



PEARLS OF LABORATORY MEDICINE

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TITLE: Antinuclear Antibody Testing

PRESENTER: Danyel H. Tacker, PhD, DABCC, FAACC

Slide 1:

Hello, my name is Danyel Tacker. I am an Associate Clinical Professor in the Department of Pathology, Anatomy, and Laboratory Medicine at West Virginia University. Welcome to this Pearl of Laboratory Medicine on “**Antinuclear Antibody Testing.**”

Slide 2:

In this Pearl, we will start with a definition of antinuclear antibodies – or, ANA – and clinical disease states with strongest association to ANA. We’ll look at different techniques employed in ANA testing – with emphasis on the gold-standard testing technique – as well as benefits and limitations of these techniques. Finally, we’ll look at current clinical recommendations for ANA testing and reporting practices.

Slide 3:

ANA are defined as autoimmune antibodies that bind to epitopes in the cell nucleus. In most healthy individuals, these components are viewed by the immune system as “self” and evade immune attack. However, in patients with a significant derangement of immune self-recognition, production of autoantibodies can result in clinical illness. Listed here are some of the major classes of nuclear components that are targeted by ANA. Keep in mind that over decades of research, over 150 discrete epitopes have been identified, making ANA a diverse group of antibodies.

Slide 4:

The American College of Rheumatology – or, ACR – strongly recommends testing for ANA in a patient only after a clearly established suspicion of an associated disease state is developed

clinically. Thus, ANA should support diagnosis largely for the conditions and indications listed in the table shown, rather than serve as a screening tool for the general population. Note that some of the conditions listed deem ANA results useful for diagnosis, whereas others have ANA positivity listed as a criterion for diagnosis. Several conditions have weaker associations to ANA positivity in terms of diagnosis, but employ ANA testing for monitoring disease status and assessing prognosis.

Slide 5:

The first of the modern ANA testing methods is the indirect immunofluorescence assay, or IFA. Briefly, ANA in the patient serum are allowed to bind to corresponding nuclear epitopes in human epithelium-derived HEp-2 cells on microscope slides. Fluorophore-conjugated detection antibodies bind to the ANA that are bound to the cells; the slide is then viewed with a fluorescence microscope. Typically, testing employs a dilution series, starting with a 1:40 or 1:80 minimal dilution that doubles with each additional dilution prepared. When positive, the staining pattern is reported along with the highest reportable dilution giving a positive fluorescence signal.

Slide 6:

Over the years, variability in IIFA testing and reporting practices pointed out a need for standardization. The International Consensus on ANA Pattern – or, ICAP – answered the call and has published several consensus documents. The ICAP initially gathered expert consensus regarding 28 distinct IIFA patterns found in ANA testing, categorizing them first by nuclear, cytoplasmic, and mitotic findings, and then by general pattern and complexity.

Since the initial release of the classification system in 2016, additional consensus documents released by ICAP have given negative results a classification number (AC-0), addressed the reporting of as-yet unidentified patterns, and named a classification for the DNA topoisomerase I pattern (AC-29).

Slide 7:

With this background in mind, here are some relatively common examples of IIFA staining patterns for ANA, with their associated ICAP classifications. ICAP's website,

www.anapatterns.org, offers free registration and excellent educational resources, as well as access to images, for HEp-2 ANA patterns. It is a long-overdue training tool, available in several languages, and created and maintained by ICAP members.

Slide 8:

Standardization of pattern reporting for ANA is important clinically. Universal language about ANA results can point providers to targeted sub-serology testing, to determine which antigens may be associated with the pattern. This in turn can point to the likely clinical disease and aid in diagnosis, evaluating prognosis, and/or monitoring patients with known disease. Shown here are the first five ICAP classification numbers, with HEp-2 IIFA pattern, likely antigens targeted, and likely disease associations.

Slide 9:

The benefits of IIFA include clinically relevant information about pattern, and relatively good clinical sensitivity.

The limitations of the IIFA technique involve specificity for disease, particularly for weakly-positive test results. Preparation is still manual at most testing sites, with specimens typically batched for cost and workflow reasons; this can prolong turn-around time and delay result reporting. IIFA automation is available, but expensive. Also, the IIFA method requires relatively advanced local technical expertise for testing and interpretation and a dark or light-shielded space for optimal visibility of fluorescence patterns.

Slide 10:

ANA's first modern evolutionary shift came with ELISA. When testing ANA with ELISA, epitopes are either cloned or extracted from source material – such as HEp-2 cells – and coated on solid-phase wells. ANA in the patient serum are captured when they bind corresponding epitopes, and an enzyme-conjugated detection antibody binds the ANA. Signal is generated from a reaction driven by the enzyme on the detection antibody, and is captured and measured with a colorimetric detector. Signal obtained from a patient specimen is compared to a decision point determined by the analytic calibration, and an index – or, cutoff – is used to determine if ANA are present or not.

EIA-based ELISA can take two different forms. The first form of ANA testing with ELISA can serve as a general screening assay; this is made possible by presenting HEp-2 cell or nuclear homogenates, or a blend of purified common antigens, in the assay wells.

The second form of ANA testing with ELISA serves to target specific antigens associated with IIFA staining patterns; this approach is called sub-serology testing and it commonly serves as the second-line test after a positive ANA pattern is detected.

Slide 11:

The benefits of ANA testing with ELISA methods include the ability to automate, avoidance of dilution series if using the test for screening, and reagents that are relatively inexpensive. Also, the level of technical expertise required to perform testing and result reporting is lower. Assay cutoffs are typically engineered for maximal clinical sensitivity, and high plate capacities allow for large batches, making for efficient work in high-volume laboratories.

The limitations of ELISA-based ANA testing include widely variable clinical performance due to lacking test standardization and differences in solid phase preparation. Also, due to the engineering of these methods to seek high clinical sensitivity, the specificity and overall accuracy of ELISA can be poor. ELISA-based ANA results yield limited clinical information beyond binary “positive or negative” outputs, so they require further testing to determine the clinical relevance of the result. Finally, ELISA performs best when automated, and the available platforms are relatively expensive.

Slide 12:

ANA testing evolved again in the early 2000’s with multiplexed immunoassay – or, MIA – approaches. MIA uses all of the same ideology as ELISA, but advances the technique by presenting epitopes on beads that are individually detectable on the analytic system.

For example, if a positive IIFA points to a need for sub-serology testing of several epitopes, ELISA-based testing would require a separate ELISA batch for each epitope, but MIA-based testing could cover most or all of these epitopes in a single test batch.

Most MIA for ANA include ten to twelve of the most commonly-detected sub-serology antigens. Fluorescence signal from one or more beads representing these markers can enable reporting for the specific antigen yielding signal.

As for ELISA-based ANA testing, MIA reports are typically qualitative, with index-based cutoffs driving the interpretation.

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The benefits of ANA testing with MIA are derived from their intrinsically automated nature and placement on random-access analyzers, giving them rapid cycle times. Also, linking signal to specific epitopes can allow for more specific, pattern-like reporting of results.

The limitations of ANA testing with MIA are also derived from the way the modern systems are designed. Primarily, only limited numbers of epitopes are currently included in MIA-based ANA methods, creating problems for clinical performance in terms of sensitivity and specificity when compared to IIFA and ELISA techniques that use HEp-2 nuclear homogenate as substrate. Consequently, MIA are not recommended as screening tests for ANA. Another feature of MIA which limits its use in ANA testing is the expense of the testing systems on the market, which are currently considered high.

Slide 14:

With all of these testing options, it would seem difficult to determine which method is the right fit for a laboratory. Something one must consider is the ACR's position statement about testing methods for ANA. This position statement, most recently reviewed and re-approved in 2015, maintains that IIFA is the gold-standard method for ANA testing. Consequently, regardless of the first-line testing involved at an institution, IIFA is likely to remain the definitive method in the eyes of specialist providers.

Also, due to the known limitations of ELISA- and MIA-based ANA methods, laboratories using these technologies in particular need to demonstrate the same or improved clinical sensitivity compared to IIFA and have data available for provider review.

Pearls of Laboratory Medicine

Antinuclear Antibody Testing

In addition to these points, the ACR also calls for standardization of ANA testing methodology, reporting practices for ANA and sub-serology testing, and naming the method used for testing in result reports.

Slide 15:

In conclusion, we have discussed ANA testing in the framework of confirming the presence of clinical disease, and assisting clinical specialists in this task.

We have explored various testing modalities in-use currently for detecting ANA – with the greatest emphasis placed on IIFA – and listed the strengths and limitations of each. We are reminded that though many methods currently exist for ANA testing, IIFA remains the gold standard for clinical utility, and that ICAP is leading the effort to standardize reporting for this method.

Finally we have reviewed the American College of Rheumatology’s call for improved laboratory transparency regarding ANA testing methods used. The most important elements to keep in focus are how the tests are used and perform clinically, since both of these have large impacts in the clinical spectrum.

Slide 16: References

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine, “**Antinuclear Antibody Testing.**”



*Better health through
laboratory medicine.*

PEARLS OF LABORATORY MEDICINE

Antinuclear Antibody Testing

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DOI:



Objectives

1. Define antinuclear antibodies (ANA).
2. Name common clinical disease states associated with ANA positivity.
3. Discuss ANA testing techniques, with emphasis on gold standard immunofluorescence testing.
4. Recall current clinical recommendations for ANA testing & reporting practices.

Define Antinuclear Antibodies (ANA)

Autoimmune antibodies that bind nuclear components^{1,2}

- Double-stranded DNA
- Small nuclear ribonucleoproteins (eg, SS-A/Ro, SS-B/La, RNP, Smith antigen)
- Enzymes (eg, topoisomerase/Scl70)
- Histone proteins
- Centromeric proteins

>150 epitopes identified to-date¹

Applying ANA to Clinical Diagnosis

Disease	ANA Positive in:	ANA Has Utility For:
Systemic Lupus Erythematosus/SLE	95 – 100%	Diagnosis (very useful)
Systemic sclerosis/scleroderma/SSc	60 – 80%	
Sjögren syndrome/SjS	40-70%	Diagnosis (somewhat useful)
Dermatomyositis, Polymyositis	30 – 80%	
Juvenile rheumatoid arthritis	20 – 50%	Monitoring, prognosis
Raynaud phenomenon	20 – 60%	
Drug-induced SLE	~100%	Diagnosis (part of criteria)
Autoimmune hepatitis	~100%	
Mixed Connective Tissue Disease/MCTD	~100%	

Adapted from: Kavanaugh et al. *Arch Pathol Lab Med* 2000;124:71-81 with permission.



Indirect ImmunoFluorescence Assay (IIFA)

ANA bind epitopes in the substrate → ANA bound with a secondary, fluorophore-labeled antibody → nuclear fluorescence if ANA present²

- Preferred substrate – Human Epithelial-2 cells
- Serial titers in 1:2 increments, starting at 1:40 or 1:80
- [Check laboratory guidelines – setting a maximal dilution may be required.]

Efforts to Standardize IIFA Reporting³⁻⁵

International Consensus on ANA Pattern, aka, ICAP

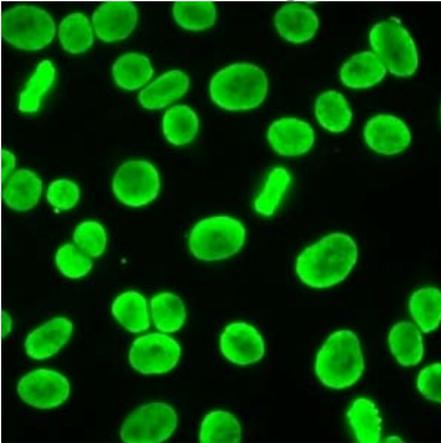
- Goal: standardize HEp-2 ANA reporting practice
- Consensus document, 2016³
 - 28 initial Anti-Cell/AC patterns
 - Nuclear, cytoplasmic, and mitotic categories
 - Organized by category, pattern, and level of training/expertise
- Regarding negative & unidentified patterns, 2018⁴; AC-0 (Negative) added
- AC-29 (DNA Topoisomerase I) added, 2018⁵

See References & www.anapatterns.org
for more information.

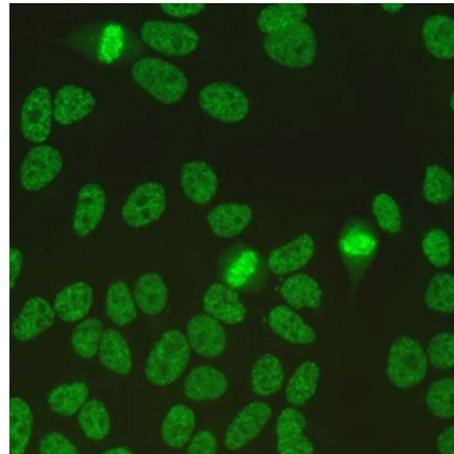


Examples of ICAP-Classified IIFA Staining Patterns

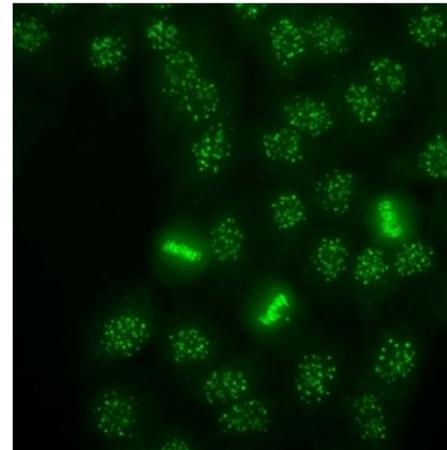
AC-1: Homogeneous



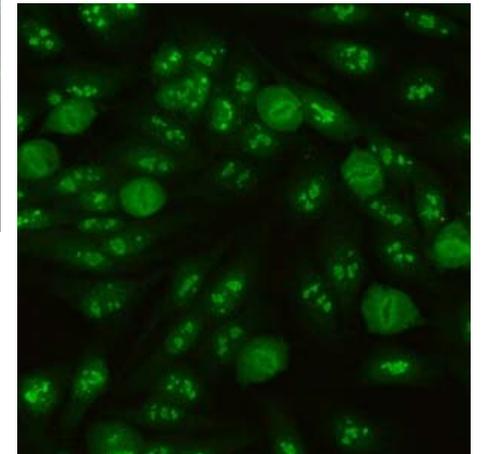
AC-2: Nuclear Dense
Fine Speckled



AC-3: Centromere



AC-9: Clumpy
Nucleolar



Images from www.anapatterns.org, with permission from Dr. Edward Chan.

Examples: Linking Pattern, Antigen, Disease with ICAP Classifications

Classification	Antigen Association(s)	Disease(s)
AC-1/Homogeneous	dsDNA, nucleosomes, histones	SLE, drug-induced lupus, juvenile idiopathic arthritis
AC-2/Nuclear Dense Fine Speckled	DFS70/LEDGF	(Rare) SjS, SSc, SLE
AC-3/Centromere	CENP-A/B (C)	Cutaneous SSc, PBC
AC-4/Nuclear Fine Speckled	SS-A/Ro, SS-B/La, Mi-2, TIF1 γ , TIF1 β , Ku	SjS, SLE, dermatomyositis
AC-5/Nuclear Large/Coarse Speckled	hnRNP, U1RNP, Sm, RNA Pol III	MCTD, SLE, SSc

PBC, primary biliary cirrhosis



Benefits & Limitations of IIFA Testing

Benefits

- Patterns correspond to disease states
- Sensitive

Limitations

- Specificity can be low, particularly at low titers
- Batched, manual preparation common
- Automation now available, but expensive
- Dark room/space & technical expertise needed

Enzyme Immunoassays (EIA) for ANA

Direct: epitopes on solid phase capture ANA in specimen → enzyme-conjugated detection antibody binds ANA → signal generated → colorimetric detection

- Semi-quantitative result vs index-based cutoff
- Qualitative interpretation – Positive, Equivocal, Negative

ANA screening by EIA

- HEp-2 cellular or nuclear homogenate coats the wells

ANA sub-serology testing by EIA

- Purified antigen in wells(eg, SS-A/Ro, SS-B/La, dsDNA...)
- Commonly used as second-level tests after initial ANA result is positive

Benefits & Limitations of EIA Testing

Benefits

- Automatable
- Relatively inexpensive
- High-capacity
- Very sensitive

Limitations

- Widely variable clinical performance
- No pattern given (if screening)
- Best when automated (expensive)



Multiplexed Immunoassay (MIA) for ANA

Direct, but in a liquid, bead-based phase

- Detection usually fluorescence-based
- Simultaneous testing for 10-12 most common ANA antigens (eg, dsDNA, SS-A, SS-B, Sm, RNP...)
- Semi-quantitative signal vs index-based cutoff
- Qualitative interpretation: Positive, Equivocal, Negative

Benefits & Limitations of MIA Testing

Benefits

- Rapid
- Automated
- Random-access
- Ability to report specific antigens/epitopes targeted
- Well-suited for basic sub-serology testing

Limitations

- Limited epitopes represented → limits clinical performance as first-level test
- Testing systems can be very expensive



Many Testing Options – (Still) One Clinical Gold Standard

American College of Rheumatology's (ACR's) Position Statement (2015)^{1, 6}

- IIFA remains the gold standard for ANA testing
- Clinical performance data needed for locally-used methods
- Data available on-request
- Non-IIFA methods used for ANA detection must be demonstrably equivalent or superior to IIFA in terms of **sensitivity**

ALSO:

- Call for standardized methodology and/or reporting
- Method used stated in the result report



Conclusion/Summary

ANA testing supports Rheumatologists and other medical specialists in their efforts to diagnose, monitor, and predict outcomes for an array of disease states, most of which are connective tissue disorders.

Though there are many approaches to ANA testing, the indirect immunofluorescence assay (IIFA) remains the gold standard.

Efforts to standardize reporting for ANA testing by IIFA are driven by ICAP.

Laboratories need to generate and provide locally-sourced clinical performance data for ANA testing methods used.

References

1. <https://www.rheumatology.org/Portals/0/Files/Methodology%20of%20Testing%20Antinuclear%20Antibodies%20Position%20Statement.pdf> [Updated 8/2015; Accessed 17 July 2018]
2. Kavanaugh A, Tomar R, Reveille J, *et al.* Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantigens to nuclear antigens. *Arch Pathol Lab Med* 2000;124:71-81.
3. Chan EKL, Damoiseaux J, de Melo Cruvinel W, *et al.* Report on the second International Consensus on ANA Pattern (ICAP) workshop in Dresden 2015. *Lupus* 2016;25:797-804.
4. Herold M, Klotz W, Andrade LEC, Conrad K, *et al.* International consensus on antinuclear antibody patterns: defining negative results and reporting unidentified patterns. *Clin Chem Lab Med* 2018;56(10):1799-1802.
5. Andrade LEC, Klotz W, Herold M, Conrad K, *et al.* International consensus on antinuclear antibody patterns: definition of the AC-29 pattern associated with antibodies to DNA topoisomerase I. *Clin Chem Lab Med* 2018;56(10):1783-8.
6. <https://www.rheumatology.org/Practice-Quality/Clinical-Support/Clinical-Practice-Guidelines> [Accessed 17 July 2018]

ACKNOWLEDGEMENT:

Thanks to Dr. Edward Chan at UFL for granting permission to use representative images of common patterns from <https://www.anapatterns.org>. [Accessed 21 September 2018]



Disclosures/Potential Conflicts of Interest

Upon Pearl submission, the presenter completed the Clinical Chemistry disclosure form. Disclosures and/or potential conflicts of interest:

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- **Consultant or Advisory Role:**
- **Stock Ownership:**
- **Honoraria:**
- **Research Funding:**
- **Expert Testimony:**
- **Patents:**

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QUESTION BANK TEMPLATE

Field	Instructions	
Stem	Write one question <i>Refer to Guide for Presenters for guidance (Page 5)</i>	Per the American College of Rheumatology, testing for antinuclear antibodies is intended for the diagnosis and/or monitoring of:
Responses	Provide 5 responses <i>Refer to Guide for Presenters for guidance (Page 5)</i>	<ul style="list-style-type: none"> A. Central neurological disorders B. Gastrointestinal diseases C. Connective tissue disorders D. Follicular thyroid disorders E. Adrenal disease
Answer	Indicate one correct response	C – Connective tissue disorders
Discussion	Provide a discussion of the correct response with main points explaining why it is the best choice	Though ANA testing is used in conjunction with a diverse array of clinical workups, the ACR's guidelines specify ANA test utility in terms of connective tissue disorders.
Source(s)	Provide the source(s) of information for further study <i>Refer to Guide for Presenters for full citation formatting (Page 3)</i>	Kavanaugh A, Tomar R, Reveille J, <i>et al.</i> Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantigens to nuclear antigens. <i>Arch Pathol Lab Med</i> 2000;124:71-81.
Difficulty	Select one level of difficulty: <i>Easy, intermediate, advanced</i>	Easy
Category	Select one category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Sub-category	Select one sub-category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Keywords	Include at least 1-2 keywords <i>Keywords should describe a subtopic to the sub-category selected. Examples include, thyroid, electrolytes, diabetes, pregnancy, etc.</i>	Antinuclear antibodies, Autoimmune disease

Question Bank Template

Field	Instructions	
Stem	Write one question <i>Refer to Guide for Presenters for guidance (Page 5)</i>	A clinical condition that relies on positive antinuclear antibody test results for diagnosis is:
Responses	Provide 5 responses <i>Refer to Guide for Presenters for guidance (Page 5)</i>	<ul style="list-style-type: none"> A. Rheumatoid arthritis B. Autoimmune hepatitis C. Multiple sclerosis D. Fibromyalgia E. Hashimoto thyroiditis
Answer	Indicate one correct response	B – Autoimmune hepatitis
Discussion	Provide a discussion of the correct response with main points explaining why it is the best choice	The American College of Rheumatology names only 3 conditions for which positive antinuclear antibody test results are an intrinsic part of the diagnostic criteria: 1) drug-induced systemic lupus erythematosus; 2) autoimmune hepatitis; and 3) mixed connective tissue disease. The other answer choices were deemed “not useful in diagnosis” in the same guideline.
Source(s)	Provide the source(s) of information for further study <i>Refer to Guide for Presenters for full citation formatting (Page 3)</i>	Kavanaugh A, Tomar R, Reveille J, <i>et al.</i> Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantigens to nuclear antigens. <i>Arch Pathol Lab Med</i> 2000;124:71-81.
Difficulty	Select one level of difficulty: <i>Easy, intermediate, advanced</i>	Advanced
Category	Select one category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Sub-category	Select one sub-category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Keywords	Include at least 1-2 keywords <i>Keywords should describe a subtopic to the sub-category selected. Examples include, thyroid, electrolytes, diabetes, pregnancy, etc.</i>	Antinuclear antibodies, Autoimmune disease

Question Bank Template

Field	Instructions	
Stem	Write one question <i>Refer to Guide for Presenters for guidance (Page 5)</i>	Which of the following test methods is considered the gold standard with regard to antinuclear antibody testing?
Responses	Provide 5 responses <i>Refer to Guide for Presenters for guidance (Page 5)</i>	<ul style="list-style-type: none"> A. Indirect Immunofluorescence Assay B. Enzyme-Linked Immunosorbent Assay C. Multiplexed Enzyme Immunoassay D. Hargraves’s method for detecting LE cells E. The test is the gold standard, not the method used
Answer	Indicate one correct response	A – Indirect Immunofluorescence Assay
Discussion	Provide a discussion of the correct response with main points explaining why it is the best choice	The American College of Rheumatology maintains that the indirect immunofluorescence assay (IIFA) technique is the gold standard for detecting antinuclear antibodies. The Hargraves method for detecting “LE cells” is historic and was never widely adopted. Both Immunoassay options are modern adaptations of IIFA that have not gained clinical acceptance as definitive tests.
Source(s)	Provide the source(s) of information for further study <i>Refer to Guide for Presenters for full citation formatting (Page 3)</i>	Kavanaugh A, Tomar R, Reveille J, <i>et al.</i> Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantigens to nuclear antigens. <i>Arch Pathol Lab Med</i> 2000;124:71-81.
Difficulty	Select one level of difficulty: <i>Easy, intermediate, advanced</i>	Easy
Category	Select one category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Sub-category	Select one sub-category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Keywords	Include at least 1-2 keywords <i>Keywords should describe a subtopic to the sub-category selected. Examples include, thyroid, electrolytes, diabetes, pregnancy, etc.</i>	Antinuclear antibodies, Autoimmune disease

Question Bank Template

Field	Instructions	
Stem	Write one question <i>Refer to Guide for Presenters for guidance (Page 5)</i>	Which technique, used for antinuclear antibody testing, relies on serial dilutions of sera and accordingly incorporates titer into the interpretation?
Responses	Provide 5 responses <i>Refer to Guide for Presenters for guidance (Page 5)</i>	<ul style="list-style-type: none"> A. Enzyme-Linked Immunosorbent Assay B. Hargraves's method for detecting LE cells C. Indirect Immunofluorescence Assay D. Multiplexed Enzyme Immunoassay E. None of these
Answer	Indicate one correct response	C – Indirect Immunofluorescence Assay
Discussion	Provide a discussion of the correct response with main points explaining why it is the best choice	Indirect immunofluorescence assay (IIFA) technique is employs slides cultured with human epithelial (HEp)-2 cells, and serial dilutions of patient sera to detect antinuclear antibodies. The serial dilution allows for reporting the highest titer in which ANA are detected.
Source(s)	Provide the source(s) of information for further study <i>Refer to Guide for Presenters for full citation formatting (Page 3)</i>	Kavanaugh A, Tomar R, Reveille J, <i>et al.</i> Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantigens to nuclear antigens. <i>Arch Pathol Lab Med</i> 2000;124:71-81.
Difficulty	Select one level of difficulty: <i>Easy, intermediate, advanced</i>	Intermediate
Category	Select one category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Sub-category	Select one sub-category (<i>Refer to list in Guide for Presenters - Page 6</i>)	Immunology
Keywords	Include at least 1-2 keywords <i>Keywords should describe a subtopic to the sub-category selected. Examples include, thyroid, electrolytes, diabetes, pregnancy, etc.</i>	Antinuclear antibodies, Autoimmune disease