Individual Enzyme-Linked Immunosorbent Assays for Antibodies to
Jo-1, Sm/RNP, SSA, SSB and Scl-70

Catalog Numbers: EIA-2782, EIA-2783, EIA-2784,
EIA-2785, EIA-2786, EIA-2787

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IVD

INTENDED USE

The DRG International Inc. ENA ELISA test systems are semi-quantitative immunoassay for the detection of IgG antibodies to Jo-1, Sm, Sm/RNP, SSA(Ro), SSB(La), and Scl-70 in human sera. When performed according to these instructions, the results of these assays may aid in the diagnosis and treatment of autoimmune connective tissue disorders. This device is for *in vitro* diagnostic use.

SIGNIFICANCE AND BACKGROUND

In recent years, it has become clear that autoantibodies to a number of nuclear constituents have proven to be useful in the diagnosis of various connective tissue diseases. The Jo-1 autoantibody is one of a family of characteristic autoantibodies seen in myositis patients (19). They are all specifically found in patients with myositis, and are all associated with a high incidence of accompanying interstitial lung disease (10). Antibodies directed against the Sm marker are highly specific for patients with SLE and are considered a diagnostic criterion for SLE (1,2). The presence of high level RNP antibodies alone are considered diagnostic of mixed connective tissue disease (MCTD) and are usually associated with a more benign disease course (3), while patients with low levels of RNP antibodies, together with other autoantibodies, may be observed in the serum of patients with progressive systemic sclerosis, Sjögren's Syndrome, and rheumatoid arthritis. The presence of RNP antibodies in the serum of SLE patients is usually associated with a lower incidence of renal involvement and a more benign disease course. To the contrary, patients with Sm antibodies experience a higher frequency of renal and central nervous system complications (4). Autoantibodies directed against SSA and SSB may be observed in patients with SLE (5-6) and Sjögren's disease (7-9). SSA antibodies are frequently present in the serum of ANA negative SLE patients, such as subacute cutaneous lupus erythematosus (12), a lupus-like syndrome associated with a homozygous C2 deficiency (13), and in a subset of patients who lack anti-dsDNA antibodies (11). Scl-70 antibodies are highly specific for scleroderma (11). They are also observed in a minority of SLE patients. Scl-70 positive scleroderma patients tend to have a more severe disease course, more internal organ involvement and diffuse rather than limited skin involvement (14). Scl-70 antibodies are rarely found in other autoimmune diseases, and thus, their detection in a patient with the recent onset of Raynaud's phenomenon is highly significant (15). The relative frequency of these autoantibodies in association with SLE and other connective tissue diseases either singly, or as multiple autoantibodies, requires an autoantibody profile assessment of each patient's serum in order to obtain the highest degree of clinical relevance in the laboratory workup of these types of patients. Until recently, autoantibodies were tested individually by indirect immunofluorescence, Ouchterlony gel diffusion, hemagglutination, radioimmunoassay, or enzyme-linked immunosorbent assay (ELISA). Although the exact etiology of autoimmune diseases is unknown, and the specific role played by autoantibodies in the onset of various autoimmune connective tissue diseases is obscure, the association and frequency of detection of these antibodies, particularly those of the IgG class, by the DRG, Inc. ENA Profile-6 ELISA test system, offers an efficient test procedure for the laboratory workup of patients

with various connective tissue diseases. The following table summarizes the various autoantibodies noted above with respect to disease association:

Antibody	Disease State	Relative Frequency of Antibody Detection %
Anti-Jo-1	Myositis	25-44% (19)
Anti-Sm	SLE	30*
Anti-RNP	MCTD,SLE	100** and >40, respectively
Anti-SSA (Ro)	SLE, Sjögren's	15 and 30-40, respectively
Anti-SSB (La)	SLE, Sjögren's	15 and 60-70, respectively
Anti-Scl-70	Systemic sclerosis	20-28*
* Highly Specific		
* *Highly specific when present alone at high titer		

PRINCIPLE OF THE ELISA ASSAY

The DRG International, Inc. ENA Profile-6 ELISA test system is designed to detect IgG class antibodies to a select group of six common extractable nuclear antigens in human sera. Wells of plastic microwell strips are sensitized by passive absorption with the specific antigen. The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in antigen coated microwells. Any antigen specific antibody will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase conjugated goat anti-human IgG (□ chain specific) is added to the wells and the plate is incubated. The conjugate will react with the specific antibody immobilized on the solid phase in step 1. The wells are washed to remove unreacted conjugate.
3. The microwells containing immobilized peroxidase conjugate are incubated with peroxidase Substrate Solution. Hydrolysis of the substrate by peroxidase produces a color change. After a period of time, the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the original test sample.

MATERIALS PROVIDED

Each kit contains the following components in sufficient quantities to perform the number of tests indicated on packaging label.

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1. **Plates –** 96 wells configured in twelve 1x8-well strips coated with inactivated antigen. Each row of the plate is coated with inactivated antigens. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
2. **Human positive serum control** – One 0.35 mL vial. Preservative added.
3. **Human negative serum control** – One 0.35 mL vial. Preservative added.
4. **Human serum calibrator** – One 0.5 mL vial. Preservative and Buffer added.
5. **Conjugate –** Conjugated (horseradish peroxidase) goat anti-human IgG (chain specific). Ready to use. One 15 ml vial with a white cap. Preservative added.
6. **Sample diluent** – One 30 mL bottle (green cap) containing Tween-20, bovine serum albumin and phosphate-buffered-saline, (pH 7.2 $\pm$ 0.2). Green solution, ready to use. *Note: Shake Well Before Use.* Preservative added.
7. **TMB** – One 15 mL amber bottle (amber cap) containing 3,3',5,5'-tetramethylbenzidine (TMB). Ready to use. Contains DMSO $\leq$ 15% (w).
8. **Stop solution** – One 15 mL amber bottle (amber cap) containing 1M H_2SO_4, 0.7M HCI. Ready to use.
9. **Wash buffer concentrate (10X)** – Dilute 1 part concentrate + 9 parts deionized or distilled water. One 100 mL bottle (clear cap) containing a 10X concentrated phosphate-buffered saline and Tween-20 solution (blue solution). Contains preservative. **NOTE**: 1X solution will have a pH of 7.2 $\pm$ 0.2.

PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. Normal precautions exercised in handling laboratory reagents should be followed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
3. The wells of the ELISA plate do not contain any viable organisms. However, the strips should be considered **POTENTIALLY BIOHAZARDOUS MATERIALS** and handled accordingly.
4. The human serum controls are **POTENTIALLY BIOHAZARDOUS MATERIALS**. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products 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 Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": current editions; and OSHA's Standard for Bloodborne Pathogens (6).
5. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20-25°C) before stating the assay**. Return unused reagents to refrigerated temperature immediately after use.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.

7. The human serum controls, Sample Diluent, Conjugate, and Wash Buffer concentrate contain a preservative (thimerosal, 0.04% (w/v) which may be toxic if ingested.
8. The Stop Solution id **TOXIC**. Causes burns. Toxic by inhalation, in contact with skin and if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
9. The TMB solution is **HARMFUL**. Irritating to eyes, respiratory system and skin.
10. The Wash Buffer concentrate is an **IRRITANT**. Irritating to eyes, respiratory system and skin.
11. Wipe bottom of plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
12. Dilution or adulteration of these reagents may generate erroneous results.
13. Reagents form other sources or manufacturers should not be used.
14. TMB solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color. To help reduce the possibility of contamination, refer to Test Procedure, Substrate Incubation section to determine the amount of TMB to be used.
15. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
16. Avoid microbial contamination in reagents. Incorrect results may occur.
17. Cross contamination of reagents and/or samples could cause erroneous results.
18. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
19. Avoid splashing or generation of aerosols.
20. Do not expose reagents to strong light during storage or incubation.
21. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
22. Wash Solution should be collected in a disposable basin. Treat the waste solution with 10% household bleach (0.5% sodium hypochlorite). Avoid exposure of reagents to bleach fumes.
23. Caution: Liquid waste at acid pH should be neutralized before adding to bleach solution.
24. Do not use Elisa plate if the indicator strip on the desiccant pouch has turned from blue to pink.
25. The calibrator must be fully reconstituted prior to performing the assay. Improper or inadequate reconstitution will produce erroneous results.
26. Do not allow the conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing sodium azide as a preservative. Residual amounts of sodium azide may destroy the conjugate's enzymatic activity.
27. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this kit.

MATERIALS REQUIRED BUT NOT PROVIDED

- ELISA microwell reader capable of reading at a wavelength of 450nm.
- Pipettes capable of accurately delivering 10 to 200□l.

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- Mulltichannel pipette capable of accurately delivering (50-200 l)
- Reagent reservoirs for multichannel pipettes.
- Wash bottle or microwell washing system.
- Distilled or deionized water.
- One liter graduated cylinder.
- Seroligical pipettes.
- Disposable pipette tips.
- Paper towels.
- Laboratory timer to monitor incubation steps.
- Disposal basin and disinfectant, (example: 10% household bleach, 0.5% sodium hypochlorite.)

STORAGE CONDITIONS

1. Store the unopened kit at 2° and 8°C.
2. **Coated microwell strips**: Store between 2° and 8°C. Extra strips should be immediately resealed with desiccant and returned to proper storage. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator strip on the desiccant pouch remains blue.
3. **Conjugate**: Store between 2° and 8°C. DO NOT FREEZE.
4. **Calibrator, Positive Control and Negative Control**: Store between 2° and 8°C.
5. **TMB**: Store between 2° and 8°C.
6. **Wash Buffer concentrate (10X)**: Store between 2 and 25°C. Diluted wash buffer (1X) is stable at room temperature (20° to 25°C) for up to 7 days or for 30 days between 2 and 8°C.
7. **Sample Diluent**: Store between 2° and 8°C.
8. **Stop Solution**: Store at 2° and 25°C.

SPECIMEN COLLECTION

1. It is recommended that specimen collection be carried out in accordance with NCCLS document M29: Protection of Laboratory Workers from Infectious Disease.
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.
3. Only freshly drawn and properly stored blood sera obtained by approved aseptic venipuncture procedures should be used in this assay (7, 8). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
4. Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored between 2° and 8°C for no longer than 48 hours. If delay in testing is anticipated, store test sera at –20°C or lower. Avoid multiple freeze/thaw cycles that may cause loss of antibody activity and give erroneous results.

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GENERAL PROCEDURE

1. Remove the individual components from storage and allow them to warm to room temperature (20-25 ^{O}C)
2. Determine the number of microwells needed. Allow six Control/Calibrator determinations (one Blank, one Negative Control, three Calibrators and one Positive Control) per run. A Reagent Blank should be run on each assay. Check software and reader requirements for the correct Controls/Calibrator configurations. Return unused strips to the resealable pouch with desiccant, seal, and returned to storage between 2° and 8 ^{O}C.

EXAMPLE PLATE SET-UP		
	1	2
A	Blank	Patient 3
B	Neg. Control	Patient 4
C	Calibrator	Etc.
D	Calibrator	
E	Calibrator	
F	Pos. Controls	
G	Patient 1	
H	Patient 2	

3. Prepare a 1:21 dilution (e.g.: 10 ul of serum + 200 □L of Sample Diluent. NOTE: Shake Well Before Use) of the Negative Control, Calibrator, Positive Control, and each patient serum.
4. To individual wells, add 100 uL of each diluted control, calibrator and sample. Ensure that the samples are properly mixed. Use a different pipette tip for each sample.
5. Add 100□l of Sample Diluent to well A1 as a reagent blank. Check software and reader requirements for the correct blank well configuration.
6. Incubate the plate at room temperature (20-25 ^{O}C) for 25 $\pm$ 5 minutes.
7. Wash the microwell strips 5X.
   A. **Manual Wash Procedure:**
      a. Vigorously shake out the liquid from the wells.
      b. Fill each well with wash buffer. Make sure no air bubbles are trapped in the wells.
      c. Repeat steps **a**. and **b**. for a total of five washes.
      d. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with 0.5% sodium hypochlorite (10% household bleach) at the end of the days run.

B. Automated Wash Procedure:

If using an automated microwell wash system, set the dispensing volume to 300-350µL/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.

8. Add 100µL of the conjugate solution to each well at the same rate and in the same order as the specimens were added.
9. Incubate the plate at room temperature (20-25^{O}C) for 25 ± 5 minutes.
10. Wash the microwells by following the procedure as previously described in step 7.
11. Add 100□L of TMB to each well, including reagent blank well, at the same rate and in the same order as the specimens were added.
12. Incubate the plate at room temperature (20-25^{O}C) for 10 to 15 minutes.
13. Stop the reaction by adding 50ul of Stop Solution to each well, including reagent blank well, at the same rate and in the same order as the TMB was added. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate several times to ensure that the samples are thoroughly mixed.
14. Set the microwell reader to read at a wavelength of 450 nm and measure the optical density (OD) of each well against the reagent blank. The plate should be read within 30 minutes after the addition of the Stop Solution.

QUALITY CONTROL

1. Each time the assay is run, the positive calibrator should be run in triplicate. A positive and negative control, and reagent blank must also be included in each assay.
2. Calculate the mean of the three positive calibrator determinations. If any of the three positive calibrator values differ by more than 15% from the mean, discard that value and calculate the mean of the remaining two values.
3. The mean OD value for the positive calibrator and the OD values for the positive and negative controls should fall within the following ranges:

	OD Range
Negative Control	≤ 0.250
Positive Calibrator	≥ 0.300
Positive Control	≥ 0.500

   a. The OD of the negative control divided by the mean OD of the positive calibrator should be ≤ 0.9.
   b. The OD of the positive control divided by the mean OD of the positive calibrator should be ≥ 1.25.
   c. If the control values are not within the above ranges, the test should be considered invalid and the test should be repeated.
4. The Positive Control and Negative Control are intended to monitor for substantial reagent failure and will not ensure precision at the assay cut-off.

5. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
6. Refer to NCCLS document C24: Statistical Quality Control for Quantitative Measurements for guidance on appropriate QC practices.

INTERPRETATION OF RESULTS

A. Calculations

1. Correction Factor

A cutoff OD value for positive samples has been determined by the manufacturer and correlated to the Calibrator. The correction factor (CF) will allow you to determine the cutoff value for positive samples and to correct for slight day-to-day variations in test results. The correction factor is determined for each lot of kit components and is printed on the Component List located in the kit box.

2. Cutoff OD Value

To obtain the cutoff OD value, multiply the CF by the mean OD of the Calibrator determined above
(CF x mean OD of Calibrator = cutoff OD value)

3. Index Values or OD Ratios

Calculate the Index Value or OD Ratio for each specimen by dividing its OD value by the cutoff OD from step 2.

Example:

Mean OD of Calibrator	=	0.793
Correction Factor	=	0.25
Cut off OD	=	0.793 x 0.25 = 0.198
Unknown Specimen	=	0.432
Specimen Index Value or OD Ratio	=	0.432 / 0.198 = 2.18

B. Interpretation:

Index Values or OD ratios are interpreted as follows:

	Index Value or OD Ration
Negative Specimens	≤ 0.90
Equivocal Specimens	0.91 to 1.09
Positive Specimens	≥ 1.10

Use the above guidelines when evaluating or interpreting patient specimens. Equivocal specimens should be repeated. Specimens which are repeatedly equivocal should be evaluated using an alternate serological method. Elevated autoantibody levels to any of the six autoantigens may be indicative of a specific rheumatic disorder. The SIGNIFICANCE AND BACKGROUND section of this package insert describes some of the more common diseases associated with elevated autoantibody levels.

NOTE: When interpreting the anti-Sm/RNP result to determine potential anti-RNP (only) activity, one must consider the anti-Sm and the anti-Sm/RNP result simultaneously.

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LIMITATION OF THE ASSAY

1. A diagnosis should not be made solely on the basis of the ENA ELISA test results.
2. Test results should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.

EXPECTED VALUES

The expected value for a normal patient is a negative result. The number of reactives, and the degree of reactivity is dependent upon parameters such as population type being tested, treatment, etc. Each laboratory should establish their own expected values based upon the specimens typically being tested.
With respect to disease-state and percent reactivity, Table 1 in the SIGNIFICANCE AND BACKGROUND section of this package insert shows the relative frequency of autoantibody activity for various rheumatic disorders.

PERFORMANCE CHARACTERISTICS

Comparative Study:
A comparative study was performed to demonstrate the equivalence of the various DRG ENA ELISA test systems to other commercially available autoantibody ELISA test systems. The performance of the DRG ELISAs was evaluated using 337* serum specimens; 152 normal donor samples from the northeastern and southeastern United States, and 185 disease-state repository samples previously characterized with respect to autoantibody activity. The results of the investigation have been summarized in Tables 1 and 2 below:

*The total population tested for anti-Jo-1 was 126; 64 normal donor samples, and 62 of the disease-state repository samples.

Table 1: Relative Sensitivity; Disease-State Specimens
N=177 Disease-State Specimens

Autoantigen	A	B	C	D	Sensitivity
Jo-1	8	8	0	8	8/8 = 100.0%
Sm	13	16	3	13	13/13 = 100.0%
Sm/RNP	46	58	11	50	46/50 = 92.0%
SSA	56	74	18	57	56/57 = 98.2%
SSB	28	34	6	29	28/29 = 96.6%
Scl-70	8	17	9	8	8/8 = 100.0%

A. Number of specimens reactive on DRG Test System.
B. Number of specimens reactive on Commercial ELISA Test System.
C. Number of discrepant specimens.
D. Number of positive specimens in the population after resolution of the discrepant specimens using alternate methodology such as gel immunodiffusion (GID), IFA, and third-party ELISA tests.

Table 2: Relative Specificity; Normal Donor Specimens

Autoantigen	E	F	G	H	Specificity
Jo-1	64	64	0	64	64/64 = 100.0%
Sm	136	137	1	137	136/137 = 99.3%
Sm/RNP	141	144	3	144	141/144 = 97.9%
SSA	146	146	0	146	146/146 = 100.0%
SSB	147	147	0	147	147/147 = 100.0%
Scl-70	151	151	0	151	151/151 = 100.0%

A. Number of specimens non-reactive on DRG Test System.
B. Number of specimens non-reactive on Commercial ELISA Test System.
C. Number of discrepant specimens.
D. Number of non-reactive specimens in the population after resolution of the discrepant specimens using alternate methodology such as gel immunodiffusion (GID), IFA, and third-party ELISA tests.

REPRODUCIBILITY

To assess the intra-assay and inter-assay variability of the test procedure, a strong positive, a low positive, and a negative sample for all of the autoantigens were tested eleven times on each of three days. The mean unit value, the standard deviation, and the percent CV were calculated for each sample. The results of this study are depicted in Tables 3 - 6 below:

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Table 3: Intra-Assay Reproducibility; "High Positive" Specimen
DRG ENA IgG ELISAs

	Day 1			Day 2			Day 3		
Antigen	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**
Jo-1	459	15	3	391	22	6	385	18	5
Sm	576	71	12	690	71	10	702	29	4
Sm/RNP	535	73	14	426	73	17	608	76	12
SSA	818	62	7	652	68	10	779	52	7
SSB	1022	120	12	881	65	7	987	67	7
Scl-70	669	95	14	626	65	10	726	93	3

Table 4: Intra-Assay Reproducibility; "Low Positive Specimen
DRG ENA IgG ELISA

	Day 1			Day 2			Day 3		
Antigen	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**
Jo-1	232	11	5	189	9	4	189	8	4
Sm	460	43	9	587	52	9	392	28	7
Sm/RNP	184	34	18	246	34	14	216	29	13
SSA	199	26	13	231	38	17	189	22	12
SSB	178	29	16	167	20	12	210	25	12
Scl-70	231	21	9	214	10	5	270	21	8

Table 5: Intra-Assay Reproducibility, Negative Specimen;
DRG ENA IgG ELISA

	Day 1	Day 2	Day 3

Antigen	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Jo-1	5	2	N/A	5	1	N/A	4	1	N/A
Sm	12	3	N/A	8	3	N/A	7	1	N/A
Sm/RNP	26	4	N/A	29	9	N/A	22	6	N/A
SSA	27	4	N/A	14	6	N/A	13	5	N/A
SSB	2	2	N/A	1	1	N/A	1	1	N/A
Scl-70	5	2	N/A	5	3	N/A	3	2	NA/

**Table 6: Intra-Assay Reproducibility;
DRG ENA Profile-6 ENA ELISA**

	Day 1			Day 2			Day 3		
Antigen	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**
Jo-1	412	38	9	203	23	11	5	2	N/A
Sm	656	85	13	479	93	19	9	3	N/A
Sm/RNP	532	97	18	216	42	19	26	7	N/A
SSA	750	95	13	207	35	17	18	9	N/A
SSB	963	108	11	185	32	17	1	1	N/A
Scl-70	674	97	14	238	30	13	5	2	N/A

CROSS REACTIVITY

Specimens negative for ANA by HEp-2 IFA and positive for IgG antibody to various antigens such as EBV-VCA, EBNA, HSV-1, HSV-2, CMV, Rubella, and/or Toxo, were tested for potential cross-reactivity using the DRG International, Inc. ENA ELISA Test System. All specimens tested were negative on the ELISAs, indicating that the potential for cross reactivity with such antibodies is not likely, and therefore, should not interfere with the results obtained.

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ABBREVIATED TEST PROCEDURE

1. Dilute Serum 1:21
2. Add diluted serum to microwell 100 uL/Well
3. ⟶ Incubate 20 to 30 minutes**.**
4. Wash
5. Add Conjugate – 100 uL/Well
6. ⟶ Incubate 20 to 30 minutes
7. Wash
8. Add TMB 100 uL/Well
9. ⟶ Incubate 10 to 15 minutes
10. Add Stop Solution 50 uL/Well-Mix
11. READ

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