24. van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* 2013;65:2737-2747.
25. Shiboski CH, Shiboski SC, Seror R, et al. 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjogren's syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Ann Rheum Dis.* 2017;76:9-16.
26. Maksymowych WP, Naides SJ, Bykerk V, et al. Serum 14-3-3eta is a novel marker that complements current serological measurements to enhance detection of patients with rheumatoid arthritis. *J Rheumatol.* 2014;41:2104-2113.
27. Singh JA, Saag KG, Bridges SL Jr, et al. 2015 American College of Rheumatology guideline for the treatment of rheumatoid arthritis. *Arthritis Rheumatol.* 2016;68:1-26.

* The CPT codes provided are based on AMA guidelines and are for informational purposes only. CPT coding is the sole responsibility of the billing party. Please direct any questions regarding coding to the payer being billed.

This test was developed and its analytical performance characteristics have been determined by Quest Diagnostics. It has not been cleared or approved by the U.S. Food and Drug Administration. This assay has been validated pursuant to the CLIA regulations and is used for clinical purposes.

Test Summary

ANA Screen, IFA, Reflex Titer/Pattern, Reflex Mplx 11 Ab Cascade With IdentRA®

Test Code: 94954

Specimen Requirements: 4 mL room-temperature serum (red-top tube [no gel]); 2 mL minimum

CPT Codes*: 86038, 86431, 86200, 83520. If the ANA IFA test is positive, reflex testing for titer and pattern (CPT code 86039) and 5 antibodies will be performed: dsDNA (CPT code 86225), Sm/RNP (CPT code 86235), RNP (CPT code 86235), Sm (CPT code 86235), chromatin (CPT code 86235). If all 5 are negative, 4 additional antibodies will be tested: SSA (CPT code 86235), SSB (CPT code 86235), Scl-70 (CPT code 86235), Jo-1 (CPT code 86235). If all 4 are negative, 2 additional antibodies will be tested: ribosomal P (CPT code 83516) and centromere B (CPT code 86235). Reflex tests are performed at additional charge.

CLINICAL USE

- Evaluate suspected autoimmune rheumatic diseases

CLINICAL BACKGROUND

Autoimmune rheumatic diseases are conditions in which the immune system attacks the joints and certain systems. They are often difficult to diagnose, as their symptoms can be vague, vary from patient to patient, and often overlap. Laboratory testing can provide useful information, but no single test provides a definitive diagnosis for any one rheumatic disease.

Diagnosis is most often based on a compilation of symptoms and signs, including clinical information and laboratory test results. Testing for antinuclear antibodies (ANAs) using an immunofluorescence assay (IFA) is a good first approach for laboratory evaluation of patients suspected of having certain autoimmune rheumatic diseases.

ANAs, a group of autoantibodies directed against diverse nuclear and cytoplasmic antigens, are associated with several autoimmune rheumatic diseases (**Table 1**). These include systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and mixed connective tissue disease (MCTD). Although various platforms can be used to detect ANAs, an IFA with HEp-2 cells remains the gold standard[1] because of its high sensitivity for several of the autoimmune rheumatic diseases.[2] The high sensitivity stems from the large number of autoantigens (up to 150) in HEp-2 cells.

The diagnostic value of ANA testing varies with the specific clinical condition (**Table 1**). For example, positive ANA results are required for diagnosis of drug-associated lupus and MCTD. Testing is also recommended when SLE or SSc are suspected. ANA testing may also be helpful in evaluating Sjögren syndrome and polymyositis/dermatomyositis.

Individuals with rheumatoid arthritis (RA) or other rheumatoid disorders may be positive or negative for ANAs.

Knowing the ANA titer can be helpful in interpreting positive ANA results. A titer of at least 1:40 is considered positive, although most patients with autoimmune disease will have higher levels. Low-positive titers (eg, 1:40) are not uncommon in healthy individuals.[6] For example, about 20% to 30% of healthy individuals have positive titers of 1:40, but this prevalence drops to <15% for titers of ≥1:80 and 5% for titers ≥1:160.[4] Nevertheless, using a low threshold of 1:40 can increase sensitivity for SLE, systemic sclerosis, and Sjögren syndrome.[6]

For patients with positive ANA screening results, nuclear and cytoplasmic antibody fluorescence patterns can be used to evaluate the likelihood of certain autoimmune diseases.[7,8] These patterns may inform the differential diagnosis, although they may not be specific for individual antibodies or diseases. For example, a homogenous nuclear pattern may be associated with SLE, drug-induced SLE/vasculitis, or juvenile idiopathic arthritis (JIA), while a diffuse cytoplasmic pattern could be consistent with SLE or an inflammatory myopathy.[1]

Given the range of conditions associated with positive ANA IFA results, positive results are not highly specific for any single condition. Tests for individual antibodies may offer greater specificity[9,10] and are often needed to help establish the diagnosis.[4,7] The combination of the highly sensitive ANA IFA screen, followed by specific autoantibody testing when the IFA is positive, can help maximize sensitivity as well as specificity. Testing *tiers* of specific antibodies following a positive ANA IFA result, as depicted in the **Figure**, can help speed the evaluation. **Table 2** summarizes the sensitivity of individual antibodies across disorders.

The ANA Screen, IFA, Reflex Titer/Pattern, Reflex Mplx 11 Ab Cascade with IdentRA® includes ANA IFA and a tiered cascade

of specific autoantibodies (**Figure**). The "IdentRA" components of the test include widely used laboratory markers for RA—rheumatoid factor (RF) and cyclic citrullinated peptide (CCP) antibodies[11]—as well as serum 14-3-3η protein, a biomarker for diagnosis of RA.[26] Used in conjunction with RF and CCP antibody testing, 14-3-3η improves sensitivity for diagnosis of early and established RA (**Table 3**).[26] In 21% to 67% of seronegative RA patients, 14-3-3η was found to be positive. Since a positive result for any of these RA markers facilitates timelier diagnosis of ANA-negative RA, simultaneous testing can enable prompt initiation of disease-modifying therapy before significant joint erosion develops. Although RA is not generally associated with a high prevalence of ANA, occasional patients may present with features of RA and SLE ("rhupus") and be positive for ANA and IdentRA analytes. RA can be present as a comorbid condition in patients with other autoimmune rheumatic diseases, and also in patients with suspected rheumatic diseases who test negative for ANAs.

INDIVIDUALS SUITABLE FOR TESTING

- Individuals with signs and symptoms associated with autoimmune disease(s)

METHOD

- CCP antibody and 14-3-3η testing performed using ELISA-based assays
- RF testing performed with immunonephelometry
- ANA screening conducted using an IFA performed with HEp-2 cells
  - Negative ANA IFA results at the 1:40 dilution terminate the reflex cascade
  - Positive ANA IFA results at the 1:40 dilution prompt reflex to
    - Reporting of the corresponding antibody fluorescence pattern
    - Titer is determined by serial dilution until the pattern cannot be observed, or to a dilution of 1:1280; specimens may be titrated to endpoint upon request
    - The 3-tier autoantibody reflex cascade (**Figure**) is performed using a multiplex immunoassay

Table 1. Utility of Antinuclear Antibody IFA Test by Condition[3-5]

Condition	Comments/Recommendations[a]
Drug-associated lupus	• Positive ANA part of the diagnostic criteria • ANA useful for symptomatic people who are taking a drug associated with drug-induced lupus
Mixed connective tissue disease (MCTD)	• Positive ANA part of the diagnostic criteria • ANA recommended when clinical suspicion of MCTD • Follow-up with RNP antibody recommended to confirm diagnosis
Autoimmune hepatitis	• Positive ANA part of diagnostic criteria • Positive ANA often seen in patients with diverse liver disease; does not exclude other hepatic diseases
Systemic lupus erythematosus (SLE)	• ANA sensitivity 93%, specificity 57% • Best initial test when clinical suspicion of SLE is strong • SLE unlikely if ANA negative • Specific antibody tests recommended as follow-up to positive ANA
Systemic sclerosis (SSc)	• ANA sensitivity 85%, specificity 54% • ANA recommended when clinical suspicion of SSc • If negative, consider other fibrosing illnesses (eg, eosinophilic fasciitis, linear scleroderma)
Sjögren syndrome	• Sensitivity 48%, specificity 52% (if ANA titer ≥320, sensitivity = 72.8% [95% CI, 67.5–77.7%] and specificity = 80.4% [95% CI, 76.9–84.0]) • Can help clarify whether an underlying connective tissue disease exists when Sjögren syndrome suspected
Polymyositis/ dermatomyositis	• ANA sensitivity 61%, specificity 63% • Positive ANA provides weak evidence of disease even when combined with clinical suspicion • Must consider other connective tissue diseases (SLE or overlap syndrome) regardless of ANA status

ANA, antinuclear antibody test; RNP, ribonucleoprotein.
[a] The American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines[3]

Table 2. Autoantibody Prevalence (%) in Rheumatic and Related Diseases[a 3,5,11-13,15-23]

Antibody	SLE	MCTD	Sjögren Syndrome	Systemic Sclerosis	Polymyositis	CREST Syndrome[b]	Neurologic SLE
ANA	93	100	48	85	61	70	NA
CENP-B	3-12	7[c]	<2	27[d]	<2	66	
Chromatin	37-73	>80	12	14	8		
dsDNA	57-62	0-8	11-20	8	10-43		
Jo-1	<2	7[e]	<2	<2	17		
Rib P		7	<2	<2	<2		9-30
RNP	22-48	>80	12	14	8		
Scl-70	2-3	7[f]	<2	16[d]	<2		
Sm	20-30	8	4	0	10		
Sm/RNP	30	54-94	9	4	9		
SS-A/Ro	33-52	13	>80	23	42		
SS-B/La[g]	13-27	<2	>80	5	<2		

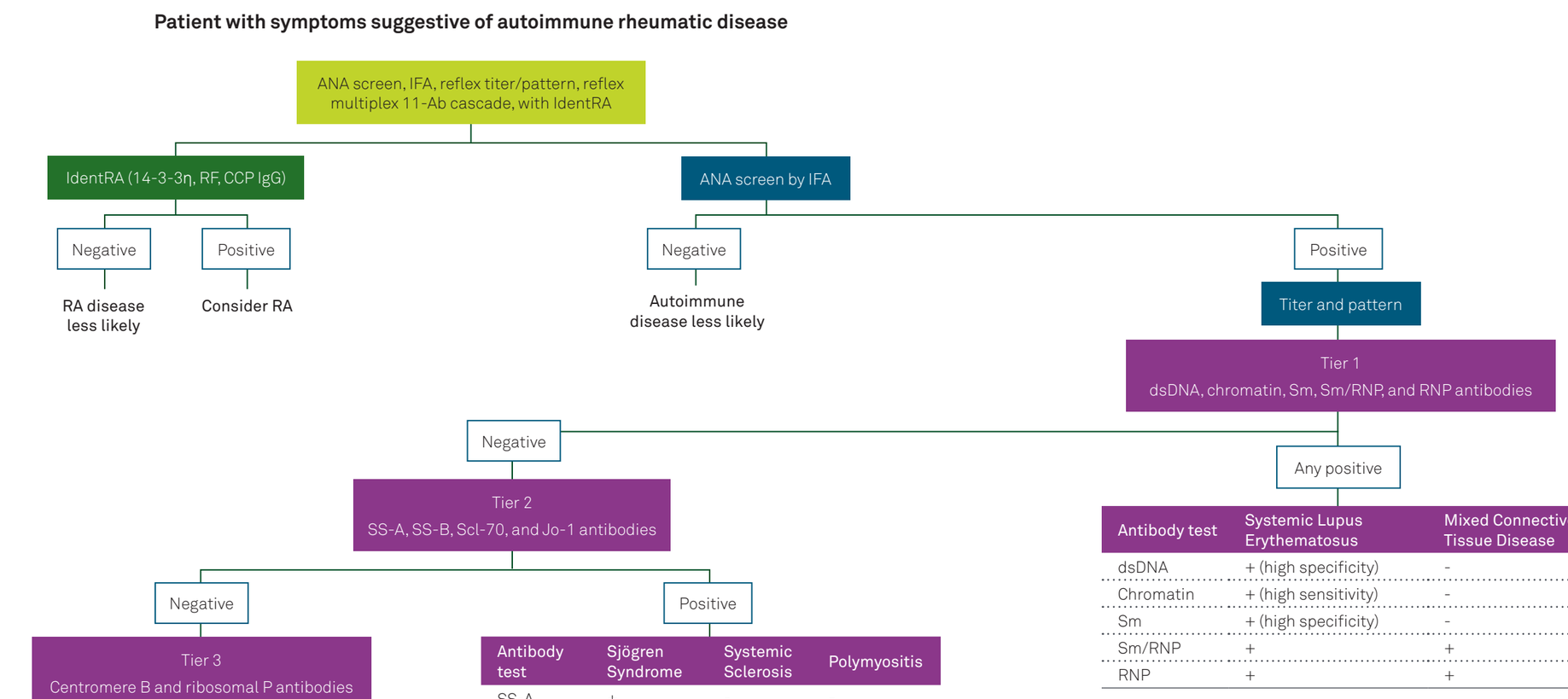
ANA, antinuclear antibody; CENP-B, centromere B; dsDNA, double-stranded DNA; Jo-1, histidyl-tRNA synthetase; NA, not available; MCTD, mixed connective tissue disease; Rib P, ribosomal P; RNP, ribonucleoprotein; Scl-70, scleroderma (topoisomerase 1); SLE, systemic lupus erythematosus; Sm, Smith; Sm/RNP, Smith/ribonucleoprotein; and SS-A, SS-B, Sjögren antibodies A and B.
[a] Highlighted antibodies represent diagnostic criteria for the disease. Note that antibodies whose presence is a diagnostic criterion do not always correspond to those with the highest prevalence in that disease.
[b] CREST syndrome is a variant of systemic sclerosis defined by the presence of calcinosis cutis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia. Also known as limited cutaneous scleroderma.
[c] Typically in cases with features of polymyositis.
[d] The presence of scleroderma-related antibodies (centromere, Scl-70, or RNA polymerase III antibodies) is not necessary or sufficient for diagnosis, but is useful for classification in the absence of diagnostic clinical findings ("clear skin thickening of the fingers extending proximal to the metacarpophalangeal joints"[24]).
[e] Especially in cases with features of muscle inflammation.
[f] Especially in cases with features of systemic sclerosis.
[g] SS-B is no longer included in consensus classification criteria for primary Sjögren syndrome.[25]

Table 3. Sensitivity and Specificity of RF, CCP Antibody, and 14-3-3η for Detecting RA[26]

Markers	Early RA[a] (n=99)		Established RA[a] (n=135)	
	Sensitivity	Specificity	Sensitivity	Specificity
RF	57	85	84	85
CCP antibody	59	99	79	99
14-3-3η	64	93	77	93
RF and CCP antibody[b]	72	84	88	84
RF, CCP antibody, and 14-3-3η[b]	78	78	90	78

Early rheumatoid arthritis (RA) indicates disease history of 6 months or less. Established RA indicates disease history of >6 months.
RF, rheumatoid factor; CCP, cyclic citrullinated peptide.
[a] Comparison with healthy controls.
[b] Results considered positive for RA if any of the biomarkers are positive.

Figure. ANA Screen, IFA, Reflex Titer/Pattern, Reflex Multiplex 11-Ab Cascade, With IdentRA® (test code 94954)



Antibody test	CREST Syndrome	Neuropsychiatric SLE
Centromere B	+	-
Ribosomal P	-	+

Antibody test	Sjögren Syndrome	Systemic Sclerosis	Polymyositis
SS-A	+	-	-
SS-B	+	-	-
Scl-70	-	+[a]	-
Jo-1	-	-	+

[a] Detection of RNA polymerase III and centromere antibodies may also contribute to classification of systemic sclerosis

Antibody test	Systemic Lupus Erythematosus	Mixed Connective Tissue Disease
dsDNA	+ (high specificity)	-
Chromatin	+ (high sensitivity)	-
Sm	+ (high specificity)	-
Sm/RNP	+	+
RNP	+	+

If ANA IFA is positive, and no positive specific antibodies are detected, correlate with clinical findings and consider other autoimmune diseases and tests.

Sub-panels that can be ordered separately include test code 91472 (dark green fill), test code 249 (blue fill), and test code 16814 (blue fill plus purple fill).

The acronym CREST refers to a syndrome defined by presence of calcinosis cutis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia. CCP indicates cyclic citrullinated peptide; dsDNA, DNA; IFA, immunofluorescence assay; Jo-1, histidyl-tRNA synthetase; RA, rheumatoid arthritis; RF, rheumatoid factor; Sm/RNP, Smith/ribonucleoprotein; SS-A and -B, Sjögren syndrome A and B; and Scl-70, systemic sclerosis (topoisomerase I).

This figure was developed by Quest Diagnostics based in part on references 2,9, and 11-14. It is provided for informational purposes only and is not intended as medical advice. A physician's test selection and interpretation, diagnosis, and patient management decisions should be based on his/her education, clinical expertise, and assessment of the patient.

- Aside from ANA titer and pattern, panel components may be ordered separately (**Table 4**).
- Reflex tests are performed at additional charge and are associated with an additional CPT code(s).

REFERENCE RANGES

ANA IFA Screen: Negative

ANA Titer:

- Negative: <1:40
- Low antibody level: 1:40-1:80
- Elevated antibody level: >1:80

RF: <14 IU/mL

CCP antibody:

- Negative: <20 Units
- Weak positive: 20-39 Units
- Moderate positive: 40-59 Units
- Strong positive: >59 Units

14-3-3η: <0.2 ng/mL

INTERPRETIVE INFORMATION

A positive ANA result in conjunction with clinical suspicion suggests, but does not necessarily confirm, the presence of an autoimmune disease. Positive results are not uncommon in healthy individuals (particularly as they age) and those with certain infectious diseases or cancer.[12] In cases with strong clinical suspicion, specific antibody testing may be appropriate even if the ANA IFA result is negative.

A positive ANA IFA result with a positive result for 1 or more of the specific antibodies may suggest the presence of a certain autoimmune disease (**Table 2**). If the ANA is positive but the 11 antibodies tested in the cascade antibody tests are negative, the patient may still have an autoimmune disease other than those typically associated with the antibodies tested. Tests for other autoimmune diseases may be considered if clinically indicated; these include autoimmune hepatitis, primary biliary cholangitis, autoimmune thyroiditis, Addison's disease, pernicious anemia, autoimmune neuropathies, vasculitis, celiac disease, and bullous disease.

A negative ANA IFA result suggests the absence of many autoimmune diseases, but does not rule them out. Thus, specific autoantibody testing may be considered for patients with negative ANA IFA results if clinical suspicion is high (eg, Jo-1 for suspected polymyositis/dermatomyositis, SS-A for suspected Sjögren syndrome).

A positive result on the RF, CCP antibody, or 14-3-3η tests generally supports a diagnosis of early or established RA, or potential subclinical RA (**Table 3**). In ANA-negative patients, positive results are consistent with early RA.[27] A negative result on all 3 tests is consistent with absence of RA (sensitivity = 78%), although early RA cannot be ruled out (**Table 3**). If clinically indicated, a negative test result may also be followed up with tests for other autoimmune diseases.

Table 4. Individual Tests Included in the ANA Screen, IFA, Reflex Titer/Pattern, Reflex Mplx 11-Ab Cascade With IdentRA®

Test Code	Test Name	CPT Code
249	ANA Screen, IFA, with Reflex to Titer and Pattern[a]	86038
16814	ANA Screen, IFA, Reflex Titer/Pattern, and Reflex to Multiplex 11 Ab Cascade[a] (**Figure**)	86038
255	DNA (ds) Antibody	86225
38567	Sm/RNP Antibody	86235
19887	RNP Antibody	86235
37923	Sm Antibody	86235
34088	Chromatin (Nucleosomal) Antibody	86235
38568	Sjögren's Antibody (SS-A)	86235
38569	Sjögren's Antibody (SS-B)	86235
4942	Scleroderma Antibody (Scl-70)	86235
5810	Jo-1 Antibody	86235
34283	Ribosomal P Antibody	83516
16088	Centromere B Antibody	86235
4418	Rheumatoid Factor	86431
11173	Cyclic Citrullinated Peptide (CCP) Antibody (IgG)	86200
91455	14-3-3 eta Protein[b]	83520

[a] Reflex tests are performed at additional charge and are associated with an additional CPT code(s).
[b] This test was developed and its analytical performance characteristics have been determined by Quest Diagnostics. It has not been cleared or approved by the U.S. Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. This assay has been validated pursuant to the CLIA regulations and is used for clinical purposes.

References

1. Agmon-Levin N, Damoiseaux J, Kallenberg C, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *Ann Rheum Dis.* 2014;73:17-23.
2. American College of Rheumatology. Methodology of testing for antinuclear antibodies (position statement). www.rheumatology.org/Portals/0/Files/Methodology%20of%20Testing%20Antinuclear%20Antibodies%20Position%20Statement.pdf. Published January 2009. Updated August 2015. Accessed December 5, 2017.
3. Solomon DH, Kavanaugh AJ, Schur PH, et al. Evidence-based guidelines for the use of immunologic tests: antinuclear antibody testing. *Arthritis Rheum.* 2002;47:434-444.
4. Kavanaugh A, Tomar R, Reveille J, et al. Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. American College of Pathologists. *Arch Pathol Lab Med.* 2000;124:71-81.
5. Shiboski SC, Shiboski CH, Criswell L, et al. American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the Sjogren's International Collaborative Clinical Alliance cohort. *Arthritis Care Res (Hoboken).* 2012;64:475-487.
6. Tozzoli R, Bizzaro N, Tonutti E, et al. Guidelines for the laboratory use of autoantibody tests in the diagnosis and monitoring of autoimmune rheumatic diseases. *Am J Clin Pathol.* 2002;117:316-324.
7. Satoh M, Vazquez-Del Mercado M, Chan EK. Clinical interpretation of antinuclear antibody tests in systemic rheumatic diseases. *Mod Rheumatol.* 2009;19:219-228.
8. Chan EK, Damoiseaux J, Carballo OG, et al. Report of the first international consensus on standardized nomenclature of antinuclear antibody HEp-2 cell patterns 2014-2015. *Front Immunol.* 2015;6:412.
9. Satoh M, Chan EK, Sobel ES, et al. Clinical implication of autoantibodies in patients with systemic rheumatic diseases. *Expert Rev Clin Immunol.* 2007;3:721-738.
10. Kumar Y, Bhatia A, Minz RW. Antinuclear antibodies and their detection methods in diagnosis of connective tissue diseases: a journey revisited. *Diagn Pathol.* 2009;4:1.
11. Aletaha D, Neogi T, Silman AJ, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* 2010;62:2569-2581.
12. Petri M, Orbai AM, Alarcon GS, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum.* 2012;64:2677-2686.
13. Cappelli S, Bellando Randone S, Martinovic D, et al. "To be or not to be," ten years after: evidence for mixed connective tissue disease as a distinct entity. *Semin Arthritis Rheum.* 2012;41:589-598.
14. Stinton LM, Fritzler MJ. A clinical approach to autoantibody testing in systemic autoimmune rheumatic disorders. *Autoimmun Rev.* 2007;7:77-84.
15. Hoffman IE, Peene I, Meheus L, et al. Specific antinuclear antibodies are associated with clinical features in systemic lupus erythematosus. *Ann Rheum Dis.* 2004;63:1155-1158.
16. Cervera R, Vinas O, Ramos-Casals M, et al. Anti-chromatin antibodies in systemic lupus erythematosus: a useful marker for lupus nephropathy. *Ann Rheum Dis.* 2003;62:431-434.
17. Colglazier CL, Sutej PG. Laboratory testing in the rheumatic diseases: a practical review. *South Med J.* 2005;98:185-191.
18. Scholz J, Grossmann K, Knutter I, et al. Second generation analysis of antinuclear antibody (ANA) by combination of screening and confirmatory testing. *Clin Chem Lab Med.* 2015;53:1991-2002.
19. Moder KG, Wener MH, Weisman MH, et al. Measurement of antinuclear antibodies by multiplex immunoassay: a prospective, multicenter clinical evaluation. *J Rheumatol.* 2007;34:978-986.
20. Binder SR, Genovese MC, Merrill JT, et al. Computer-assisted pattern recognition of autoantibody results. *Clin Diagn Lab Immunol.* 2005;12:1353-1357.
21. Mouthon L, Dunogue B, Guillevin L. Diagnosis and classification of eosinophilic granulomatosis with polyangiitis (formerly named Churg-Strauss syndrome). *J Autoimmun.* 2014;48-49:99-103.
22. Tur BS, Suldur N, Ataman S, et al. Anti-neutrophil cytoplasmic antibodies in patients with rheumatoid arthritis: clinical, biological, and radiological correlations. *Joint Bone Spine.* 2004;71:198-202.
23. van Paassen P, Damoiseaux J, Tervaert JW. Laboratory assessment in musculoskeletal disorders. *Best Pract Res Clin Rheumatol.* 2003;17:475-494.