in the active period of SLE and at a low titer in the nonactive period. Raynaud's phenomena and nephropathy are reported more frequently in SLE patients testing positive for anti-Sm. (14)

SSA antibody is an ENA antibody present in high frequency (88-96%) in the sera of patients with Sjögren's Syndrome (SS) and SLE (24-60%). (16) SSA is also sometimes found in patients with Rheumatoid Arthritis, Myositis and Scleroderma. SSB antibody is an ENA antibody present in high frequency (71-87%) in Sjögren's Syndrome and in lower frequency in SLE (9-35%). (16) It has been confirmed that the SSA and SSB antibodies are identical with anti-Ro and anti-La respectively. (17) SSB antibodies are frequently associated with SSA antibodies and are rarely detected alone. SSB antibodies are seen almost exclusively in women; and, the presence of both SSA and SSB antibodies in mothers and infants are associated with skin lesions, neonatal lupus and congenital heart block. (16)

Scl-70 antibody is an ENA antibody present in high specificity in the sera of patients with Progressive Systemic Sclerosis (PSS). (15) The antigen of anti-Scl-70 is identified with topoisomerase I, one of the nuclear enzymes, thus Scl-70 antibodies are also called topoisomerase I antibodies. (18) Scl-70 antibodies, as well as centromere antibodies, in the sera of patients are essential for diagnosis of PSS. PSS is classified as two types; diffuse scleroderma and limited scleroderma. Scl-70 antibodies are present specifically in diffuse scleroderma and centromere antibodies are present in limited scleroderma. (19) Therefore, detection of both antibodies is useful for classification of PSS. Rarely, Scl-70 antibody is found in SLE and MCTD patients. (16)

Among the methodologies available to detect anti-ENA's are Immunoblot, Double Immunodiffusion (DID), Immunoprecipitation (IPP) and Enzyme-linked Immunosorbent Assay (ELISA). The ELISA tests are specific, sensitive, and due to their objectivity and rapidity, suitable for testing samples from patients with suspected connective tissue diseases. The RhiGene MESACUP-2 TESTS for Sm, RNP, SSA, SSB and Scl-70 each use recombinant protein (20), native purified protein (21) or both for the solid phase antigen. Therefore, the tests show specificity for detection of specific autoantibodies present in high frequency in the sera of patients

with certain connective tissue diseases.

PRINCIPLE OF PROCEDURE

The RhiGene MESACUP-2 TESTS utilize the commonly employed heterogeneous noncompetitive indirect solid-phase method of enzyme-linked immunosorbent assays (ELISA). The procedure is carried out in three basic reaction steps.

Brief Assay Procedure

	Add the following to microwells:	Sera are reacted with antigen-coated microtiter wells.
<Sample incubation>	1. Diluted sample (1:101) 100 µl	Antibodies, if present, will react with the antigens forming
(20-25 ºC) 60 min.		stable antigen-antibody complexes. If no antibodies are
	2. Wash 4X	present, the complexes will not form and the serum
		components will be washed away.
<Conjugate incubation>	3. Conjugate solution 100 µl	
(20-25 ºC) 60 min.		HRP conjugate is added to bind with the complexes
	4. Wash 4X	formed in step one. If no complexes are formed in step
		one, the conjugate will be washed away.
<Substrate incubation>	5. Substrate 100 µl	
(20-25 ºC) 30 min		TMB is added to produce a color change when reacted
	6. Stop solution 100 µl	with the complexes previously formed. After terminating
		the reaction by adding stop solution, the color intensity is
		measured photometrically. The intensity corresponds to
		the amount of antibody, if present, in the sera.
	7. Read and interpret	

REAGENTS AND STORAGE

ANTIGEN SUBSTRATE 96 wells coated with antigen produced from recombinant purified proteins and native purified proteins. Plates for each individual assay are coated as follows:

Sm: Native purified proteins of Sm-D nuclear antigens
RNP: Recombinant proteins 70kDa, A, C and *in vitro* transcribed U1 RNA
SSA: Purified native SSA/Ro
SSB: Recombinant protein 50kDa
Scl-70: Recombinant protein 70kDa

The twelve, 1 X 8 well breakaway strips packaged in a strip holder and sealed in a foil envelope with desiccant, are stable at 2-8°C until the labeled expiration date. Once the envelope is opened, unused strips are stable for 60 days stored at 2-8°C in the properly resealed envelope.

CONJUGATE REAGENT One vial containing 15 ml of horseradish peroxidase conjugated goat anti-human IgG, IgA and IgM (heavy chain specific), HEPES, saline, Bromphenol Blue, p-Hydroxyphenylacetic acid, Proclin 150, BSA at a pH of 6.5±0.2. Ready to use. Stable at 2-8°C until labeled expiration date.

CALIBRATOR 1 Two vials containing 1.5 ml of Sample Diluent. Ready to use-make no further dilution. Stable at 2-8°C until labeled expiration date.

CALIBRATOR 2 Two vials containing 1.5 ml each of antigen-specific positive human serum with Sample Diluent. Ready to use-make no further dilution. Stable at 2-8°C until labeled expiration date.

POSITIVE CONTROL SERUM One vial containing 0.2 ml of antigen-specific positive human serum with sodium azide and/or thimerosal. Stable at 2-8°C until labeled expiration date.

NEGATIVE CONTROL SERUM One vial containing 0.2 ml of ENA negative human serum with sodium azide and/or thimerosal. Stable at 2-8°C until labeled expiration date.

SAMPLE DILUENT Two 50 ml bottles containing Phosphate buffered saline, Tween-20, casein enzymatic hydrolysate, milk casein, E. coli extract, sodium azide, phenol red at a pH of 7.4±2. Ready to use. Stable at 2-8°C until labeled expiration date.

WASH BUFFER CONCENTRATE (10X) One 100 ml bottle containing sodium dihydrogen phosphate, sodium chloride and Tween-20 as a 10X concentrate. Stable at 2-8°C until labeled expiration date. The 1X solution is at a pH of 7.4± 0.2.

SUBSTRATE SOLUTION One 20 ml bottle containing 3,3',5,5'-tetramethylbenzidine dihydrochloride/hydrogen peroxide (TMB/H_2O_2). Ready to use. Stable at 2-8°C until labeled expiration date

STOP SOLUTION One 20 ml bottle containing 1N Sulfuric acid solution (H_2SO_4). Ready to use. Stable at 2-8°C until labeled expiration date.

WARNINGS AND PRECAUTIONS

1. This product is for *in vitro* diagnostic use only.
2. Control sera are derived from human serum, in which HBsAg, HCV antibody and HIV antibody cannot be detected. No test method, however, can guarantee the absence of these or any other infectious agents. These reagents and all patient samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Center for Disease Control/National Institute of Health manual, "Biosafety in Microbiology and Biomedical Laboratories", 1993 and FDA LABELING GUIDELINES FOR IN VITRO DIAGNOSTIC REAGENT MANUFACTURERS, DEC. 1985.
3. Matching lot numbers of microtiter antigen wells, conjugate reagent, calibrators, positive and negative controls must be used together in the assay. Do not substitute reagents from kits with different lot numbers or from other manufacturers.
4. All breakaway microtiter wells, which are not immediately required should be returned to the zip lock pouch with desiccant and must be carefully resealed to avoid moisture absorption. Wells that have been processed must be discarded.

5. Do not use kit components beyond their stated expiration dates.
6. Refrigeration (2-8°C) of kit immediately upon arrival will insure stability until labeled expiration date.
7. Do not expose the kit components to direct sun during storage or when in use during the assay. Store all kit components at 2-8°C.
8. All reagents must be brought to room temperature (20-25°C) before starting the assay. Do not open protective envelope with antigen microtiter wells before equilibration to room temperature to prevent moisture condensation in the wells. Return unused reagents to refrigerator promptly after use.
9. Previously frozen samples after thawing should be thoroughly mixed prior to testing.
10. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
11. Assay diluent may form precipitate. Before use in test, bring diluent to room temperature and mix well to dissolve precipitate. If all precipitate does not dissolve, allow the remaining precipitate to settle and avoid when using diluent. It does not affect test results.
12. Wash buffer concentrate may look turbid from crystal formation at 2-8°C, but this does not cause inconsistent results. Prior to preparation of the 1X wash buffer working solution, bring to room temperature and mix thoroughly to dissolve.
13. Avoid microbial contamination of all reagents and samples or incorrect results may occur.
14. Cross contamination of reagents and/or samples could cause false results. Carefully pipette samples and reagents without foaming or splashing to avoid cross contamination of microtiter wells. Mix reagents by gentle inversion. Do not vortex or shake.
15. Contamination of the TMB substrate solution with conjugate or other oxidants will cause the solution to change color prematurely.
16. Do not expose any of the reagents to sodium hypochlorite (bleach) solutions, or strong odors from these solutions. Exposure to the bleach may cause the biological activity of these reagents to be destroyed.
17. Strict adherence to specified assay instructions is essential. Incubation temperatures above or below normal room temperature (20-25°C), incubation outside the time period range, and inaccurate dilution of samples may yield erroneous results.
18. When adding reagents to antigen wells add them at the same rate and in the same order specimens, calibrators and controls were added. Then, before incubation, cover wells with a plate sealer and shake gently on counter to ensure an even spread of samples or reagents throughout the antigen well.
19. The wells must be rinsed with wash solution properly to avoid false positive or negative results.
20. Blot plates free of any residual wash solution before adding conjugate or substrate solution to prevent inconsistent color development. Do not allow wells to become dry during the assay procedure.
21. Ensure that the bottom of the plate is clean and dry, and that no air bubbles are present on the surface of the liquid in the wells before reading the plate.
22. In accordance with the principles of good laboratory practice, it is strongly recommended that all clinical specimens and assay materials, including the antigen microtiter wells, should be considered potentially infectious and handled and disposed of with all necessary precautions. Disposable implements (pipettes, tips, tubes, etc.) used in the test should be soaked in 0.5% sodium hypochlorite solution for at least one hour or autoclaved at 121°C for 30 minutes prior to discarding.
23. Liquid waste containing acid must be neutralized with base (e.g. sodium bicarbonate) before decontamination with sodium hypochlorite (bleach).
24. Acid-containing liquid waste that has been neutralized and other liquid waste should be collected and decontaminated by adding a sufficient volume of sodium hypochlorite to obtain a final concentration of 0.5%. A 30-minute exposure to 0.5% sodium hypochlorite is necessary to ensure effective decontamination.
25. Do not pipette by mouth. Care should be taken to avoid splashing and generation of aerosols. Avoid contact of reagents with eyes, skin, mucous membranes, and clothing. Wear disposable gloves, eye protection and lab apparel while handling specimens and performing the assay. Wash hands thoroughly when finished. If contact occurs, immediately flush with copious amounts of water.
26. Control sera and Sample Diluent contain sodium azide (0.1%) as a preservative. Azide may react with copper or lead in plumbing system to form explosive metal azides. Therefore, always flush with ample amounts of water when disposing of materials containing azide into a drain. Sodium azide is a poison and may be toxic if ingested.

SPECIMEN COLLECTION

Blood should be collected fasting or at least one hour after meals to avoid lipemic serum. Aseptically collect 5-8 ml of blood by approved venipuncture procedures. Allow the blood to clot at room temperature (20-25°C) and separate serum as soon as possible to minimize hemolysis. No anticoagulants or preservatives should be added. Avoid using sera exhibiting a high degree of lipemia, hemolysis, icterus or microbial growth. These conditions may cause aberrant test results. Do not use heat-inactivated serum as this affects test result.

Specimens should be stored refrigerated (2-8°C) and tested within one week of collection. If testing is delayed, store in aliquots at -20°C for up to one month or at -70°C or lower for longer periods. Avoid repeated freezing and thawing which may cause denaturation of protein and loss of antibody activity to yield erroneous results. Do not store in a self-defrosting freezer.

When testing multiple samples on the same patient to look for a significant change in antibody level, the specimens must be tested simultaneously in the same assay.

MATERIALS PROVIDED

1. Antigen-coated Microwells
2. Conjugate
3. Calibrators
4. ENA Negative Control
5. Antigen-specific Positive Control
6. Sample Diluent
7. Wash Buffer (10X Concentrate)
8. TMB Substrate Solution
9. Stop Solution
10. Sample Worksheet

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of reading at a duel wavelength of 450nm and 630nm
2. Calibrated multichannel 100 µl micropipette
3. Calibrated single-channel 10 µl micropipette

4. Disposable pipette tips
5. Reagent reservoirs for multichannel pipettes
6. Wash bottle or plate washer instrument
7. Distilled water-CAP Type one or equivalent
8. One liter graduate cylinder
9. 1.1 ml microtubes with 12 X 8 rack for sample dilution
10. Timer with alarm capable of measuring ± 1 second
11. Disposable serological pipettes, 1 ml and 5 ml
12. Disposal basin and bleach
13. Plate sealers
14. Paper towels
15. Felt tip marking pen

PROCEDURE

PREPARATION OF REAGENTS

1. Remove all kit test materials from refrigeration and allow them to warm to room temperature (20-25°C) prior to use. Return unused materials to refrigerator (2-8°C) promptly after use.
2. Prepare Worksheet:
   a. Determine number of samples, controls and calibrators to be tested. Each sample and control requires one antigen-coated well. Calibrators are to be run in duplicate.

   b. On the worksheet, indicate the location of the samples, controls and calibrators to be placed in the microtiter wells.
   Note: Depending on available software, check for correct sample, control and calibrator configuration.
3. Antigen Microtiter Wells:
   a. Remove the required number of microtiter wells and place in holder.
   Note: To prevent moisture condensation in the wells, do not open protective envelope with the antigen microtiter wells before equilibration to room temperature.
   b. Promptly return unused strips to their packaging with desiccant, seal and return to refrigerator (2-8°C). After the envelope has been opened, the unused strips are stable for 60 days.
4. Wash Solution:
   a. Prior to preparation, bring 10X concentrate to room temperature and mix thoroughly to dissolve residual crystals that may be presen

   b. Prepare a 1:10 dilution of the Wash Buffer Concentrate with distilled water prior to use in assay.
   Note: Do not dilute all of the 100 ml concentrate at one time if more than 2 weeks is anticipated to use up entire kit.
   c. For example: add 100 ml of 10X concentrate to 900 ml of distilled water. Mix thoroughly.
   d. The diluted wash solution is stable for 2 weeks at 2-8°C.
5. Other Reagents: Calibrators, Sample Diluent, Conjugate Reagent, TMB Substrate Solution and Stop Solution are ready to use with no additional preparation needed.

PREPARATION OF SAMPLES AND CONTROLS

1. If samples and controls are refrigerated, remove and allow to come to room temperature. If samples are frozen, after thawing, they should be thoroughly mixed.
   Note: Do not use heat-inactivated sera. Avoid using sera exhibiting a high degree of lipemia, hemolysis, icterus or microbial growth.
2. Prepare a 1:101 dilution of each patient serum and the Positive and Negative Controls. To the 1.1 ml microtubes in the 12 X 8 rack, add 10 µl of each sample to 1 ml of the Sample Diluent. Mix well.
3. Diluted samples may be stored at 2-8°C for up to 3 days.

TEST PROCEDURE STEPS

1. **SAMPLE APPLICATION** Using the multi-channel pipettor, transfer 100 µl of each diluted sample, Positive and Negative Controls into the appropriate microtiter wells of the antigen test plate. Add Calibrators directly to appropriate wells with the single channel pipettor. (Do not dilute Calibrators.)

2. **SAMPLE INCUBATION** Cover wells with a plate sealer and incubate at room temperature (20-25°C) for 60 ± 5 minutes.

3. **WASH 1** Remove plate sealer. Aspirate or discard the well contents. To wash, fill the well with Wash Solution and then aspirate or discard the contents completely. Wash 4 times. Tap the plate on a paper towel to remove any remaining Wash Solution.
   Note: If using an automated wash instrument, set the dispensing volume at 300-350µl. Set the wash cycle for 4 washes with no delay between washes. When wash cycle is complete, invert plate on a paper towel to remove any remaining wash solution.

4. **CONJUGATE APPLICATION** Measure needed amount of Conjugate directly from bottle with a disposable serological pipette into a new reagent reservoir. Using the multichannel pipette, add 100 µl of the Conjugate to each well at the same rate and in the same order as the samples, controls and calibrators. Discard any remaining Conjugate Reagent in the reservoir. Do not return any leftover reagent to the Conjugate bottle.

5. **CONJUGATE INCUBATION** Cover the wells with the plate sealer and incubate at room temperature (20-25 °C) for 60 ± 5 minutes.

6. **WASH 2** Remove the plate sealer and wash the microtiter plate following the same procedure as the first wash in step 3.

7. **SUBSTRATE APPLICATION** Measure needed amount of TMB Substrate Solution directly from bottle with a disposable serological pipette into a new reagent reservoir (different than conjugate reservoir). Using the multichannel pipette, add 100 µl of the solution to each well at the same rate and in the same order as the samples, controls and calibrators. Discard any remaining Substrate Solution

in the reservoir. Do not return any leftover solution to the reagent bottle.

8. **SUBSTRATE INCUBATION** Cover wells with plate sealer and incubate at room temperature (20-25°C) for 30-35 minutes.

9. **STOP REACTION** Remove the plate sealer. Using a disposable serological pipette, measure out the Stop Solution into a new reagent reservoir (different from the conjugate or substrate reservoir). Using the multichannel pipette, add 100 µl of the solution to each well at the same rate and in the same order as the samples, controls and calibrators. Discard any remaining Stop Solution in the reservoir. Do not return any leftover solution to the reagent bottle.

10. **READING** Reading on a plate reading spectrophotometer should be done as quickly as possible after stopping reaction (within 30 minutes). Read the absorbance of each well at 450 nm. If a dual wavelength plate reader is available, set the test wavelength at 450 nm and the reference at 630 nm. An air blank is used for the initial blanking of the reader.

11. **CALCULATION OF RESULTS** For results calculated by reader program or manually, use the following formula:

    Cut off index (U/ml) = (A_{450} <Sample> - A_{450} <Mean Neg. Calibrator>) / (A_{450} <Mean Pos. Calibrator> - A_{450} <Mean Neg. Calibrator>) X 100

    A_{450} is the abbreviation of absorbance value (O.D.) at 450nm.

    For Example: A_{450} (O.D.) of sample = 0.643, Mean A_{450} (O.D.) of Neg. Calibrator = 0.021, Mean A_{450} (O.D.) of Pos. Calibrator = 1.194

    (0.643 - 0.021) / (1.194 - 0.021) X 100 = 53.026 U/ml , → 53.026 U/ml is interpreted as Positive

QUALITY CONTROL

Each time the assay is performed, the Positive Control, Negative Control and Calibrators must be included within each test run.
The index values for the Calibrators must meet the following criteria: respective ranges:

Calibrators	Mean O.D. Range at A_{450}
Negative Calibrator	Less than 0.100
Positive Calibrator	Greater than 0.700

The Positive and Negative Controls must give the following results:

Autoantigen	Index Value - U/ml	
	Positive Control	Negative Control
Sm	>100	<7
RNP	>60	<15
SS-A	>100	<10
SS-B	>100	<15
Scl-70	>100	<16

If the results for Calibrators and Controls do not meet their respective specifications, the assay should be considered invalid and should be repeated. If, upon repeat testing, the Control and Calibrator results are still incorrect, do not report patient results.

INTERPRETATION OF RESULTS

The results of the RhiGene MESACUP-2 TEST are interpreted as follows:

Autoantigen	Interpretation – Index Value (U/ml)		
	Negative	Equivocal	Positive
Sm	<7	7-29	≥30
RNP	<15	15-21	≥ 22
SS-A	<10	10-29	≥30
SS-B	<15	15-24	≥25
Scl-70	<16	16-23	≥24

TROUBLESHOOTING
To ensure consistent performance, possible problems, their causes and the solutions to correct or prevent them are mentioned below:

1. Calibrator and/or Control values incorrect - Before repeating assay, review the procedural items such as reagent temperatures, incubation temperatures, incubation time periods, and washing times or set-up of autowasher. Review handling techniques such drying of antigen wells after wash steps and splashing of reagents to cause cross contamination. Re-QC pipetting device if volume accuracy is in question.
2. No color development in substrate - Make sure samples and controls have been added during dilution preparation. Make sure conjugate has been added properly; or, perhaps kit was stored at elevated temperatures for an extended period of time causing deterioration of conjugate. Conjugate may have become contaminated which could also cause deterioration. Review proper storage instructions and the handling and preparation of reagents.
3. Substrate color too light - Use of kit beyond its stated expiration date can be a cause for poor color development in the wells. Check proper storage conditions.
4. Substrate color too dark - The appearance of darker colors throughout the antigen wells is usually an indication that the wells were not properly washed particularly after the incubation with the enzyme conjugate. Review instructions.

LIMITATIONS OF THE PROCEDURE

1. As with other diagnostic test procedures, the results obtained with the RhiGene MESACUP-2 TESTS serve only as an aid to diagnosis and should not be interpreted as diagnostic by itself. The results should be interpreted in conjunction with clinical evaluation of the patient along with other diagnostic procedures by medical authorities.
2. SSB antibodies are frequently found with SSA antibodies; presence of SSB antibodies alone is rare. Testing for anti-SSA and anti-SSB for diagnostic purposes should be done simultaneously. The results should be interpreted with caution and related to the clinical findings.
3. Sm antibodies are frequently found with RNP antibodies; presence of Sm antibodies alone is rare. Testing for anti-Sm and anti-RNP for diagnostic purposes should be done simultaneously. The results should be interpreted with caution and related to the clinical findings.
4. Some autoantibodies may be present in some healthy donors at the time of testing. Relatives of patients with autoimmune disease have also been found to have such autoantibodies.
5. Positive test results may not be valid in persons who have received blood transfusions or various blood products within the past several months.
6. Test results on specimens from immunosuppressed patients and pregnant women may be difficult to interpret.

EXPECTED VALUES

A negative result is the expected value for a normal patient. Various autoantibodies detected with the RhiGene MESACUP-2 TESTS are summarized in TABLE 1 with respect to their associated diseases and relative frequency of occurrence. (16)

TABLE 1 - Autoantibodies and Disease Associations (16)

ANTIBODY	DISEASE STATES	RELATIVE FREQUENCY
Anti-Sm	SLE	15-30% (1)
Anti-RNP	MCTD SLE Polymyositis/Scleroderma	95-100% (2) 30-40% 2-24%
Anti-SSA (Ro)	Sjögren's Syndrome SLE Polymyositis/Scleroderma	88-96% 24-60% 5-10%
Anti-SSB (La)	Sjögren's Syndrome SLE	71-87% 9-35%
Anti-Scl-70	Systemic Sclerosis	20-59% (1)

(1) Highly specific marker antibody, (2) Highly specific when present alone at high titers.

Serum samples from 99 (50 for RNP) healthy blood donors in the Mid-central United States (and Japan for RNP) were assayed with the RhiGene MESACUP-2 TESTS, The results in TABLE 2 show the calculated expected values (%) interpreted as negative:

TABLE 2 – Summary of Results on Normal Donors

Results:	Sm	RNP	SSA	SSB	SCL-70
Positive	0	1	0	0	0
* Equivocal	1	1	1	0	1
Negative	98	48	98	99	98
Expected Values:	100% (98/98)	98% (48/49)	100% (98/98)	100% (99/99)	100% (98/98)

* Equivocal results are excluded from calculations

Each laboratory should establish and maintain its own expected values based on samples from the populations they typically encounter.

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SPECIFIC PERFORMANCE CHARACTERISTICS

COMPARATIVE STUDIES

To investigate the relative specificity and sensitivity of the RhiGene MESACUP-2 TESTS, serum samples were compared with other commercially available ENA ELISA test systems. Specificity was evaluated using 164 negative samples from Japan and the United States*. Sensitivity was evaluated using specific disease-state characterized sera from Japan for each type of ENA. The results of these studies summarized in TABLES 3-7 show the calculated relative sensitivity, relative specificity and overall agreement. (* different group of samples were used for RNP)

TABLE 3: Summary of Relative Comparison Testing: **MESACUP-2 Test Sm**					Rel. Sens.	Rel. Spec.
		Positive	Negative	Equivocal	94.4%	90%
Predicate EIA kit	Positive	17	0	1*	17/18	
	Negative	11*	218	13 *		218/242
	Equivocal	1**	0	0	**Excluded from calculations	

Relative overall agreement is 90.4% (235/260)
* Discrepants: 12 of 12 positive by RhiGene ELISA were positive by third ELISA method
14 of 14 equivocal by RhiGene ELISA were negative by third ELISA method

TABLE 4: Summary of Relative Comparison Testing: **MESACUP-2 Test RNP**					Rel. Sens.	Rel. Spec.
		Positive	Negative	Equivocal	99.6%	91.6%
Predicate EIA kit	Positive	226	1*	0	226/227	
	Negative	15*	163	3**		163/178
	Equivocal	10**	1 **	2**	**Excluded from calculations	

Relative overall agreement is 92.5% (234/253)
*Discrepants: One negative by RhiGene MESACUP-2 TEST RNP was negative by in-house DID
12 of 15 positive by RhiGene MESACUP-2 TEST RNP were positive by in-house DID.

TABLE 5: Summary of Relative Comparison Testing: **MESACUP-2 Test SSA**					Rel. Sens.	Rel. Spec.
		Positive	Negative	Equivocal	100%	93.9%
Predicate EIA kit	Positive	109	0	0	109/109	
	Negative	2 *	168	9 *		168/179
	Equivocal	0	0	0		

Relative overall agreement is 96.2% (277/288)
*Discrepants: 2 of 2 positive by RhiGene ELISA were negative by third ELISA method
9 of 9 equivocal by RhiGene ELISA were negative by third ELISA method

TABLE 6: Summary of Relative Comparison Testing: **MESACUP-2 Test SSB**					Rel. Sens.	Rel. Spec.
		Positive	Negative	Equivocal	82.3%	96%
Predicate EIA kit	Positive	51	6 *	5 *	51/62	
	Negative	5 *	218	4 *		218/227
	Equivocal	0	1 **	1 **	**Excluded from calculations	

Relative overall agreement is 93.1% (269/289)
*Discrepants: 1 of 5 positive by RhiGene ELISA was positive by third ELISA method
6 of 7 negative by RhiGene ELISA were negative by third ELISA method
9 of 9 equivocal by RhiGene ELISA were negative by third ELISA method

TABLE 7: Summary of Relative Comparison Testing: **MESACUP-2 Test Scl-70**					Rel. Sens.	Rel. Spec.
		Positive	Negative	Equivocal	96.7%	99.4%
Predicate EIA kit	Positive	29	1 *	0	29/30	
	Negative	0	163	1 *		163/164
	Equivocal	0	0	0		

Relative overall agreement is 99% (192/194)
*Discrepants: 1 negative by RhiGene ELISA was negative by third ELISA, 1 equivocal by RhiGene ELISA was negative by third ELISA

REPRODUCIBILITY (Precision) STUDIES

Interlot precision of the RhiGene MESACUP-2 TESTS was evaluated by testing three (3) samples: strong positive, low positive and negative. Each sample was tested in duplicate five (5) times on three (3) different lots. The results are summarized in TABLE 8.

TABLE 8 - Summary of Interlot Precision

Elisa Assay	Specimens:	Strong Positive			Low Positive			Negative		
	Lots:	Mean	SD	% CV	Mean	SD	% CV	Mean	SD	% CV
Sm	Lot 1	165.13	3.38	2.05	53.45	1.60	2.99	0.18	0.08	NR
	Lot 2	165.39	0.96	0.58	56.17	2.14	3.80	0.17	0.10	NR
	Lot 3	158.39	2.11	1.33	56.69	0.78	1.37	0.16	0.24	NR
Precision between 3 lots		162.97	3.97	2.43	55.43	1.74	3.14	0.17	0.01	NR
RNP	Lot 1	146.88	1.34	0.91	66.79	1.58	2.36	0.54	0.09	NR
	Lot 2	145.84	2.88	1.98	66.53	0.59	0.88	0.54	0.07	NR
	Lot 3	145.07	1.34	0.92	65.66	1.37	2.09	0.22	0.09	NR
Precision between 3 lots		145.93	0.74	0.51	66.33	0.48	0.73	0.43	0.15	NR
SSA	Lot 1	180.74	15.87	8.8	36.76	1.47	4.00	-0.07	0.11	NR
	Lot 2	160.20	11.46	7.16	35.70	2.91	8.14	0.33	0.18	NR
	Lot 3	168.61	8.28	4.91	35.79	0.88	2.45	0.37	0.81	NR
Precision between 3 lots		169.85	10.33	6.10	35.33	0.71	2.01	0.21	0.24	NR
SSB	Lot 1	205.31	3.36	1.64	52.92	0.64	1.22	0.33	0.14	NR
	Lot 2	213.65	2.29	1.07	51.57	1.04	2.01	0.34	0.15	NR
	Lot 3	217.08	4.85	2.24	49.36	1.53	3.10	0.03	0.14	NR
Precision between 3 lots		212.01	6.06	2.86	51.28	1.80	3.51	0.23	0.17	NR
Scl-70	Lot 1	249.88	5.11	2.05	73.35	1.65	2.23	0.07	0.21	NR
	Lot 2	248.17	6.40	2.58	81.02	2.33	2.88	0.02	0.13	NR
	Lot 3	246.31	3.60	1.46	80.24	1.51	1.88	-0.37	0.26	NR
Precision between 3 lots		248.12	1.79	0.72	78.20	4.22	5.40	-0.10	0.24	NR

NR: Not Relevant

Intralot precision of the RhiGene MESACUP-2 TESTS was evaluated by testing three (3) samples: strong positive, low positive and negative. Each sample was tested in duplicate five (5) times on the same assay lot on three (3) different days. The results are summarized in TABLE 9. Additional intralot precision testing was performed by testing each of the three (3) samples eight (8) times on different strips within the same plate and on different plates from the same lot and also repeated in different runs. Each mean value represents 64 individual values. The results are summarized in TABLE 10.

TABLE 9 - Summary of Intralot Precision

Elisa Assay	Specimens:	Strong Positive			Low Positive			Negative		
	Times:	Mean	SD	% CV	Mean	SD	% CV	Mean	SD	% CV
Sm	Day 1	170.88	1.66	0.97	51.77	1.85	3.58	-0.14	0.20	NR
	Day 2	170.75	3.42	2.00	54.84	0.73	1.32	-0.02	0.07	NR
	Day 3	165.13	3.38	2.05	53.43	1.61	3.02	0.18	0.08	NR
Precision between 3 days		168.92	3.28	1.94	53.34	1.54	2.88	0.01	0.16	NR
RNP	Day 1	160.20	3.94	2.46	21.80	1.43	6.56	0.59	0.08	NR
	Day 2	142.85	6.50	4.55	23.69	1.15	4.84	0.56	0.07	NR
	Day 3	157.44	6.23	3.96	22.91	1.01	4.41	0.56	0.07	NR
Precision between 3 days		153.50	7.58	4.94	22.80	0.78	3.42	0.57	0.01	NR
SSA	Day 1	178.24	17.6	9.88	39.24	3.10	7.90	0.11	0.07	NR
	Day 2	187.42	5.99	3.20	37.69	0.83	2.20	-0.92	2.50	NR
	Day 3	181.14	13.77	7.60	36.76	1.47	4.00	-0.07	0.11	NR
Precision between 3 days		182.27	4.70	2.58	37.15	2.41	6.49	-0.29	0.55	NR
SSB	Day 1	204.98	2.89	1.41	52.91	1.77	3.34	0.03	0.21	NR
	Day 2	205.31	3.36	1.64	52.92	0.64	1.22	0.33	0.14	NR
	Day 3	208.63	3.05	1.46	53.21	1.00	1.88	0.22	0.16	NR
Precision between 3 days		206.30	2.02	0.98	53.01	0.17	0.32	0.19	0.15	NR
Scl-70	Day 1	252.64	3.78	1.50	75.94	1.89	2.49	0.01	0.10	NR
	Day 2	249.88	5.11	2.05	73.35	1.65	2.30	0.07	0.21	NR
	Day 3	242.10	2.71	1.12	72.06	1.65	2.29	-0.25	0.14	NR
Precision between 3 days		248.20	5.47	2.20	73.78	1.98	2.68	-0.06	0.17	NR

TABLE 10 - Summary of Additional Intralot Precision (same lot, between strips, between plates, between runs)

	Specimen:	Strong Positive			Low Positive			Negative		
	Antigen	Mean	SD	% CV	Mean	SD	% CV	Mean	SD	% CV
Overall Precision on 64 Tests/Each Specimen:	Sm	165.39	4.38	2.65	51.19	1.98	3.86	0.03	0.13	NR
	RNP	143.99	4.62	3.21	66.77	2.57	3.85	0.53	0.11	NR
	SSA	203.62	1.69	0.83	48.12	0.68	1.41	-0.04	0.36	NR
	SSB	221.50	1.69	0.77	53.57	0.06	0.10	-0.08	0.14	NR
	Scl-70	270.34	0.31	0.11	88.16	1.89	2.14	0.25	0.17	NR

CROSS REACTIVITY STUDIES

Sera containing high levels of antibodies to potentially cross reactive antigens were assayed on each of the RhiGene MESACUP-2 TESTS. Results indicate that Sm antibodies are most likely to be present with RNP antibodies, and SSB antibodies present with SSA antibodies. Therefore, testing simultaneously for anti-Sm with anti-RNP or anti-SSA with anti-SSB is recommended. Anti-RNP, anti-SSA and anti-Scl-70 showed no cross-reactivity to alternate autoimmune antigens. Refer to TABLE 11 for data.

TABLE 11

# Samples	Autoantibody	Sm	RNP	SSA	SSB	Scl-70
5	Sm	+	*	-	-	-
5	RNP	-	+	-	-	-
5	SSA	-	-	+	-	-
5	SSB	-	-	*	+	-
5	SCL-70	-	-	-	-	+

*Note: All five samples were positive for anti-Sm with the RNP antigen and anti-SSB positive with the SSA antigen

In addition, studies with other autoimmune and infectious diseases were also performed to investigate the potential for positive reactions due to cross-reactive antibodies. A total of forty-two (42) samples negative for ANA by IFA (HEp-2 cells) were tested using the five RhiGene MESACUP-2 TESTS. Twenty-seven (27) samples were positive for infectious disease antibodies against antigens such as EBV-VCA, HSV, CMV, Measles, Mumps, VZV, Toxo, Borrelia (Lyme's Disease); and fifteen (15) samples were positive for antibodies to various other autoimmune diseases such as ANCA, AMA, ASMA, and APA. Most samples tested were negative on the RhiGene MESACUP-2 TESTS except one AMA serum positive for anti-SSA. These results indicate that cross reactivity between the ELISA antigens and the antibodies from most other disease states is likely to be minimal when using the five RhiGene MESACUP-2 TESTS. Results should always be interpreted in conjunction with clinical evaluation of the patient along with other diagnostic procedures.

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