ANA HEp-2 Test System

FA2500A

IVD

CE

Rx Only

INTENDED USE

ZEUS IFA™ ANA HEp-2 Test System is an indirect immunofluorescence assay for the qualitative detection and semi-quantitative determination of IgG anti-nuclear antibodies in human serum by manual fluorescence microscopy or with ZEUS dIFine®. The presence of anti-nuclear antibodies can be used in conjunction with other serological tests and clinical findings to aid in the diagnosis of systemic lupus erythematosus and other systemic rheumatic diseases.

ZEUS dIFine® is an automated instrument consisting of a fluorescent microscope and software that acquires, interprets, stores, and displays digital images of stained indirect immunofluorescence slides. ZEUS dIFine® can only be used with FDA cleared or approved ZEUS *in vitro* diagnostic assays that are indicated for use on this instrument. All suggested results obtained with ZEUS dIFine® must be confirmed by a trained operator.

SIGNIFICANCE AND BACKGROUND

The indirect fluorescent antibody (IFA) technique method has been used extensively for detecting the presence of ANA in the sera of patients with systemic lupus erythematosus (SLE), and other clinically similar connective tissue disorders (1 - 5). In addition, ANA may be associated with numerous drug-induced lupus syndromes (6 - 7) which clinically mimic the spontaneous form of SLE. The IFA technique was adapted to ANA testing by several investigators (8 - 9) following the basic methods originally described by Coons (10). ANA are primarily composed of IgG antibodies; however, IgA and IgM ANA may also be detected (11). It is now recognized that many sources of nuclear material may be employed as a substrate for ANA testing. Although most of the original ANA research was performed using rat or mouse liver or kidney tissue section substrate, the use of human or animal embryonic tissue cell culture substrates has provided a reliable and easy to interpret alternative substrate for ANA testing. The HEp-2 cell line is a recommended substrate for detecting centromere antibody, which is highly indicative of the CREST variant of progressive systemic sclerosis (27). There are several different patterns of nuclear and cytoplasmic immunofluorescence. **ZEUS dIFine® has been programmed to suggest eight common ANA patterns. These eight patterns and the basis for them can be evaluated by ZEUS dIFine® and manual reader are as follows:**

1. **Homogeneous** - Homogeneous or diffuse staining patterns of the nucleus is consistent with autoantibodies to native DNA (nDNA) histones and/or deoxyribonucleoprotein (DNP) (12, 13). The chromosomes of the mitotic cells (dividing cells) are important indicators of a homogeneous pattern because they will stain as irregularly shaped masses with more intensely stained outer edge
2. **Speckled** - The speckled pattern is the most commonly observed ANA pattern. A uniform "true speckled" pattern may be seen with centromere antibodies in cells not in division. A clumpy speckled pattern may be seen with antibodies to n-RNP, Sm, and SSB/La.
   Fine speckled pattern, chromosome-negative: Numerous small and uniform points of fluorescence uniformly scattered throughout the nucleus. The nucleoli will generally appear unstained. The mitotic cells may demonstrate a few speckles in their cytoplasm, but the chromosomes will be negative.
   Coarse speckled pattern, chromosome-negative: Medium-sized points of fluorescence will be scattered throughout the nuclei with distinct nuclear margins. Larger-sized points of fluorescence may also be observed; however, they are too numerous and variable in size to be identified as a nucleolar pattern. The chromosomes in the mitotic cells will be negative.
3. **Centromere** - *Discrete speckled, chromosome (centromere specificity) positive:* The chromosomes will be positive in mitotic cells; in fact, the discrete speckles will only be clustered in the chromosome mass clearly demonstrating the various stages of mitosis. The centromere pattern has been recognized to be associated with the CREST syndrome, which is a milder variant of progressive systemic sclerosis (PSS). The centromere pattern will demonstrate discrete and uniform points of fluorescent speckles scattered throughout the nucleus. Mitotic cells will be positive, demonstrating a clustering of the centromeres in the chromosomes in different arrangements according to the mitotic stage. Harmon, et al (17) demonstrated that serum samples containing highly monospecific anti-SSA/Ro gave an IF-ANA test pattern of discrete nuclear speckles on a wide variety of human cells and tumor nuclei. Such serum samples with monospecific anti-SSA/Ro produced very little cytoplasmic staining of substrate cells. A distinct, large, variable speckled pattern of 3 to 10 large speckles in the nucleus has been described. These patients with large, variable speckles have undifferentiated rheumatic disease syndromes with IgM antihistone H-3 antibody (18).
4. **Nucleolar** - The nucleolar pattern demonstrates a homogeneous or speckled staining of the nucleolus. This pattern is often associated with a dull, homogeneous fluorescence in the rest of the nucleus. The chromosomes in the mitotic cells will be negative. The nucleolar pattern suggests autoantibodies to 4 - 6S RNA. The nucleolar fluorescence will appear as homogeneous, clumped, or speckled, depending on the antigen to which the autoantibody reacts. Antinucleolar antibodies occur primarily in the sera of patients with scleroderma, systemic lupus erythematosus, Sjögren's syndrome, or Raynaud's phenomenon (19).
5. **Nuclear Dots** – A small number of nuclear dots ranging from 6 to 20 are most commonly associated with primary biliary cirrhosis.
6. **Nuclear Membrane** - *Antinuclear membrane (nuclear laminae):* The antinuclear membrane pattern appears as a rim around the nucleus and resembles a rim pattern; however, it is distinguished from the rim pattern by the fact that the metaphase chromosome stage is negative. This autoantibody is important to report because it is recognized to be associated with autoimmune liver disease
7. **Cytoplasmic (Ribosomal)**- The ribosomal pattern is characterized by numerous cytoplasmic speckles with the highest concentration around the nucleus. It is distinguished from the mitochondrial pattern because of the smaller specks and higher density. The significance of the pattern is unknown.
8. **Cytoplasmic (Mitochondrial)** - The pattern will characteristically have numerous cytoplasmic speckles with the highest concentration in the peri-nuclear area. The pattern can be observed in interphase and mitotic cells. The clinical significance of AMA is most frequently an association with primary biliary cirrhosis, especially when the AMA is a high titer.

The additional patterns below cannot be identified by ZEUS dIFine® and can only be identified by a trained operator.

Additional Nuclear Patterns

1. *Spindle fiber pattern, chromosome-positive:* The spindle fiber pattern is unique to cells undergoing mitosis where only the spindle apparatus fluoresces. This pattern has a "spider web" appearance extending from the centriole to the centromeres. The pattern is suggestive of autoantibodies to the microtubules and its significance is unclear.
2. *Midbody pattern:* The midbody pattern is a densely staining region near the cleavage furrow of telophase cells, that is, in the area where the two daughter cells separate. The clinical significance of the pattern is unknown; however, the pattern has been recognized in selected patients with systemic sclerosis.
3. *Centriole pattern:* The centriole pattern is characterized by two distinct points of fluorescence in the nucleus of the mitotic cells or one distinct point of fluorescence in the resting cell. The significance of this pattern is not known; however, it has been observed in PSS.
4. *Proliferating cell nuclear antigen (PCNA) pattern:* The proliferating cell nuclear antigen pattern is observed as a fine to course nuclear speckling in 30 - 60% of the cells in interphase, and a negative staining of the chromosome region of mitotic cells. The PCNA is very specific for patients with SLE but not detected in other connective tissue disease disorders.
5. *Peripheral (Rim)* - The nuclei stain predominantly at their periphery. The chromosomes of the mitotic cells stain as irregularly shaped masses with more intensely stained outer edges. This pattern is often seen with autoantibodies to nDNA (3, 14 - 16). If the chromosomes of the mitotic cells are negative, then the pattern would be suggestive of autoantibodies to the nuclear membrane and not to nDNA, and not reported as a peripheral pattern (see nuclear membrane interpretation above).

Additional Cytoplasmic Patterns

1. *Golgi apparatus pattern:* The golgi apparatus pattern is characterized by positive cytoplasmic staining that is concentrated on only one side of the perinuclear region. The clinical significance is uncertain, but reports in the literature have suggested an association with SLE and Sjögren's Syndrome.
2. *Lysosomal pattern:* The lysosomal pattern is observed as a few discrete speckles sparsely spaced throughout the cytoplasm. The pattern is observed in the cytoplasm of interphase and mitotic cells. The clinical significance is unknown.
3. *Cytoskeletal pattern:* The cytoskeletal pattern is characterized by a distinct "spider web" or fibrous appearance throughout the cell. It has been reported to be associated with autoimmune liver disease (anti-smooth muscle).

PRINCIPLE OF THE ASSAY

The ZEUS IFA ANA HEp-2 Test System is designed to detect the presence of circulating ANA in human sera. The assay employs tissue cell culture substrate and goat anti-human immunoglobulin adjusted for optimum use and free of nonspecific background staining. The reaction occurs in two steps:

1. Step one is the sample incubation where any ANA present in the patient sample may bind to the cell substrate, forming an antigen-antibody complex. Other serum components are subsequently washed away.
2. Step two is the Conjugate incubation where the anti-human immunoglobulin labeled with FITC is allowed to react with any human immunoglobulin that bound to the substrate during the sample incubation. This will form a stable antigen-antibody-Conjugate complex at the location where the initial patient antibody bound to the cell substrate. Excess Conjugate is subsequently washed away. The results of the assay can be visualized using a properly equipped fluorescent microscope or ZEUS dIFine®. Any positive reactions will appear as apple-green fluorescent staining within the cell. If the sample had no specific ANA, there will be no distinct nuclear staining of the cell.

TEST SYSTEM COMPONENTS

Materials Provided:

Each Test System contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label. **NOTE: Conjugate and Controls contain a combination of Proclin (0.05% v/v) and Sodium Azide (<0.1% w/v) as preservatives. Zorba-NS® contains Sodium Azide (<0.1% w/v) as a preservative.**

• • •	1. ANA HEp-2 Substrate Slides: Twenty, 12-well Slides with absorbent blotter and desiccant pouch.
CONJ	2. Conjugate: Goat anti-human IgG labeled with fluorescein isothiocyanate (FITC). Contains phosphate buffer with BSA and counterstain. One, white-capped 15mL amber bottle, containing 12mL. Ready to use.
CONTROL **+**	3. Positive Control (Human Serum): Will produce positive apple-green, homogeneous, staining of the cell nucleus. One, 0.5mL, red-capped, vial. Ready to use.
CONTROL **-**	4. Negative Control (Human Serum): Will produce no detectable nuclear staining. One, 0.5mL, green-capped, vial. Ready to use.
DIL **SPE**	5. Zorba-NS® Sample Diluent: Four, 25mL, green-capped, bottles containing phosphate-buffered-saline. Ready to use.
BUF **PBS**	6. Phosphate-buffered-saline (PBS): pH 7.2 ± 0.2. Empty contents of each buffer packet into one liter of distilled or deionized water. Mix until all salts are thoroughly dissolved. Ten packets, sufficient to prepare 10 liters.
MNTMED	7. Mounting Media (Buffered Glycerol): One, clear-capped 15 mL clear bottle, containing 12mL. Ready to use.

NOTES:

1. **The following components are not Test System Lot Number dependent and may be used interchangeably with the ZEUS IFA Test Systems, as long as the product numbers are identical: Zorba-NS® (Product Number: FA025), Mounting Media (Product Number: FA0009SB), and PBS (Product Number: 0008S).**
2. **Test System also contains a Component Label containing lot specific information inside the Test System box.**

PRECAUTIONS

1. For *In Vitro* diagnostic use.
2. Follow normal precautions exercised in handling laboratory reagents. 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 Slide do not contain viable organisms. However, consider the Slide **potentially bio-hazardous materials** and handle accordingly.
4. The Controls are **potentially bio-hazardous 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 Bio-safety 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 edition; and OSHA's Standard for Bloodborne Pathogens (20).
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 starting the assay**. Return unused reagents to their original containers immediately and follow storage requirements.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual PBS, by blotting, before adding Conjugate. Do not allow the wells to dry out between incubations.
7. The Conjugate, Zorba NS® and Controls contain Sodium Azide at a concentration of <0.1% (w/v). Sodium Azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions on hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing Sodium Azide. This preservative may by toxic if ingested.
8. Dilution or adulteration of these reagents may generate erroneous results.
9. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
10. Avoid microbial contamination of reagents. Incorrect results may occur.
11. Cross contamination of reagents and/or samples could cause erroneous results.
12. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
13. Avoid splashing or generation of aerosols.
14. Do not expose reagents to strong light during storage or incubation.
15. Allowing the slide packet to equilibrate to room temperature prior to opening the protective envelope will protect the wells and blotter from condensation.
16. Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant (i.e.:10% household bleach - 0.5% Sodium Hypochlorite). Avoid exposure of reagents to bleach fumes.
17. 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 Test System.
18. Do not apply pressure to slide envelope. This may damage the substrate.

19. The components of this Test System are matched for optimum sensitivity and reproducibility. Reagents from other manufacturers should not be interchanged. Follow Package Insert carefully.
20. Unopened/opened components are stable until the expiration date printed on the label, provided the recommended storage conditions are strictly followed. Do not use beyond the expiration date. Do not freeze.
21. Evans Blue Counterstain is a potential carcinogen. If skin contact occurs, flush with water. Dispose of according to local regulations.
22. Do not allow slides to dry during the procedure. Depending upon lab conditions, it may be necessary to place slides in a moist chamber during incubations.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Properly equipped fluorescence microscope or ZEUS dIFine® instrument.
2. Small serological, Pasteur, capillary, or automatic pipettes.
3. Disposable pipette tips.
4. Small test tubes, 13 x 100mm or comparable.
5. Test tube racks.
6. Staining dish: A large staining dish with a small magnetic mixing set-up provides an ideal mechanism for washing Slides between incubation steps.
7. Cover slips, 24 x 60mm, thickness No. 1.
8. Distilled or deionized water.
9. 1 Liter Graduated Cylinder.
10. Laboratory timer to monitor incubation steps.
11. Disposal basin and disinfectant (i.e.: 10% household bleach – 0.5% Sodium Hypochlorite).

The following filter systems, or their equivalent, have been found to be satisfactory for routine use with transmitted or incident light darkfield assemblies:

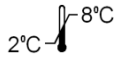
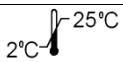
Transmitted Light		
Light Source: Mercury Vapor 200W or 50W		
Excitation Filter	Barrier Filter	Red Suppression Filter
KP490	K510 or K530	BG38
BG12	K510 or K530	BG38
FITC	K520	BG38
Light Source: Tungsten – Halogen 100W		
KP490	K510 or K530	BG38

Incident Light			
Light Source: Mercury Vapor 200, 100, 50 W			
Excitation Filter	Dichroic Mirror	Barrier Filter	Red Suppression Filter
KP500	TK510	K510 or K530	BG38
FITC	TK510	K530	BG38
Light Source: Tungsten – Halogen 50 and 100 W			
KP500	TK510	K510 or K530	BG38
FITC	TK510	K530	BG38

SPECIMEN COLLECTION

1. ZEUS Scientific recommends that the user carry out specimen collection in accordance with CLSI document M29: Protection of Laboratory Workers from Occupationally Acquired Infectious Diseases. 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.
2. Only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures with this assay (30, 31). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.
3. 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 - 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 which may cause loss of antibody activity and give erroneous results. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine stability criteria for its laboratory (38).

STORAGE CONDITIONS

	Unopened Test System.
	Mounting Media, Conjugate, Zorba-NS®, Slides*, Positive Control and Negative Control.
	Rehydrated PBS (Stable for 30 days).
	Phosphate-buffered-saline (PBS) Packets.
*Once opened, Slides must be used that day. Other ready-to-use reagents, except PBS, may be used until their stated expiration date.	

ASSAY PROCEDURE

1. Remove Slides from refrigerated storage and allow them to warm to room temperature (20 - 25°C). Tear open the protective envelope and remove Slides. **Do not apply pressure to flat sides of protective envelope.**
2. Identify each well with the appropriate patient sera and Controls. **NOTE: The Controls are intended to be used undiluted**. Prepare a 1:40 dilution (e.g.: 10µL of serum + 390µL of Zorba-NS®) of each patient serum.
   Dilution Options:
   a. Users may titrate the Positive Control to endpoint to serve as a semi-quantitative (1+ Minimally Reactive) Control. In such cases, the Control should be diluted two-fold in PBS, **not Zorba-NS®.** When evaluated by ZEUS Scientific, an endpoint dilution is established and printed on the Positive Control vial (± one dilution). It should be noted that due to variations within the laboratory (equipment, etc.), each laboratory should establish its own expected end-point titer for each lot of Positive Control.
   b. When titrating patient specimens, initial dilutions should be prepared in Zorba-NS® and all subsequent dilutions should be prepared in PBS only. **Titrations must not be prepared in Zorba-NS.**
3. With suitable dispenser (listed above), dispense 20 - 40µL of each Control and each diluted patient sera in the appropriate wells.
4. Incubate Slides at room temperature (20 - 25°C) for 20 - 30 minutes.
5. Gently rinse Slides with PBS. **Do not direct a stream of PBS into the test wells.**

6. Wash Slides for two, five minute intervals, changing PBS between washes. Slides may soak during each wash for up to five minutes. **NOTE: For those using automated washers, set the washer to wash each well three times with a soak of zero to five minutes.**
7. Remove Slides from PBS one at a time. Invert Slide and key wells to holes in blotters provided. Blot Slide by wiping the reverse side with an absorbent wipe. CAUTION: Position the blotter and Slide on a hard, flat surface. Blotting on paper towels may destroy the Slide matrix. **Do not allow the Slides to dry during the test procedure**.
8. Add 20 - 40µL of Conjugate to each well.
9. Repeat steps 4 through 7.
10. Apply 3 - 5 drops of Mounting Media to each Slide (between the wells) and coverslip. Mounting Media must be added within two hours of completing the last wash cycle.
11. Examine Slides immediately with an appropriate fluorescence microscope. This may be accomplished manually using a traditional fluorescent microscope or with ZEUS dIFine®. If using ZEUS dIFine®, please refer to the instrument manual for operating instructions. **It is recommended that Slides be examined on the same day as testing.**
    NOTES:
    a. **If it is not possible to view the Slides immediately, Slides may be stored for up to 48 hours at 2 - 8°C.**
    b. **If Slides will not be examined within a 48-hour period, seal coverslip with clear nail polish and store in refrigerator.**

QUALITY CONTROL

1. Every time the assay is run, a Positive Control, a Negative Control, and a Buffer Control must be included.
2. It is recommended that the Controls be read prior to evaluating the test samples. If the Controls do not appear as described, results may be invalid.
    a. Negative Control - characterized by the absence of specific fluorescence and a red, or dull green, background staining of all cells due to counterstain.
    b. Positive Control (homogeneous pattern) - characterized by apple-green fluorescence. The homogeneous staining pattern is a diffused uniform staining of the entire nucleus.
3. Additional Controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
    NOTES:
    a. **Non-specific reagent trapping may exist. It is important to adequately wash Slides to eliminate false positive results.**
    b. **The intensity of the observed fluorescence may vary with the microscope and filter system used.**
    c. **Non-nuclear staining of the cell substrate may be observed with some human sera.**

INTERPRETATION OF RESULTS

1. The interpretation of the results depends on the pattern observed, the titer of the autoantibody, and the age of the patient. The elderly, especially women, are prone to develop low-titered autoantibodies (<1:80) in the absence of clinical autoimmune disease. Experience suggests that a 1:40 dilution is a good dilution to screen for ANA. Low-titer positive results may occur in apparently healthy persons; therefore, the ANA results must always be interpreted in light of the patient's total clinical presentation.
2. Titers less than 1:40 are considered negative.
3. Positive test: A positive reaction is the presence of any pattern of nuclear apple-green staining observed at a 1:40 dilution based on a 1+ to 4+ scale of staining intensity. 1+ is considered a weak reaction and 4+ a strong reaction. All sera positive at 1:40 should be titered to endpoint dilution. This is accomplished by making 1:40, 1:80, 1:160, etc. serial dilutions of all positives. The endpoint titer is the highest dilution that produces a 1+ positive reaction.
4. Homogeneous patterns with peripheral accentuation are frequently found in sera from patients with SLE.

	Disease Most Frequently Found In	**Reference**
Homogeneous: High Titer Low Titer	 SLE Rheumatoid Arthritis and other diseases	 (3, 8, 9, and 16) (1)
Centromere	CREST Syndrome variant of PSS	(27)
Speckled	Scleroderma, Raynaud's Syndrome, Sjögren's Syndrome, Mixed connective tissue disease	(34 - 36)
Nucleolar	Scleroderma	(37)
Peripheral	SLE	(2, 8, 9, and 16)

LIMITATIONS OF THE ASSAY

1. The ZEUS IFA ANA HEp-2 Test System is a laboratory diagnostic aid and it's results are not diagnostic by itself. Positive ANA may be found in apparently healthy individuals. It is therefore imperative that ANA results be interpreted in conjunction with other serological tests and clinical findings.
2. SLE patients undergoing steroid therapy may have negative test results (40).
3. Many commonly prescribed drugs may induce ANA (6, 7).
4. One autoantibody pattern may partially or completely obscure the diagnostic features of the other. In such instances, it is necessary to titrate the serum.
5. No definitive association between the pattern of nuclear fluorescence and any specific disease state is intended with this product.

EXPECTED RESULTS

The expected value in the normal population is negative or less than 1:40. However, apparently healthy individuals may contain ANA in their sera (36). This percentage increases with aging, particularly in the 7th decade of life.

PERFORMANCE CHARACTERISTICS

NOTE: When establishing Performance Characteristics of the ZEUS IFA ANA HEp-2 Test System, Slides were interpreted using three different methods as outlined below:

Interpretation Method:
Method A. Method A was a completely manual interpretation method. It was accomplished using a traditional fluorescent microscope equipped with objective and ocular lenses. Determining the qualitative outcome and the pattern were accomplished using trained laboratory technicians.
Method B. Method B was accomplished by scanning the slides using ZEUS dIFine® and subsequently having a trained laboratory technician interpret the resulting digital image (qualitative and pattern if positive) appearing on the computer monitor.

Method C. Method C is the ***suggested outcome*** predicted by ZEUS dIFine®; Method C predicts the qualitative result and, if positive, the pattern if it is one of the eight patterns the system has been programmed to call. If Method C is "UNC" (uncertain), the level of fluorescence measured by ZEUS dIFine is borderline between positive and negative. Method C must be "validated" or accepted by the laboratory technician or modified or invalidated completely. For purposes of this study and the data presented below, **Method C** is logged "AS IS" without any modification by the laboratory technician(s). It is therefore presented for *informational purposes only*.

1. Analytical Performance Studies:

a. Linearity:

Strong positive samples were identified for each of the following eight ANA patterns: Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial). Each of the samples was assayed at 1:40 and titered to 1:20480 and interpreted by all three methods noted above. This study was conducted internally at ZEUS Scientific. The endpoints for each sample and each method are presented below:

Sample	Method A	Method B	Method C
Homogeneous	1:1280	1:1280	1:1280
Speckled	1:5120	1:5120	1:2560
Centromere	1:5120	1:5120	1:5120
Nucleolar	1:2560	1:5120	1:2560
Nuclear Dots	1:640	1:640	1:640
Nuclear Membrane	1:2560	1:2560	1:2560
Cytoplasmic (Ribosomal)	1:320	1:320	1:320
Cytoplasmic (Mitochondrial)	1:10240	1:10240	1:10240

Summary: In 100% of the cases, the pattern called was as expected and in agreement regardless of the method of interpretation. Likewise, the endpoint determination was as expected ± one dilution for 100% of the determinations regardless of the method of interpretation.

b. Lot-to-Lot Reproducibility:

A moderately positive sample and a strong positive sample were identified for each of the following eight ANA patterns: Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial). Additionally, nine negative samples were included bringing the total group to 25 specimens. This group of 25 specimens was assayed on three different lots of ZEUS IFA™ ANA HEp-2 Test System and interpreted via all three methods noted above. Additionally, all sixteen positive members were titered to endpoint and interpreted via all three methods. This study was conducted internally at ZEUS Scientific.

Results:

i. **Qualitative Agreement:** There was 100% agreement in the qualitative results at the screening dilution of all 25 specimens across all three kit lots, and there was 100% agreement across all three interpretation methods regardless of reagent kit lot.

ii. **Endpoint Titer Agreement:** All 16 positive specimens resulted in same endpoint titers ± one dilution regardless of reagent kit lot or method interpretation.

iii. **Pattern Agreement:** For dilutions yielding a positive result across all three interpretation methods, there was 100% pattern agreement for lot one and lot two for all three interpretation methods. For lot three there was 100% pattern agreement between Method A and Method B, with one homogenous mid positive specimen as the only outlier when comparing Method C versus Methods A and B.

c. Reference Range Study:

One hundred and eighty random serum samples were acquired from healthy donors in Northeastern US. The samples were assayed at the screening dilution of 1:40 and interpreted via all three methods. Any samples producing a positive result at 1:40 were titered and interpreted via all three methods. The results of the screening test are summarized below:

Method	Number of Positives	% Positives	Number of Negatives	% Negatives	Number of Uncertain	% Uncertain
A	19	10.56	161	89.44	NA	NA
B	19	10.56	161	89.44	NA	NA
C	14	7.78	152	84.44	14	7.78

d. Ten-day Repeatability Study:

A low positive sample (~1:40 endpoint), a mid-positive sample (~1:160 to 1:320 endpoint) and a strong positive sample ($\geq$ 1:640 endpoint) were identified for each of the following eight ANA patterns: Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial). One ANA negative specimen was also included bringing the group of specimens to a total of 25. These 25 specimens were assayed (1:40 screening dilution) at ZEUS Scientific in triplicate, on ten different days producing 30 results per sample. The slides were interpreted by all three methods.

For the within-method evaluation, the results of the qualitative agreement and the pattern agreement are depicted below: there was 100% qualitative agreement in all samples when read using Methods A and B. For Method C, 22 out of 25 specimens showed 100% qualitative agreement; homogenous low positive, nucleolar low positive, and nuclear membrane low positive specimens did not have complete qualitative agreement. Additionally, there was 100% pattern agreement within Methods A and B. For method C, there was 100% pattern agreement for 21 out of 25 specimens with outliers observed in homogenous low positive, nucleolar low positive and nuclear membrane low positive and nuclear membrane high positive specimens.

Table 3: Within-Method Qualitative Result Agreement

Sample	Method A Agreement (95% CI)	Method B Agreement (95% CI)	Method C Agreement (95% CI)
Homogenous Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	76.7% (59.1 - 88.2%)
Homogenous Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Homogenous High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	96.7% (83.3 - 99.4%)
Nucleolar Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondria Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Sample	Method A Agreement (95% CI)	Method B Agreement (95% CI)	Method C Agreement (95% CI)
Ribosomal High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Negative	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Table 4: Within-Method Pattern Result Agreement

Sample	Method A Agreement (95% CI)	Method B Agreement (95% CI)	Method C Agreement (95% CI)
Homogenous Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	73.3% (55.6 - 85.8%)
Homogenous Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Homogenous High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	96.7% (83.3 - 99.4%)
Nucleolar Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondria Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	86.67% (70.3 - 94.7%)
Nuclear Membrane Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	96.7% (83.3 - 99.4%)
Nuclear Dots Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Sample	Method A Agreement (95% CI)	Method B Agreement (95% CI)	Method C Agreement (95% CI)
Ribosomal Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

For the between-method agreement, there was 100% qualitative and pattern agreement between Method A versus Method B. However, only 22 out of 25 specimens showed 100% qualitative in the comparisons between Method B vs Method C and Method A vs Method C; the discrepant samples were the homogenous low positive, nucleolar low positive and nuclear membrane low positive specimens. Additionally, only 21 out of 25 specimens showed 100% pattern agreement in the comparisons between Method B vs Method C and Method A vs Method C; the discrepant samples were the homogenous low positive, nucleolar low positive and nuclear membrane low positive and nuclear membrane high positive specimens.

Table 5: Between-Method Qualitative Result Agreement

Sample	Method A vs Method B Agreement (95% CI)	Method A vs Method C Agreement (95% CI)	Method B vs Method C Agreement (95% CI)
Homogenous Low Positive	100% (88.7 – 100%)	76.67% (59.1 - 88.2%)	76.67% (59.1 - 88.2%)
Homogenous Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Homogenous High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar Low Positive	100% (88.7 – 100%)	96.67% (83.3 - 99.4%)	96.67% (83.3-99.4%)
Nucleolar Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondria Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	90% (74.4 - 96.5%)
Nuclear Membrane Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Sample	Method A vs Method B Agreement (95% CI)	Method A vs Method C Agreement (95% CI)	Method B vs Method C Agreement (95% CI)
Nuclear Dots High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Negative	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Table 6: Between Method Pattern Result Agreement

Sample	Method A vs Method B Agreement (95% CI)	Method A vs Method C Agreement (95% CI)	Method B vs Method C Agreement (95% CI)
Homogenous Low Positive	100% (88.7 – 100%)	73.3% (55.6 - 85.9%)	73.3% (55.6 - 85.9%)
Homogenous Mid Positive	100% (88.65 – 100%)	100% (88.65 – 100%)	100% (88.7 – 100%)
Homogenous High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Speckled High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Centromere Mid Positive	100% (88.65 – 100%)	100% (88.65 – 100%)	100% (88.7 – 100%)
Centromere High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nucleolar Low Positive	100% (88.7 – 100%)	96.7% (83.3 - 99.4%)	96.7% (83.3 - 99.4%)
Nucleolar Mid Positive	100% (88.65 – 100%)	100% (88.65 – 100%)	100% (88.7 – 100%)
Nucleolar High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondria Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Mitochondrial High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane Low Positive	100% (88.7 – 100%)	86.7% (70.3 - 94.7)	86.7% (70.3 - 94.7%)
Nuclear Membrane Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Membrane High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	96.7% (83.3 - 99.4%)
Nuclear Dots Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Nuclear Dots High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

Sample	Method A vs Method B Agreement (95% CI)	Method A vs Method C Agreement (95% CI)	Method B vs Method C Agreement (95% CI)
Ribosomal Low Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal Mid Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)
Ribosomal High Positive	100% (88.7 – 100%)	100% (88.7 – 100%)	100% (88.7 – 100%)

e. Five-day, Multi-Site Reproducibility Study:

A low positive sample (~1:40 endpoint), a mid-positive sample (~1:160 - 1:320 endpoint) and a strong positive sample (≥ 1:640 endpoint) were identified for each of the following eight ANA patterns: Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial). One ANA negative specimen was also included bringing the group of specimens to a total of 25. These 25 specimens were assayed (1:40 screening dilution) in triplicate, two times per day on five different days and three different laboratories, producing 30 results per sample, per location. The Slides were interpreted by all three methods. At each site, Method A and Method B were also interpreted by two separate laboratory technicians. The results of the qualitative agreement and the pattern agreement are depicted below:

i. **Overall Qualitative Result Agreement**

a. **Within Method:** Within method qualitative agreement for Method A and Method B was 100% for all three sites and all technicians. For Method C, there was 100% agreement for 24 out of 25 specimens at all sites and all technicians: one nuclear membrane low positive specimen being the only discrepant sample at site 2.

b. **Between Method:** There was 100% qualitative agreement for all 25 specimens between Method A versus Method B and Method A versus Method C. Notably, there was 100% qualitative agreement between Method B vs Method C for 24 out of 25 specimens with the nuclear membrane low positive specimen being an outlier at site 2.

ii. **Pattern Result Agreement**

a. **Within Method:** Within method pattern agreement for Method A and Method B was 100% for all three sites and all technicians. For Method C, there was 100% agreement for 24 out of 25 specimens with centromere mid specimen as the only outlier.

b. **Between Method:** There was 100% pattern agreement for all 25 specimens between Method A vs B for all sites and all technicians. For Method A vs Method C, there were two discrepant samples: a centromere mid specimen at Site 1 and nuclear membrane low specimen at Site 2. Similarly, for Method B vs Method C, there were two discrepant samples: a centromere mid specimen at Site 1 and a nuclear membrane low specimen at Site 2.

f. Interference Study:

A mid-positive sample (~1:160 to 1:320 endpoint) and a strong positive sample (≥ 1:640 endpoint) were identified for each of the following eight ANA patterns: Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial). One ANA negative specimen was also included bringing the group of specimens to a total of 17. These 17 specimens were spiked with two different concentrations (low spike and high spike) of twelve different possible interferrents as outlined in the table below. All specimens were evaluated on the ZEUS IFA™ ANA HEp-2 Test System and interpreted by all three methods noted above.

	Interferent Testing Concentrations	
Interferent	**Low**	**High**
Intralipids	3 mg/mL	7.50 mg/mL
Cholesterol	1.5 mg/mL	2.5 mg/mL
Albumin	35 mg/1mL	50 mg/1mL
Ibuprofen	0.5 mg/mL	2 mg/mL
Prednisone	0.2 mg/mL	0.8 mg/mL
Hydroxychloroquine	0.006 mg/mL	0.2 mg/mL
Simvastatin	4 ug/mL	20 ug/mL
Cyclophosphamide	1 mg/mL	4 mg/mL
Rheumatoid Factor	200 U/mL	400 U/mL
Hemoglobin	100 mg/mL	200 mg/mL
Triglycerides	1.5 mg/mL	5 mg/mL
Bilirubin	0.01 mg/mL	0.15 mg/mL

Results: For methods A and B, neither the qualitative agreement or the resulting pattern were affected by the addition of the possible interferents. Method C yielded one uncertain result in a low positive nuclear membrane sample when spiked with Albumin. Therefore, it can be concluded that the results of the ZEUS IFA™ ANA HEp-2 Test System are not at significant risk of erronous results from the presence of the interferents tested.

A second interference study was conducted that focused on the assay cut off and included some additional potential interferents. Samples were selected for each of the eight ANA patterns (Homogeneous, Speckled, Centromere, Nucleolar, Nuclear Dots, Nuclear Membrane, Cytoplasmic (Ribosomal) and Cytoplasmic (Mitochondrial)) that exhibited an endpoint titer of 1:40-1:80 (near the assay cut off) as well as one ANA negative specimen. These nine specimens were spiked at two different concentrations of the interferents as listed below.

	Interferent Testing Concentrations	
Interferent	**Low**	**High**
Bilirubin (unconjugated)	0.02 mg/mL	0.15 mg/mL
Cholesterol (total)	1.5 mg/mL	2.2 mg/mL
Triglycerides	1 mg/mL	2.5 mg/mL
Albumin	35 mg/mL	52 mg/mL
Hemoglobin	100 mg/mL	200 mg/mL
Intralipids	2.0 mg/mL	20 mg/mL
Rheumatoid Factor	200 U/mL	400 mg/mL

Cyclophosphamide	0.183 mg/mL	0.549 mg/mL
Ibuprofen	0.073 mg/mL	0.219 mg/mL
Hydroxychloroquine	0.006 mg/mL	0.024 mg/mL
Simvastatin	0.0000277 mg/mL	0.000083 mg/mL
Prednisone	0.000033 mg/mL	0.000099 mg/mL
Azathioprine	0.00086 mg/mL	0.00258 mg/mL
Diltiazem	0.0003 mg/mL	0.0009 mg/mL
Mycophenolate mofetil	0.012 mg/mL	0.048 mg/mL
Rituximab	0.5 mg/mL	2 mg/mL
Belimumab	2 mg/mL	8 mg/mL

Results: Again, neither the qualitative agreement or the resulting pattern were affected by the addition of the possible interferents, regardless of the method of interpretation. Therefore, it can be concluded that the results of the ZEUS IFA™ ANA HEp-2 Test System are not at significant risk of erronous results from the presence of the interferents tested.

2. Clinical Performance Study Design:

A total of 380* clinically characterized specimens were acquired as outlined in the table below. These 380 specimens were aliquoted and sent to three different laboratories for evaluation by ZEUS IFA™ ANA HEp-2 Test System. All three labs evaluated the Slides via all three methods noted above. Additionally, slide interpretation Method A and Method B were repeated twice at each clinical site by two different laboratory technicians. At each clinical site, the 380 specimens were first screened at a 1:40 dilution for an assessment of the qualitative status of the 380 specimens. Any specimen generating a positive result at the 1:40 screening dilution (for any of the three interpretation methods) was then titered and interpreted via all three methods again to determine the endpoint as well as the pattern. *** NOTE: Site two tested a fewer number of Celiac and Vasculitis specimens resulting in a total of 350 specimens at site two.**

Results were used to assess clinical specificity (potential cross reactivity), clinical sensitivity, qualitative agreement between methods within sites, Method C interpretation between sites (i.e., ZEUS dIFine™ to ZEUS dIFine™ instrument comparison), endpoint titer agreement and pattern agreement.

ANA-Associated Diseases	***n*, All Sites**
Connective Tissue Diseases:	
Systemic Lupus Erythematosus (SLE)	40
Sjogren's Syndrome (SS)	30
Scleroderma	20
Autoimmune Myositis (AM)	20
Mixed Connective Tissue Disease (MCTD)	20
CREST	20
Other ANA-Associated Autoimmune Diseases:	
Autoimmune Hepatitis (AIH)	20
Drug-Induced Lupus (DIL)	20
Total	190

Non- ANA-Associated Diseases	***n*, Site 1 and Site 3**	***n*, Site 2**
Other Autoimmune Diseases:		
Celiac Disease	22	10
ANCA-associated Vasculitis	28	10
Crohn's Disease	10	10
Rheumatoid Arthritis	30	30
Autoimmune Thyroiditis	30	30
Inflammatory Bowel Disease (IBD)	10	10
Ulcerative Colitis	10	10
Other Diseases:		
Malignancy/Cancer	20	20
Fibromyalgia	10	10
Infectious Diseases	10	10
Total	190	160

3. Clinical Sensitivity and Clinical Specificity:

The clinical sensitivity was calculated at each site on SLE separately, and on the combination of the connective tissue diseases (CTD: SLE+ SS + scleroderma+ CREST+ MCTD+ AM) plus other ANA-associated diseases (autoimmune liver hepatitis + Drug-induced Lupus). Specificity was calculated using the non-ANA-associated diseases total control population.

a. Clinical Performance at Site 1

Diagnostic Sensitivity and Specificity			% Sensitivity (95% CI) SLE (n = 40)	% Sensitivity 95% CI) CTD + ANA-Associated Diseases (n = 190)	% Specificity (95% CI) Non- ANA-Associated Diseases (n = 190)
Site 1	Method A	Technician A	52.5% (37.5 - 67.1%)	53.2% (46.1 - 60.1%)	75.8% (69.2 - 81.3%)
	Method A	Technician B	57.5% (42.2 - 71.5%)	60.0% (52.9 - 66.7%)	73.2% (66.4 - 79.0%)
	Method B	Technician A	55.0% (39.8 - 69.3%)	54.7% (47.6 - 61.6%)	75.8% (69.2 - 81.3%)
	Method B	Technician B	52.5% (37.5 - 67.1%)	57.9% (50.8 - 64.7%)	74.2% (67.6 - 79.9%)
	Method C	dIFine	55.0% (39.8 - 69.3%)	57.4% (50.3 - 64.2%)	67.4% (60.4 - 73.6%)

b. Clinical Performance at Site 2

Diagnostic Sensitivity and Specificity			% Sensitivity (95% CI) SLE (n = 40)	% Sensitivity (95% CI) CTD + ANA-Associated Diseases (n = 190)	% Specificity (95% CI) Non- ANA-Associated Diseases (n = 160)
Site 2	Method A	Technician A	50.0% (35.2 - 64.8%)	51.6% (44.5 - 58.6%)	83.1% (76.6 - 88.1%)
	Method A	Technician B	55.0% (39.8 - 69.3%)	56.3% (49.2 - 63.2%)	80.0% (73.1 - 85.5%)
	Method B	Technician A	52.5% (37.5 - 67.1%)	56.8% (49.7 - 63.7%)	81.3% (74.5 - 86.5%)
	Method B	Technician B	55.0%	58.4%	80.6%

			(39.8 - 69.3%)	(51.3 - 65.2%)	(73.8 - 86.0%)
	Method C	dIFine	52.5% (37.5 - 67.1%)	55.8% (48.7 - 62.7%)	74.4% (67.1 - 80.5%)

c. Clinical Performance at Site 3

Diagnostic Sensitivity and Specificity			% Sensitivity (95% CI) SLE (n = 40)	% Sensitivity (95% CI) CTD + ANA-Associated Diseases (n = 190)	% Specificity (95% CI) Non- ANA-Associated Diseases (n = 190)
Site 3	Method A	Technician A	47.5% (32.9 - 62.5%)	53.2% (46.1 - 60.1%)	76.8% (70.4 - 82.3%)
	Method A	Technician B	55.0% (39.8 - 69.3%)	57.4% (50.3 - 64.2%)	74.2% (67.6 - 79.9%)
	Method B	Technician A	45.0% (30.7 - 60.2%)	52.6% (45.6 - 59.6%)	78.9% (72.6 - 84.1%)
	Method B	Technician B	57.5% (42.2 - 71.5%)	56.3% (49.2 - 63.2%)	74.7% (68.1%- 80.4%)
	Method C	dIFine	55.0% (39.8 - 69.3%)	54.2% (47.1 - 61.1%)	67.5% (63.1 - 76.1%)

Sensitivity among the SLE cohort ranged from a low of 45.0% to a high of 57.5% across all three methods, across all three sites. The prevalence of ANA observed in this SLE cohort seemed lower than what is typically observed. According to the Rheumatologists who helped with the collection of these specimens, this may be because the majority of these patients were on strong immunosuppressive treatments and either had low disease activity or were in remission. This low prevalence of ANA observed was further confirmed using another FDA-cleared, commercial ANA HEp-2 IFA product. Clinical sensitivity among the CTD + ANA-Associated Diseases cohort ranged from a low of 51.6% to a high of 60.0% across all three methods, across all three sites. Clinical specificity among the Non-ANA-Associated Diseases cohort ranged from a low of 67.4% to a high of 83.1% across all three methods, across all three sites. If one averages all methods of interpretation across all three sites, the clinical sensitivity in the SLE group averaged 53.2%, the clinical sensitivity in the CTD + ANA-Associated Diseases group averaged 55.7% and the clinical specificity in the Non-ANA-Associated Diseases group averaged 75.9%.

4. Frequency of ANA Detected by Disease Group:

In addition to a positive or negative determination, Method C may result in an "Uncertain" call if ZEUS dIFine® could not identify the result as clearly positive or clearly negative. Uncertain results were considered negative for this summary below (i.e., were **not** positive). The table below shows the percentage of each disease category that was called qualitatively positive by each technician at each site by each method. This table summarizes the frequency of ANA found in each disease category and the consistency of that ANA determination across methods.

Site 1:

Diseases	Technician A		Technician B		
	Method A	Method B	Method A	Method B	Method C
ANA-Associated Diseases					
SLE	52.5%	55.0%	57.5%	52.5%	55.0%
Sjögren's	50.0%	50.0%	56.7%	53.3%	53.3%
Scleroderma	75.0%	70.0%	80.0%	80.0%	80.0%
Autoimmune Myositis	50.0%	55.0%	65.0%	60.0%	60.0%
MCTD	40.0%	40.0%	50.0%	55.0%	50.0%
CREST	80.0%	80.0%	80.0%	80.0%	80.0%
Autoimmune Hepatitis	45.0%	50.0%	60.0%	55.0%	50.0%
Drug-Induces Lupus	35.0%	40.0%	35.0%	35.0%	35.0%
Non-ANA Associated Diseases					
Autoimmune Thyroiditis	23.3%	16.7%	16.7%	16.7%	16.7%
Cancer	15.0%	15.0%	20.0%	20.0%	15.0%
Celiac Disease	45.5%	45.5%	45.5%	45.5%	45.5%
Crohn's Disease	30.0%	30.0%	30.0%	30.0%	30.0%
Fibromyalgia	30.0%	40.0%	50.0%	40.0%	40.0%
Infectious Disease	5.0%	5.0%	5.0%	5.0%	5.0%
Inflammatory Bowel Disease	40.0%	30.0%	40.0%	20.0%	20.0%
Rheumatoid Arthritis	16.7%	23.3%	23.3%	23.3%	16.7%
Ulcerative Colitis	30.0%	30.0%	50.0%	60.0%	40.0%
Vasculitis	17.9%	14.3%	14.3%	14.3%	14.3%

Site 2:

Diseases	Technician A		Technician B		
	MethodA	Method B	Method A	Method B	Method C
ANA-Associated Diseases					
SLE	50.0%	52.5%	55.0%	55.0%	52.5%
Sjögren's	53.3%	53.3%	56.7%	56.7%	53.3%
Scleroderma	75.0%	80.0%	75.0%	80.0%	80.0%
Autoimmune Myositis	55.0%	65.0%	65.0%	70.0%	60.0%
MCTD	30.0%	45.0%	35.0%	45.0%	45.0%
CREST	75.0%	80.0%	80.0%	80.0%	75.0%
Autoimmune Hepatitis	40.0%	45.0%	45.0%	45.0%	45.0%
Drug-Induces Lupus	35.0%	40.0%	40.0%	40.0%	40.0%
Non-ANA Associated Diseases					
Autoimmune Thyroiditis	13.3%	16.7%	16.7%	16.7%	16.7%
Cancer	10.0%	15.0%	15.0%	15.0%	15.0%
Celiac Disease	20.0%	30.0%	20.0%	30.0%	30.0%
Crohn's Disease	30.0%	30.0%	30.0%	30.0%	30.0%

Fibromyalgia	40.0%	40.0%	40.0%	40.0%	40.0%
Infectious Disease	5.0%	5.0%	5.0%	5.0%	5.0%
Inflammatory Bowel Disease	20.0%	20.0%	20.0%	20.0%	20.0%
Rheumatoid Arthritis	16.7%	16.7%	16.7%	16.7%	16.7%
Ulcerative Colitis	40.0%	40.0%	60.0%	40.0%	40.0%
Vasculitis	0.0%	0.0%	10.0%	10.0%	0.0%

Site 3

Diseases	Technician A		Technician B		
	MethodA	Method B	Method A	Method B	Method C
ANA-Associated Diseases					
SLE	47.5%	45.0%	55.0%	57.5%	52.5%
Sjögren's	46.7%	50.0%	60.0%	53.3%	50.0%
Scleroderma	75.0%	75.0%	80.0%	75.0%	75.0%
Autoimmune Myositis	60.0%	60.0%	60.0%	60.0%	60.0%
MCTD	45.0%	45.0%	45.0%	45.0%	45.0%
CREST	80.0%	80.0%	75.0%	75.0%	75.0%
Autoimmune Hepatitis	45.0%	40.0%	50.0%	50.0%	45.0%
Drug-Induces Lupus	35.0%	35.0%	35.0%	35.0%	35.0%
Non-ANA Associated Diseases					
Autoimmune Thyroiditis	16.7%	13.3%	20.0%	16.7%	16.7%
Cancer	20.0%	20.0%	20.0%	25.0%	15.0%
Celiac Disease	45.5%	45.5%	45.5%	45.5%	45.5%
Crohn's Disease	30.0%	20.0%	30.0%	30.0%	30.0%
Fibromyalgia	40.0%	40.0%	50.0%	50.0%	40.0%
Infectious Disease	5.0%	5.0%	5.0%	5.0%	5.0%
Inflammatory Bowel Disease	30.0%	30.0%	30.0%	30.0%	20.0%
Rheumatoid Arthritis	23.3%	16.7%	26.7%	20.0%	16.7%
Ulcerative Colitis	40.0%	40.0%	60.0%	60.0%	40.0%
Vasculitis	10.7%	10.7%	10.7%	14.3%	14.3%

The frequency of ANA detected in the Celiac Disease cohort seemed higher than expected. There is literature supporting elevated ANA in celiac disease patients (39). Additionally, these specimens were tested on another FDA-cleared, commercial ANA HEp-2 IFA and the ANA was confirmed on that product.

5. Interpretation Method Comparisons:

There were 350 clinical samples that were tested at all three clinical sites. There were 30 additional clinically characterized celiac disease and vasculitis specimens that were tested at site one and site three. Finally, there were an additional 12 retrospectively collected specimens that exhibited the nuclear membrane or nuclear dots pattern that were tested at sites one and three. With these 392 specimens, there were a total of 2268 instances where one could compare the results of Method A versus Method B, Method A versus Method C, and Method B versus Method C. A summary of those qualitative comparisons appears in the tables below:

a. **Method A vs Method B Qualitative Comparison**

Method A vs Method B		Positive Sample Agreement (95% CI)	Negative Sample Agreement (95% CI)	Total Sample Agreement (95% CI)
Site 1	Technician A	95.6% (152/159) (91.2 - 97.9%)	95.7% (223/233) (92.3 - 97.7%)	95.7% (375/392) (93.2 - 97.3%)
	Technician B	94.9% (168/177) (90.6 - 97.3%)	98.6% (212/215) (95.9 - 99.5%)	96.9% (380/392) (94.7 - 98.2%)
Site 2	Technician A	99.2% (124/125) (95.6 - 99.9%)	93.8% (211/225) (89.8 - 96.3%)	95.7% (335/350) (93.1 - 97.4%)
	Technician B	98.6% (137/139) (94.9 - 99.6%)	97.6% (206/211) (94.6 - 98.95%)	98.0% (343/350) (95.9 - 99.0%)
Site 3	Technician A	95.5% (148/155) (90.9 - 97.8%)	98.7% (234/237) (96.4 - 99.65%)	97.5% (382/392) (95.4 - 98.6%)
	Technician B	95.9% (162/169) (91.7 - 97.9%)	97.8% (218/223) (94.9 - 99.0%)	96.9% (380/392) (94.7 - 98.2%)

b. **Combined Qualitative Agreement for Method A vs Method B All Sites/All Technicians**

		Method A	
		Positive	Negative
Method B	Positive	891	40
	Negative	33	1304

Positive Percent Agreement = 96.43% (891 / 924) 95% Confidence Interval = 95.03 - 97.45%
Negative Percent Agreement = 97.02% (1304 / 1344) 95% Confidence Interval = 95.97 - 97.81%
Total Percent Agreement = 96.78% (2195 / 2268) 95% Confidence Interval = 95.97 - 97.43%

c. **Method A vs Method C Qualitative Comparison**

Since Method C yielded uncertain results for a lot of specimens, positive and negative percent agreement was calculated using both the applicable scenarios:

Uncertain Counted Towards Positives

Method A vs Method C		Positive Sample Agreement (95% CI)	Negative Sample Agreement (95% CI)	Total Sample Agreement (95% CI)
Site 1	Technician A	97.5% (155/159) (93.7 - 99.0%)	80.3% (187/233) (74.7 - 84.9%)	87.2% (342/392) (83.6 - 90.2%)
	Technician B	98.3% (174/177) (95.1 - 99.4%)	87.4% (188/215) (82.4 - 91.2%)	92.4% (362/392) (89.3 - 94.6%)
Site 2	Technician A	100% (125/125) (97.0 - 100.0%)	85.3% (192/225) (80.1 - 89.4%)	90.6% (317/350) (87.1 - 93.2%)
	Technician B	100% (139/139) (97.3 - 100.0%)	91.0% (192/211) (86.4 - 94.2%)	94.6% (331/350) (91.7 - 96.5%)

Site 3	Technician A	100% (155/155) (97.6 - 100.0%)	87% (206/237) (82.0 - 90.7%)	92.1% (361/392) (88.9 - 94.4%)
	Technician B	98.8% (167/169) (95.8 - 99.7%)	91.9% (205/223) (87.6 - 94.8%)	94.9% (372/392) (92.3 - 96.7%)

d. Results for Method A vs Method C for all sites and all technicians combined presented below:

		Method A	
		Positive	Negative
Method C	Positive	915	174
	Negative	9	1170

Positive Percent Agreement: 99.03 % (915/924) 95% CI: 98.39 - 99.55%
Negative Percent Agreement: 87.05 % (1170/1344) 95% CI: 85.15 - 88.74%
Total Percent Agreement: 91.93 % (2085/2268) 95% CI: 90.74 - 92.98%

Uncertain Counted Towards Negatives

Method A vs Method C		Positive Sample Agreement (95% CI)	Negative Sample Agreement (95% CI)	Total Sample Agreement (95% CI)
Site 1	Technician A	93.1% (148/159) (88.0 - 96.1%)	94.0% (219/233) (90.2 - 96.4%)	93.6% (367/392) (90.8 - 96.5%)
	Technician B	90.4% (160/177) (85.2 - 93.9%)	99.1% (213/215) (96.7 - 99.7%)	95.2% (373/392) (92.6 - 96.9%)
Site 2	Technician A	99.2% (124/125) (95.1 - 99.9%)	94.7% (213/225) (90.9 - 96.9%)	96.3% (337/350) (93.8 - 97.8%)
	Technician B	95.0% (132/139) (89.9 - 97.5%)	98.1% (207/211) (95.2 - 99.3%)	96.9% (339/350) (94.5 - 98.2%)
Site 3	Technician A	96.1% (149/155) (91.8 - 98.2%)	97.1% (230/237) (94.4 - 98.1%)	96.7% (379/392) (94.4 - 98.1%)
	Technician B	90.5% (153/169) (85.2 - 94.1%)	98.7% (220/223) (96.1 - 99.5%)	95.2% (373/392) (92.6 - 96.9%)

e. Results for Method A vs Method C for all sites and all technicians combined presented below:

		Method A	
		Positive	Negative
Method C	Positive	866	42
	Negative	58	1302

Positive Percent Agreement: 93.72 % (866/924) 95% CI: 91.97 - 95.11%
Negative Percent Agreement: 96.88% (1302/1344) 95% CI: 95.80 - 97.68%
Total Percent Agreement: 95.59 % (2168/2268) 95% CI: 94.67 - 96.36%

f. **Method B vs Method C Qualitative Comparison**

Since Method C can yield an uncertain (UNC) result in addition to a positive or negative qualitative result, the agreement between methods were calculated using the UNC samples considered positive and then considered negative:

Table 15: UNC considered as positive for each site and each technician

Method B vs Method C		Positive Sample Agreement (95% CI)	Negative Sample Agreement (95% CI)	Total Sample Agreement (95% CI)
Site 1	Technician A	100.0% (162/162) (97.7 - 100.0%)	83.0% (191/230) (77.7 - 87.3%)	90.1% (353/392) (86.7 - 92.7%)
	Technician B	99.4% (170/171) (96.8 - 99.9%)	86.4% (191/221) (81.3 - 90.3%)	91.8% (360/392) (88.7 - 94.2%)
Site 2	Technician A	100.0% (138/138) (97.3 - 100.0%)	90.6% (192/212) (85.9 - 93.8%)	94.3% (330/350) (91.3 - 96.3%)
	Technician B	100.0% (142/142) (97.4 - 100.0%)	92.3% (192/208) (87.9 - 95.2%)	95.4% (334/350) (92.7 - 97.2%)
Site 3	Technician A	99.3% (150/151) (96.3 - 99.9%)	85.5% (206/241) (80.5 - 89.4%)	89.9% (356/392) (86.5 - 92.5%)
	Technician B	99.4% (166/167) (96.7 - 99.9%)	91.6% (206/225) (87.2 - 94.5%)	94.9% (372/392) (92.3 - 96.7%)

Table 16: Combined Qualitative Agreement Between Method B and Method C for All Sites/All Technicians (UNC as Positives):

		Method B		
		Positive	Negative	Total
Method C	Positive	928	160	1088
	Negative	3	1177	1180
	Total	931	1337	2268

Positive Percent Agreement: 99.7 % (928/931) 95% CI: 99.1 - 99.9%
Negative Percent Agreement: 88.0 % (1177/1337) 95% CI: 86.2 - 89.7%
Total Percent Agreement: 92.8% (2105/2268) 95% CI: 91.7 - 93.8%

Table 17: UNC Considered as Negative for Each Site and Each Technician

Method B vs Method C		Positive Sample Agreement (95% CI)	Negative Sample Agreement (95% CI)	Total Sample Agreement (95% CI)
Site 1	Technician A	95.7% (155/162) (91.4 - 97.9%)	97.0% (223/230) (93.9 - 98.5%)	96.4% (378/392) (94.1 - 97.9%)
	Technician B	94.2% (161/171) (89.6 - 96.8%)	99.6% (220/221) (97.5 - 99.9%)	97.2% (381/392) (95.1 - 98.4%)
Site 2	Technician A	98.6% (136/138) (94.9 - 99.60%)	100.0% (212/212) (98.2 - 100.0%)	99.4% (348/350) (97.9 - 99.8%)
	Technician B	95.8% (136/142) (91.1 - 98.1%)	100.00% (208/208) (98.2 - 100.0%)	98.3% (344/350) (96.3 - 99.2%)
Site 3	Technician A	98.0% (148/151) (94.3 - 99.3%)	96.7% (233/241) (93.6 - 98.3%)	97.2% (381/392) (95.1 - 98.4%)
	Technician B	92.8% (155/167) (87.9 - 95.8%)	99.56% (224/225) (97.5 - 99.9%)	96.68% (379/392) (94.4 - 98.1%)

Table 18: Combined Qualitative Agreement Between Method B and Method C for All Sites/All Technicians (UNC as Negatives):

		Method B		
		Positive	Negative	Total
Method C	Positive	891	17	908
	Negative	40	1320	1360
	Total	931	1337	2268

Positive Percent Agreement: 95.7% (891/931) 95% CI: 94.2 - 96.8%
Negative Percent Agreement: 98.7% (1320/1337) 95% CI: 98.0 - 99.2%
Total Percent Agreement: 97.5% (2211/2268) 95% CI: 96.8 - 98.1%

In all cases, the qualitative agreement between interpretation methods is quite high indicating that all three methods (manual microscope, digital read of the ZEUS dIFine® scanner and automated call from the ZEUS dIFine® scanner) correlate well with each other and exhibited few discrepancies.

Additionally, pattern determinations were correlated across different interpretation methods; samples that had no pattern (i.e., 'negative') or samples that yielded uncertain results (i.e., 'UNC') were included in the pattern agreement analyses between the different interpretation methods are presented in the table below:

		Method A vs Method B Percent Agreement (%) (# Agree/Total #) [95% CI]	**Method A vs Method C** Percent Agreement (%) (# Agree/Total #) [95% CI]	**Method B vs Method C** Percent Agreement (%) (# Agree/Total #) [95% CI]
Site 1	Technician A	98.5 (386/392 [96.7 - 99.3%]	84.2 (330/392) [80.2 - 87.5%]	83.9 (329/392) [80.0 - 87.2%]
	Technician B	97.7 (383/392) [95.7 - 98.8%]	85.0 (333/392) [81.1 - 88.2%]	85.2 (334/392) [81.4 - 88.4%]
Site 2	Technician A	96.9 (339/350) [94.5 - 98.2%]	86.0 (301/350) [82.0 - 89.3%]	86.9 (304/350) [82.9 - 90.0%]
	Technician B	97.7 (342/350) [95.6 - 98.8%]	86.6 (303/350) [82.6 - 89.8%]	87.1 (305/350) [83.2 - 90.3%]
Site 3	Technician A	98.47 (386/392) [96.7 - 99.3%]	88.3 (346/392) [84.7 - 91.1%]	88.5 (347-392) [85.0 - 91.3%]
	Technician B	98.2 (385/392) [96.4 - 99.1%]	88.0 (345/392) [84.4 - 90.9%]	87.5 (343/392) [83.9 - 90.4%]

Combining all three sites together resulted in the following pattern agreement:

	Pattern Agreement Number/Total Samples	Percent Agreement (%)	95% CI
Method A vs Method B	2221/2268	98.0	97.3-98.4%
Method A vs Method C	1958/2268	86.3	84.9-87.7%
Method B vs Method C	1962/2268	86.5	85.0-87.9%

Samples that were positive by any method at each site were titrated to endpoint and interpreted by all three methods. Samples that were qualitatively positive by all three methods at any one site could be compared for endpoint agreement. At site one, there were 156 specimens titered to endpoint. At site two there were 133 specimens titered to endpoint and at site three there were 154 specimens titered to endpoint. Considering there were two technicians at each site to read by both method A and method B, this resulted in 886 times comparisons in endpoint determinations could be made between Method A versus Method B, Method A versus Method C and Method B versus Method C. The number of times those endpoint determinations matched each other plus or minus one, two-fold dilution is depicted in the table below:

Combined Endpoint Titer Agreement (All Sites/All Technicians)			
Interpretation	[± 1 dilution]/Total Samples	Percent Agreement (%)	95% CI
Method A vs. Method B	880/886	99.32	98.53 - 99.69%
Method A vs. Method C	879/886	99.21	98.38 - 99.62%
Method B vs. Method C	880/886	99.32	98.53 - 99.69%

This comparison shows that the endpoint determination between methods is high and there were very few instances where the endpoint dilution determined by one method resulted in an endpoint dilution that was greater than ± one dilution by another method.

Taken altogether, these data demonstrate that the auto-call pattern identified by ZEUS dIFine® (Method C) agrees with Method A and/or Method B (non-automated pattern identification methods) for the vast majority of the samples. However, it is still the responsibility of the trained operator to make the final decision.

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