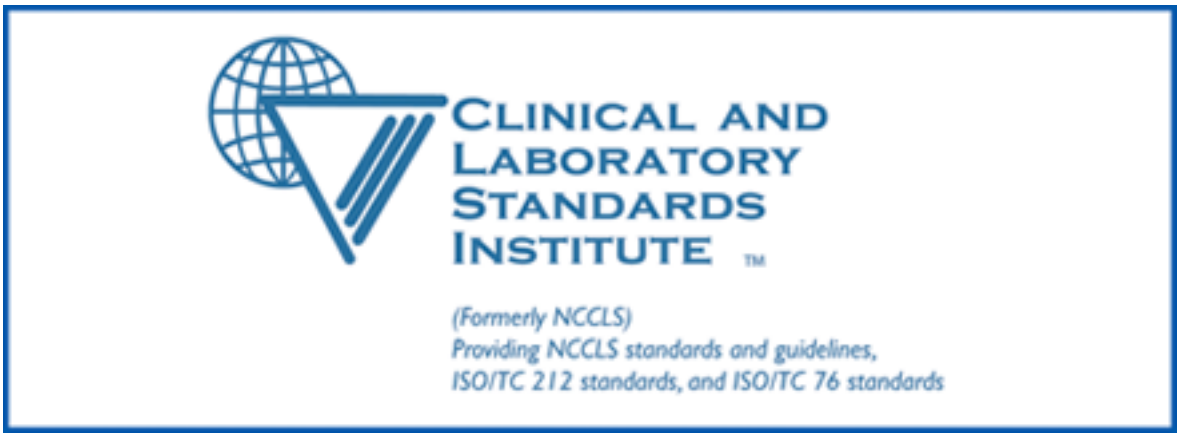
I/LA2-A
Vol. 16 No. 11
Replaces I/LA2-T
Vol. 13 No. 3

December 1996

Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline



This document addresses the criteria for immunofluorescence ANA testing, including test components, quantification of results, and classification criteria.

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Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline

Abstract

Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline (NCCLS document I/LA2-A) provides guidance for laboratorians who perform immunofluorescence tests for autoantibodies to nuclear antigen to detect diseases. Topics addressed include substrate and fixative variations, fluorescence-labeled conjugates, reference intervals, test results, and criteria for classification of systemic lupus erythematosus.

[NCCLS. *Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline*. NCCLS document I/LA2-A (ISBN 1-56238-311-6). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087, 1996.]

I/LA2-A
ISBN 1-56238-311-6
December 1996 ISSN 0273-3099

Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline

Volume 16 Number 11

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Suggested Citation

[NCCLS. *Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline*. NCCLS document I/LA2-A (ISBN 1-56238-311-6). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087, 1996.]

Proposed Guideline

August 1986

Tentative Guideline

April 1993

Approved Guideline

December 1996

ISBN 1-56238-311-6
ISSN 0273-3099

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Contents

Foreword

Tests for the detection of antinuclear antibodies are helpful in the evaluation of several systemic rheumatic diseases, such as systemic lupus erythematosus (SLE), discoid lupus erythematosus, mixed connective tissue diseases (MCTD), systemic sclerosis, Sjögren's syndrome, polymyositis, dermatomyositis, and rheumatoid arthritis. Identification of autoantibodies has proven to be useful in the diagnosis, management, and treatment of these diseases.

In developing this guideline, the area committee defined quality assurance as the practice that encompassses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. The area committee believes that this guideline addresses some of the critical issues related to IF-ANA testing, including: criteria for the immunofluorescence ANA test (Section 3); substrate and fixative variations (Section 4); fluorescence-labeled conjugates (Section 5); microscope optics (Section 6); semiquantitation of results (Section 7); establishment of reference intervals (Section 8); reporting of test results (Section 9); reference sera for ANA tests (Section 10); ANA-negative SLE (Section 11); revised criteria for the classification of SLE (Section 12); and special considerations for the use of other laboratory tests for the detection of ANA (Section 14).

The area committee acknowledges the help and input of the Standards Committee of the Association of Medical Laboratory Immunologists (AMLI) chaired by Lynn Burek, PhD, of Johns Hopkins University. Gerald Miller, PhD, has written a detailed draft of the AMLI document, and many of the suggestions found in the draft have been incorporated into this guideline.

Universal Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with "universal precautions." Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention. NCCLS document, M29-T2, *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue—Second Edition; Tentative Guideline*, deals specifically with all aspects of this issue.

Key Words

Autoantibodies, antinuclear antibodies, indirect immunofluorescence, nuclear antigen, systemic lupus erythematosus (SLE), quality assurance.

Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline

1 Introduction

The rheumatic diseases are characterized by the presence of one or more autoantibodies that react with components of the nucleus, cytoplasm, or surface of cells. The rheumatic diseases listed below vary with the type of autoantibodies and the extent and severity of lesions in the various organ systems.[1-6]

- Systemic lupus erythematosus (SLE).
- Discoid lupus erythematosus (DLE).
- Drug-induced lupus erythematosus (LE).
- Mixed connective tissue disease (MCTD).
- Sjögren's syndrome.
- Scleroderma/CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly elangiectasia) syndrome.
- Rheumatoid arthritis.
- Dermatomyositis and polymyositis.
- Other connective tissue disease syndromes that have been poorly defined as to clinical category. Includes syndromes associated with infectious diseases (such as Lyme disease), tumors, and drug reactions.

Over the past 10 years, there has been a progressive characterization of the immunochemical and molecular nature of various auto-antigens. An increased number of antigen–antibody systems associated with specific diseases have been identified. The terms “autoantibodies to nuclear antigens” or “antinuclear antibodies” (ANAs) have gained widespread use as generic descriptions of a group of autoantibodies. Several features of ANAs and their relationship to the rheumatic diseases have been reported.[1-6] Some of the ANAs have been used as diagnostic markers. Such ANAs include antinative DNA and anti-Sm in SLE, and anti-Scl-70 in diffuse scleroderma with lung disease; anticentromere in CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia); and anti-tRNA synthetases in dermatomyositis and polymyositis. Other ANAs are also found in several of the other diseases, such as hepatic disorders, tumors, vasculititis, etc., and can differ markedly in prevalence from the systemic rheumatic diseases. Such ANAs include anti-histones in SLE and drug-induced lupus; anti-U1 ribonucleoprotein (RNP) in SLE and MCTD; and anti-SS-A/Ro and anti-SS-B/La in SLE and Sjögren's syndrome.

2 Scope

The ANAs are associated with many immunologic disorders; however, they are the essential hallmark of systemic rheumatic diseases.

The significance of ANAs is as follows:

- Useful for screening and diagnostic evaluation of systemic rheumatic diseases. A negative test result is helpful in ruling out the possibility of SLE.
- Some of these diseases have distinct profiles of ANA. Significant changes in the levels of certain specific ANA, such as antibodies to Ds-DNA, are useful in following the causes of the diseases and their responses to therapy. Titers of IF-ANA tests do not necessarily correlate with severity of disease or response to therapy.
- ANAs can be useful as experimental reagents in the isolation of nuclear antigens, especially nonhistone antigens or basic studies in cell biology.

Indirect immunofluorescence and immunoenzyme tests are commonly used for ANA screening because these procedures are practical, sensitive, primary antigen–antibody reactions. However, the standardization of the indirect immunofluorescence antibody tests (IF-ANA) has been difficult.[7-9]

3 Requirements for the Immunofluorescence ANA Test

The difficulties associated with standardization and screening of tests for ANA have been shown in a recent College of American Pathologists (CAP) proficiency survey in diagnostic immunology.[9] There have been several attempts at standardization. Certain recommendations and guidelines can be helpful.[10] Many factors are involved in the performance of the IF-ANA tests. They include the following:

- Substrate and fixative variations (see Section 4).
- Fluorescence-labeled conjugate (see Section 5).
- Microscope optics (see Section 6).
- Relative semiquantitation of results (see Section 7).
- Establishment of reference interval (see Section 8).
- Interpretation of results (see Section 9).
- Reference sera (see Section 10).

3.1 Principles of the IF-ANA Test

The IF-ANA test utilizes the indirect fluorescent antibody technique first described by Coons and Kaplan.[11] The following steps are performed when using the IF technique for the detection of ANAs[12,13]:

(1) Mounted tissue sections or tissue culture cells, which are fixed on microscope slides, serve as the source of the nuclear antigens. Tissue culture cells are recommended as the most reproducible source of substrate tissue.

(2) Titered test serum is added, and incubation follows. Known ANA-positive and ANA-negative control sera are also added to separate substrate vials or slides.

(3) Excess serum protein is washed off.

(4) The detecting reagent, fluorescein-conjugated antihuman polyvalent immunoglobulin, is added, followed by incubation.

(5) Excess conjugate is washed off.

(6) The slides or vials are observed for the presence of specific nuclear immunofluorescence.

The ability to perform the IFA-ANA at room temperature is a convenience.[14]

3.2 Patient Specimen and Collection Procedure

Serum is the recommended specimen for the IF-ANA test. If prolonged storage is expected, preservatives should be added to the specimen.

Grossly hemolyzed or limpemic serum can produce increased nonspecific fluorescent background staining of the substrate. The serum specimen is centrifuged and stored at 4 °C. The specimen may be stored for up to 2 to 3 days at 4 °C; if a longer storage period is desired, it should be stored at –20 °C or colder. Because of the potential for freezing and thawing, do not store in a frost-free freezer. If a frost-free freezer is all that is available, then place the tube of serum in a styrofoam mailer when storing it in the freezer. Repeated freezing and thawing may cause deterioration of the test antibody specimen that is to be evaluated.

4 Substrate and Fixative Variations

Various substrates and fixative agents are now available, each with different levels of sensitivity and reactivity. The variety of substrates and commercial kits available for ANA makes comparison of data from different laboratories difficult. The most widely used and recommended substrate is the HEp-2 cells.

The following topics should be considered when performing the test and comparing the data:

4.1 Acetone Fixation

The substrate to be used in the IF-ANA test should be fixed with acetone. Nonfixation of the substrate can result in elution of certain nuclear antigens, such as SS-A/Ro and nRNP, during

washing steps, with subsequent negative or low-titer readings when high titers of ANA might actually be present.

Washing of the substrate before fixation of nucleoprotein (NP), or the use of ethanol or methanol for substrate fixation, can dissolve the SS-A/Ro antigen.[1,15]

4.2 Reference Intervals

Substrates vary in their qualitative and quantitative content of antigen, which affects sensitivity and specificity. Therefore, each laboratory must establish its own reference intervals using the kit intended for routine use. This allows for more consistent clinical interpretations.

Certain nuclear antigens—such as DNA, histones, Sm, nuclear RNP, and SS-B/La—are present in abundance in most cells. Therefore, antibodies directed against these specificities can be easily detected, no matter what substrate is used in the test. In contrast, other nuclear antigens—such as SS-A/Ro, PCNA, RANA, and Ku antigens—are present in low concentrations in certain tissues. Therefore, no single substrate is best for detection of all ANA.[1,2,15]

4.3 SS-A/Ro Antigen

The SS-A/Ro antigen is present in undetectable amounts when mouse or rat tissues are used as substrates. However, the SS-A/Ro antigen is present in detectable quantities in human epithelioid cell lines, such as Hep-2 or KB cells, when these substrates have been fixed in acetone.[15]

Bylund and Nakamura[10] have shown the importance of detection of SS-A/Ro autoantibodies in complete screening for autoantibodies to nuclear antigens. The detection of anti-SS-A requires implementation of, and adherence to, several technical and quality assurance recommendations. With use of the appropriate substrate cells containing the SS-A/Ro antigen, many of the so-called "ANA-negative" lupus erythematosus patients have a positive test result when the indirect immunofluorescent test is used.

4.4 Anticentromere Antibody

In the detection of anticentromere antibodies, a tissue culture cell line is necessary.[16]

4.5 RANA

The rheumatoid arthritis associated nuclear antigen (RANA) is present only in cells that contain the Epstein–Barr virus genome.[1,5]

5 Fluorescence-Labeled Conjugates

The labeled antibody is usually a fluorescein isothiocyanate (FITC) conjugate. To calculate the F/P ratio on the basis of fluorescein as FITC, fluorescein and protein concentrations are determined .[17-19]

It is recommended that the F/P ratio of the conjugates used be approximately 3.0. Conjugates with higher F/P ratios demonstrate more nonspecific fluorescein staining. Also, conjugates with higher F/P ratios can demonstrate higher IF-ANA titers than those obtained with conjugates with a lower F/P ratio.

A specific antibody-to-protein ratio equal to or greater than 0.1 is desirable, and optimal, for IF-ANA testing. The total specific antibody content should be diluted to the range of 30 to 60 μg/mL.[20]

5.1 Working Dilution

The laboratory should evaluate the recommended working dilution of the manufacturer's fluorescein conjugate. Each laboratory should titrate conjugates using *positive sera of known titer* to establish the correct optimal working dilution required in the procedure.

The common method for determining optimal conjugate dilution is the immunofluorescence chessboard titration.[3,17,18,20] Serial dilutions of known positive control serum with an identified pattern and end point are tested with a series of conjugate dilutions. A negative serum and buffer controls are also assayed.

The optimal working dilution of the conjugate should be re-established under the following circumstances:

- Each time a new lot of conjugate is used.

- Whenever the conjugate is applied to a different substrate.

- If the optical system used to read the test is changed.

5.2 Polyvalent and IgG-Specific Conjugates

Both polyvalent (reactive with IgG, IgM, and IgA) and IgG-specific conjugates have been used in IF-ANA tests.[3,20] In SLE, 96% of patients with SLE produce antinuclear antibody of the IgG class.[21] With use of an anti-IgG-specific conjugate, the IgM-class antinuclear antibodies associated with rheumatoid arthritis, drugs, and age, which are usually of no diagnostic significance, are not detected.[12] However, 4% of patients with systemic rheumatic disease produce significant amounts of IgM ANA.[20,22]

5.3 Reference Preparation of Fluorescence-Labeled Conjugates

The International Union of Immunological Societies (IUIS) Standardization Committee and the World Health Organization (WHO) have established two WHO/IUIS International Standard Conjugate preparations. FITC-labeled sheep antihuman Ig (480010) and FITC-labeled sheep antihuman IgM (anti-u chain) are available from the WHO Immunology Unit (Geneva, Switzerland).[23-25] These conjugates may be used as checks on the specificity and performance of commercial, or individually prepared, conjugates.

The standard conjugates are used at working dilutions in place of commercially prepared conjugates on negative controls, positive controls, and patient samples. The standard conjugates are compared to the commercially prepared conjugates used on another set of negative controls, positive controls, and patient samples.

6 Microscope Optics

Because there are many different light sources and filter options available, the selection of a fluorescence microscope is important. Each laboratory should establish the ANA reference interval with its own microscope and optical set-up. An optical slide standard with graded degrees of fluorescence for optical system standardization has recently become available.[26]

The use of an optical slide standard is recommended because it helps in the standardization of microscopes within and between laboratories. The microscope slide with fluorescent beads of graded intensity may be used to standardize microscopic fluorescence intensity. These fluorescence intensity patterns should be directly comparable to results obtained in other laboratories with the use of the defined reference slide.

7 Relative Semiquantitation of Results

In the performance of relative semiquantitation, one should have uniform sections or monolayer areas of tissue culture cells that are reacted with a definite amount of serum dilution. The primary immunofluorescent conjugate should be added in excess and the end point should be read as positive, strongly positive, or negative. If quantitative end point titrations are borderline, they are considered negative. *A low-titered positive ANA control (i.e., a specimen with a positive one dilution beyond the cut-off point) should be included in each assay.*[9,10]

8 Establishment of a Reference Interval

Each laboratory should establish reference interval values for its patient population representative of patient age groups younger than 40 years and older than 40 years, and a balance of men and women.[9,10]

The incidence of positive ANA results in patients over 40 years of age gradually increases with age in the “normal population”; whereas, low positive titers of ANA tests in patients under 40 (especially women) can be significant.

The age of the patient is important. For example, a low-titered IF-ANA might be seen in 18% of the normal elderly population over age 65 years but in only 4% of a normal younger population.[27] The elderly population has a greater frequency of drug-related ANA and antihistone antibodies.

(For more information on reference intervals, refer to NCCLS document C28-A, *How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline*.)

9 Reporting of Test Results

The immunofluorescent ANA test may be rendered semiquantitative by determination of an end point dilution. The results may also be reported as positive in a particular dilution in relation to a normal specimen. The determination that results are either normal or abnormal is due to a laboratory interpretation rather than a clinical assessment because the latter includes a clinical evaluation of the patient and features other than ANA screening tests.

9.1 Negative Test Result

The result of the ANA test for autoantibodies is *negative* if no specific pattern of fluorescence is observed in the nuclei of the cells. The staining intensity of a negative test should be a nondiscernable pattern.

9.2 Positive Test Result

The result of the test for ANA is *positive* when the nuclei of the tissue section, or HEp-2 tissue culture cells, demonstrate specific patterns of fluorescence.

Note that *cytoplasmic antibody patterns* can be detected on the HEp-2 substrate and should also be considered. These cytoplasmic antibody patterns are also associated with autoimmune diseases and should also be considered. These cytoplasmic antibody patterns are also associated with autoimmune diseases and should be identified.

The staining intensity should be noted. For positive tests the intensity can be graded from 1+ to 4+.

Following is a list of several different patterns of nuclear and cytoplasmic immunofluorescence[1,2,12,14,28]:

- Homogeneous or diffuse staining of the nucleus.
- Rim and fibrillar staining of the nucleus.
- Speckled or particulate staining of the nucleus.
- Nucleolar staining.
- Unique patterns.
- Cytoplasmic.

9.2.1 Homogeneous or Diffuse Pattern

Characteristically, antibodies to histones and deoxyribonucleoprotein (DNP) demonstrate homogeneous nuclear IF. The homogeneous diffuse pattern on the HEp-2 cell substrate demonstrates a uniform staining of the nuclei; however, the nucleoli occasionally appear as negatively stained areas. The mitotic cells are important indicators of an homogeneous pattern because they stain as irregularly shaped masses with more intensely stained outer edges. This pattern is consistent with autoantibodies to native DNA (nDNA) histones and/or DNP. *Always determine whether an autoantibody is chromosome positive by the reaction with the metaphase stage of mitosis*.

9.2.2 Rim and Fibrillar Pattern

The rim and fibrillar pattern is often seen with significant levels of anti-DS DNA antibodies. The nuclei stain predominantly at their periphery. The chromosomes of the mitotic cells stain as irregularly shaped masses with more intensely stained outer edges. The pattern is consistent with autoantibodies to nDNA. If the chromosomes of the mitotic cells are negative, then the pattern is suggestive of autoantibodies to the nuclear membrane and not to nDNA.

9.2.3 Speckled Patterns

The speckled pattern is the most commonly observed ANA pattern. Speckled patterns of fine, discrete, or clumpy consistency have been described.[29] A uniform "true speckled" pattern can be seen with centromere antibodies in cells not in division. A clumpy speckled pattern can be seen with antibodies to n-RNP, Sm, and SS-B/La.

- *Fine speckled pattern, chromosome-negative*: Numerous small and uniform points of fluorescence uniformly scattered throughout the nucleus. The nucleoli generally appear unstained. The mitotic cells can demonstrate a few speckles in their cytoplasm, but the chromosomes are negative.

- *Course speckled pattern, chromosome-negative*: Medium-sized points of fluorescence are scattered throughout the nuclei with distinct nuclear margins. Larger-sized points of fluorescence can 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 are negative.

- *Discrete speckled, chromosome (centromere specificity)-positive*: Medium-sized uniform points (46) of fluorescence are scattered throughout the nuclei but with indistinct nuclear margins. The chromosomes are positive in mitotic cells; in fact, the discrete speckles are only clustered in the chromosome mass clearly demonstrating the various stages of mitosis. The discrete speckled/ centromere pattern has been reported to correlate highly with the CREST syndrome of progressive systemic sclerosis.

  Harmon and coworkers[15] demonstrated that serum samples containing highly monospecific anti-SS-A/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-SS-A/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 in mouse kidney substrate. These patients with large, variable speckles have undifferentiated rheumatic disease syndromes with IgM antihistone H-3 antibody.[30]

9.2.4 Nucleolar Pattern

Three distinct patterns of nucleolar IF (speckled, homogeneous, and clumpy) have been described in scleroderma patients with antinucleolar antibodies. Antinucleolar antibodies can be seen in patients with scleroderma, SLE, Sjögren's syndrome, or Raynaud's phenomenon.[31]

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 are negative. The nucleolar pattern suggests autoantibodies to 4-6S RNA. The nucleolar fluorescence appears as homogeneous, clumped, or speckled, depending on the antigen to which the autoantibody reacts.

9.2.5 Unique Patterns

Following are several unique patterns:

- *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; however, an association between the spindle fiber pattern and carpal tunnel syndrome has been suggested.

- *Pseudocentromere pattern*: The pseudocentromere pattern is called so because of its close resemblance to the true centromere pattern, except that the number of discrete specks range from 3 to 40 and do not cluster with the chromosomes in the mitotic cells. The significance is not known.

- *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.

- *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.

- *Proliferating cell nuclear antigen (PCNA) pattern*: The proliferating cell nuclear antigen pattern is observed as a fine to coarse nuclear speckling in 30 to 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. It has been reported that SLE patients with the PCNA pattern have a

higher incidence of diffuse glomerulonephritis.

- *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 was recently recognized to be associated with autoimmune liver disease.

9.2.6 Cytoplasmic Patterns

Following are several cytoplasmic patterns:

- *Mitochondrial (AMA) pattern*: Characteristically, the mitochondrial (AMA) pattern has numerous cytoplasmic speckles with the highest concentration around the nucleus. 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.

- *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 suggest an association with SLE and Sjögren's syndrome.

- *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.

- *Ribosomal pattern*: 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 its smaller specks and higher density. The significance of the pattern is unknown.

- *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.

9.3 Other Considerations in the Interpretation and Reporting of Results

Negative results with IF-ANA tests have been observed in patients with Sjögren's syndrome, polymyositis, rheumatoid arthritis, and scleroderma.[2,3] These patients, however, can demonstrate specific nuclear antibodies when other immunologic methods are used (see Section 4.1). When the rheumatic diseases—such as Sjögren's syndrome, polymyositis, rheumatoid arthritis, and scleroderma—are clinically suspected, one should consider other tests (see Section 14) that can identify the immunologic specificity of the autoantibody to nuclear antigen.

When interpreting and reporting a positive IF-ANA test, one should consider the following:

- Age and gender of the patient.

- History of taking any ANA-inducing drugs (i.e., phenytoin sodium, hydralazine, and others).

- The titer (dilution) of the IF-ANA versus the reference population.

- Pattern of immunofluorescence. The patterns can change when read at different dilutions. If double patterns are seen, it should be noted in the report.

- Index of suspicion of an autoimmune disease.

- Presence of malignancy.

9.3.1 Screening Dilutions

After each laboratory has established its own reference interval, a screening dilution may be established with proper controls and reference materials.

9.3.2 Interpretation of Results

The interpretation of the results depends on the pattern observed, the level of the autoantibody, and the age of the patient. The elderly, especially women, are prone to develop low-titered (dilution) autoantibodies (<1:80) in the absence of clinical autoimmune disease. In contrast, one may screen serum samples of younger patients

at a dilution of 1:40. Low-titer positive results can occur in apparently healthy persons; therefore, the ANA results must always be interpreted in light of the patient's total clinical presentation.

10 Reference Sera for ANA Tests

10.1 WHO/IUIS Reference Preparations

(a) WHO International Reference Preparation Antinuclear Factor [homogeneous 66/233 100 International Units (IU)/ampule][32]

(b) WHO/IUIS IgM Class ANA (HL)[24]

A freeze-dried batch of ANA-positive serum pooled from six patients with SLE.

The ampule contains 100 arbitrary units of ANA activity as lyophilized serum. The end point is the highest dilution showing the minimal nuclear staining distinguishable from a negative reaction. Holborow et al[24] recommend the use of the WHO International Reference Preparation 66/233 as follows:

- If the determined end point of the WHO standard 66/233 is 1:160 in a particular laboratory, then the end point dilution contains 1/160 · 100 IU per mL = 0.625 IU/mL. (The undiluted 66/233 contains 100 IU/mL.)

For another laboratory with another system, the end point might represent less or more IU per mL of ANA activity. The differences among laboratories and systems might be reduced by expressing results of test sera in IU per mL, which is calculated by multiplying the reciprocals of their dilutions at their titration end points by the number of units of activity present in the reference preparation at its titration end point in the particular laboratory.

Some of the drawbacks of the WHO 66/233 reference preparation are as follows[24]:

- WHO serum 66/233 is probably an inappropriate reference for speckled or other patterns of nuclear fluorescence.

- The ANA titer is dependent upon the total antibody content and antibody avidity. These sera, with similar total amounts of antibody but varying avidity, can give different end points.

- WHO 66/233 contains mostly IgG ANA and is thus best used in the comparison of test sera that contain ANA activity of the IgG class.

10.2 Reference Sera of Defined ANA Specificity

The Committee on ANA Serology of the Arthritis Foundation, in collaboration with the Centers for Disease Control and Prevention, has made available reference sera and associated protocols. Reference sera for antibodies to DNA, SS-A, SS-B, RNP, Sm, Scl-70, Jo-1, nucleolar, homogeneous, centromere, and speckled patterns may be obtained from the AF/CDC ANA Reference Laboratory at the CDC.[33,34] Studies show that standardization of the indirect immunofluorescent ANA test can be improved with the use of reference ANA sera.[8]

10.2.1 Preparation of Reference Sera[33,34]

Sera from patients undergoing therapeutic plasmapheresis were screened for desired antinuclear reactivities. $CaCl_2$ (0.01 mol/L) and epsilon-amino-n-caproic acid (0.013 mol/L) were added to citrated plasma from single donors. The recalcified plasma was allowed to clot at 4 °C overnight, centrifuged, and stored at –70 °C until lyophilization. Volumes of 0.50 mL were dispensed into borosilicate ampules, freeze-dried, and sealed with butyl rubber stoppers under reduced pressure.

The vial contents are sterile, as determined by United States Pharmacopeia (USP)-approved methods of sterility testing; free of hepatitis-B surface antigen as determined by radio-immunoassay; and negative for rheumatoid factor as determined by latex agglutination test and radioimmunoassay. Mean dry weights; water content, as determined by the Karl Fischer residual moisture test; results of formal thermal stability studies; and data related to their practical stability for routine laboratory use are provided with the ampules. The new lots of standards should also be tested by the manufacturer for the presence of human immunodeficiency virus (HIV) antibodies. Sera that is positive for HIV antibodies should not be used.

10.2.2 Methods of Assay

Methods of assay include the fluorescence antinuclear antibody technique using a variety of tissue sections and monolayer tissue culture cells; commercial filter systems; Crithidia assays for anti-dDNA; and double immunodiffusion, counterimmunoelectrophoresis, and hemagglutination methods for anti-Sm, anti-nRNP, and anti-SS-B/La. Immunoblotting, Western blotting, and variations—such as checkerboard immunoblotting methods—have also been reported.

10.2.3 Consensus Evaluation

Triplicate ampules were distributed to committee members for testing. In addition to the indicated specificities, all sera were screened for other ANA-specific reactivities by the methods currently in use in members' laboratories. Taken together, the methodologies used encompass a wide range of techniques for detection of these specificities.

10.3 College of American Pathologists Reference Preparation For Anti-SSA/Ro Antibody

The CAP Standards Committee, in cooperation with the CAP Immunology Resource Committee, prepared a monospecific reference serum containing only anti-SS-A/Ro ANA. The CAP reference preparation (Lot #C103BG) has a positive immunodiffusion precipitin antibody titer 1:4 to 1:32 to SSA/Ro. This reference serum should be used to test IF-ANA test substrate cell lots for the presence of the SS-A/Ro antigen. In addition, the monospecific anti-SS-A/Ro reference serum can be used for specific identification of unknown anti-SS-A/Ro by immunodiffusion analyses.[10]

11 "ANA-Negative" Lupus Erythematosus

Clinical cases of patients with lupus erythematosus (LE) with negative IF-ANA test results as a result of conventional testing were initially reported by Fessel[35] and Gladman et al.[36] Subsequently, several other reports on ANA-negative LE were published.[37-39]

The exact prevalence of patients with LE who are ANA-negative is difficult to determine because it is dependent upon the substrate employed to detect antinuclear antibodies. Weiss et al.[40] used mouse liver as a substrate and found that 5 to 10% of patients fulfilling the American Rheumatism Association (ARA) criteria for diagnosis of SLE failed to demonstrate significant titers of antinuclear antibodies.

As previously discussed, autoantibody to SS-A/Ro is present in high frequency in a clinical subset of lupus called subacute cutaneous lupus erythematosus (SCLE). Many patients with SCLE have been falsely labeled as having "ANA-negative" lupus. It is now known that many of these so-called "ANA-negative" LE patients demonstrate a positive IF-ANA test result on substrate of human tumor KB and Hep-2 cells that contain the SS-A/Ro antigen[41] (see Section 4.1).

Anti-SSA/Ro antibodies can be present in the absence of traditional ANAs, with SLE seen in persons genetically deficient in C4 and occasionally other complementary deficiencies.[42-43] This combination may be more common in black persons.[44-45] In addition, C4 deficiency may be associated with increased susceptibility to development of SLE upon treatment with hydralazine.[46] These patients, if female, are likely to deliver infants with congenital heart block or lupus dermatitis.[47]

Another study has shown that certain IF-ANA-negative patients with SLE and prominent cutaneous disease possess only anti-SS DNA antibodies.[37,40,41] These anti-SS-DNA antibodies are directed toward the presence of a pyrimidine base buried internally in native DNA. These antigenic determinants are not detected in routine IF-ANA tests because SS-DNA determinants are not normally expressed in intact cell nuclei of the tissue substrate.

Maddison et al[37] reports an extensive study of 66 patients in whom clinical features of SLE developed and who had repeated negative IF-ANA test results in their sera over an extended period of time. Only 24 of 66 patients described fulfilled four or more of the 1971 preliminary criteria for SLE as proposed by the ARA. They found that 41 of the 66 patients had precipitating antibodies to SS-A/Ro and 18 of the remaining patients had antibody to single-stranded DNA. Further studies of sera from patients with SLE who have demonstrated negative IF-ANA test results with mouse liver

substrates showed that 66% of these patients were positive for nuclear staining by a more sensitive human KB epithelial tissue culture substrate.[37]

12 1982 Revised Criteria for Classification of SLE

In 1971, a subcommittee of the ARA published a report on "The Preliminary Criteria for the Classification of Systemic Lupus Erythematosus."[48] The 1971 criteria have been used as the basis for classification of patients in many clinical reports. However, the 1971 criteria do not incorporate the current serologic tests widely used in the diagnosis and management of SLE. These important markers include antibody to DNA, IF-ANA, serum complement, and other serologic and immunopathologic assays.

A subcommittee for SLE criteria of ANA, chaired by Eng Tan, published revised criteria for the classification of SLE (Table 1) in 1982.[49] The 1982 revised criteria added fluorescence antinuclear antibody, and antibody to native DNA and Sm antigen. Raynaud's phenomenon and alopecia, which were listed in the 1971 criteria, were not included in the 1982 criteria because of their low sensitivity and specificity. When compared with the 1971 criteria, the 1982 revised criteria showed gains in sensitivity and specificity. The 1982 criteria were 96.7% sensitive and 96% specific when tested with SLE in control patients.

For the purpose of identifying patients in clinical studies, a person is classified as having SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

13 Summary of Important Quality Assurance Procedures

The following guidelines are recommended in the performance of IF-ANA tests:

- The laboratory should establish a range of reference values for 95% of the non-rheumatic disease population with representative patients from age groups younger than 40 years and older than 40 years.
- In the performance of quantitative tests, one should have a uniform substrate that is reactive with the defined serum dilution. The primary immunofluorescent conjugate should be added in excess.
- Each lot of immunofluorescent conjugate should be titrated with a positive serum of *known titer* to establish the correct optimal working dilution required in the test procedure.

Table 1. 1982 Revised Criteria for Classification of Systemic Lupus Erythematosus

- Malar rash.
- Discoid rash.
- Photosensitivity.
- Oral ulcers.
- Arthritis.
- Serositis.
- Renal disorder.
- Neurological disorder.
- Hematologic disorder.
- Immunologic disorder.
  - Positive LE cell preparation.
  - Anti-DNA: antibody to DNA in abnormal titer.
  - Anti-Sm: presence of antibody to Sm nuclear antigen.
  - False-positive serologic test result for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
- Antinuclear antibody: an abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome.

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- Each lot number of substrate of KB or Hep-2 cells should be tested with a low-titer, known-positive antibody to SS-A/Ro to determine if the SS-A antigen is present in the substrate. The lot number should also be tested with a negative control.

- The end point should be read as “positive,” “strongly positive,” or “negative” and reported with semiquantitative end point titrations. A borderline result should be considered negative.

- In reporting the immunofluorescent ANA, the result of the end point titer of a specimen should be reported as “positive” with the particular dilution in relation to the normal range. The interpretation of normal or abnormal would be an interpretation of an individual laboratory value.

- A low-titered, ANA-positive control serum should be included in each assay run.

14 Special Considerations in the Use of Other Laboratory Tests for the Detection of ANA

The procedures most commonly used in clinical laboratory screening tests for intracellular and nuclear antibodies are the IF-ANA and the immunoenzyme tests. Both are sensitive to primary antigen–antibody tests.

The secondary definitive tests for specific identification of ANA utilize immunodiffusion, immunoprecipitin, particle agglutination, immunoenzyme, immunoblotting, and radioimmunoassay methods.[2,5,10]

Immunoprecipitin and double immunodiffusion analysis have been used to determine the specificity of several ANAs. Assay specificity in double immunodiffusion generally depends on the quality of the control serum used in the procedure, as well as the nature of the antigen preparation. The immunodiffusion tests are not very sensitive. The positive tests by immunodiffusion have a high degree of specificity as a diagnostic marker in certain rheumatic diseases.

Commercial immunodiffusion kits are available for the detection of antibodies to RNP, Sm, SS-A/Ro, SS-B/La, and ScL-70, as well as other less prevalent markers.

An increasing number of enzyme immunoassays (EIAs) have been developed over the past decade with use of standard purified and recombinant antigens.[42,43] Many of these EIAs have proven to be more sensitive than comparable immunodiffusion methods. Thus, because of the *high sensitivity of EIAs, one needs to determine the reference interval of normal patients and determine the proper cut-off values*.

Compared with immunodiffusion tests, the enzyme-linked immunosorbent assay (ELISA) tests showed much greater sensitivity but had lower specificity.[48,49] Further, the ELISA tests were frequently positive at low titers in the sera of patients with rheumatic diseases other than SLE. For example, the presence of antibodies to Sm, as assayed by immunodiffusion, is considered to be highly specific for SLE. However, in qualitative ELISA tests, the Sm antibody was positive in 23% of 54 patients with RA, 25% of 24 patients with systemic sclerosis, 9% of 11 patients with polymyositis, and 2% of 59 normal patients.[49] The question of whether increasing sensitivity and decreasing specificity might result in false-positive test results should be considered.

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Summary of Comments and Committee Responses

I/LA2-T: *Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Tentative Guideline*

General

1. The detection of anti-SS-A/Ro appears to be essential to the maximum sensitivity of the ANA test. Why, then, has the subcommittee avoided recommending HEp-2 or KB cells for screening the substrate?

- **Section 4 has been modified and now recommends the use of HEp-2 cells.**

2. The clinical significance of all staining patterns should be included.

- **Staining patterns of IF-ANA are indicative of the antibody specificities. Further definitive tests should be performed to determine the specificity of the antibodies. The clinical significance and interpretation of specific antibodies to nuclear cytoplasmic antigens is done best in the context of the profile of specific autoantibodies present in the patient.**

Section 1

3. The list does not include hepatic disorders, vasculititis, or Wegener's.

- **Section 1 has been revised to address this comment.**

Section 2.0

4. I dispute that the titer of ANA is usually useful in following a patient's course. ANA is most useful in diagnosis because of high sensitivity (but low specificity).

- **Section 2 has been revised.**

Section 4.3

5. The following phrase should be added to the end of the second sentence, "when these substrates have been fixed in acetone."

- **The phrase was added to Section 4.3.**

Section 5.0

6. The number "3.0" is not a range. An appropriate range should be suggested or the words "in the range of" should be deleted.

- **This was corrected in Section 5.**

Section 5.1

7. Many manufacturers of fluorescein conjugate consider the F/P ratio to be proprietary information and furnish a ready-to-use or a lyophilized, product. Is this acceptable for the laboratory if controls of known titer are within the acceptable range of ±1 tube dilution?

- **Section 5 describes the recommended fluorescein-labeled conjugates with an F/P ratio. However, if the kit is FDA-approved, the laboratory will have to evaluate the background staining accompanying the negative control and positive control of a known titer. If the background continues to remain high and the controls, as used on the patient specimens, are difficult to read, then the laboratory should call the manufacturer for information, or change to kits that provide helpful information.**

Section 5.3

8. Briefly describe how reference preparations can be used as checks on commercially prepared conjugates.

- **This information was incorporated into Section 5.3.**

Section 6.0

9. This section does not emphasize that the optical slide standard is highly recommended to be used in conjunction with specific antibody reference standards by the manufacturer.

- **Section 6 has been revised to incorporate this comment.**

Section 8.0

10. I am not sure of the scientific basis of using age 40 as a cut-off point.

- **See Section 8 (revised) for an explanation.**

Section 9.0

11. Most laboratories report out ANA titers as dilutions. For example, 1:40 indicates a dilution, not a titer. The definition of a titer is the reciprocal of the end-point dilution. Also, I have noticed that some laboratories do not give an end-point titer and report as >1:320. Does the subcommittee believe this to be appropriate?

- **Yes, some laboratories reported >1:320, and it is appropriate if it is followed-up with specific identification and some form of quantitation of the specific antibody.**

12. The reporting of double patterns should be addressed.

- **Section 9.3 has been revised.**

13. We are currently undecided about whether to report nuclear membrane, centriole, and other non-ANA results to clinicians. In some cases, it may cause confusion, but it may be helpful in others. We currently do not mention them because, for most, the clinical significance is unknown. Does the subcommittee have any recommendations?

- **The infrequent and "non-ANA" results should be reported. These odd patterns can be indicative of diseases other than SLE or other systemic rheumatic diseases.**

Section 9.1

14. Move the information about cytoplasmic antibodies into Section 9. It does not belong with the negative ANA information. Should these antibodies be reported (not just identified)?

- **Section 9.2 has been revised.**

Section 9.2

15. Include a description of staining intensity or grading for the qualitative designations of 4+ down to negative.

- **Descriptions of staining intensity have been incorporated into Sections 9.1 and 9.2.**

Section 9.2.1

16. "Supposedly responsible for LE cell prep"—is all this detail necessary?

- **The subcommittee believes that it is important information.**

17. The comments on metaphase should be included in Section 9.2, with the general discussion of positive-result information.

- **These comments are best included in Section 9.2.1 because positive results include ANA, cytoplasmic, and other antibodies.**

Section 9.2.2

18. As a technologist, I was confused by the recommendation that the interpretation of the nuclear membrane pattern as "not reported as a positive ANA." Section 9.2.5, item (7), states that [regarding the antinuclear membrane pattern], "this autoantibody is important to report because it was recently recognized to be associated with autoimmune liver disease." In this context, why would one not report an ANA as negative and then report a pattern and a titer?

- **This suggestion is good, and the phrase "not reported as positive ANA" has been deleted from Section 9.2.2. The nuclear membrane pattern should be reported as the autoantibodies are to the nuclear lamins.**

19. There should be a greater distinction made between the homogeneous and rim pattern descriptions. Adding something about how translucent the rim pattern should be would be helpful.

- **The classical rim pattern is caused by autoantibodies to Ds-DNA and one would see staining of the nuclear rim, coarse fibrillar ANA, and the chromosomes of the mitotic figures. One can see a rim pattern with autoantibodies to nuclear lamins; however, these antinuclear lamins do not stain the chromosomes of the mitotic cells.**

One cannot definitely determine the specificity of the autoantibody by the pattern alone. Specific secondary definitive tests must be employed for identification of the autoantibody specificity.

Section 9.2.4

20. The third paragraph is redundant in that it repeats what is stated in paragraph one.

- **The third paragraph was deleted.**

Section 9.2.5.2

21. The centromere pattern is already addressed in Section 9.2.3.3.

- **The centromere pattern in Section 9.2.5.2 was deleted.**

Section 9.2.5.2

22. This information should be incorporated into Section 9.2.2.

- **This information was deleted from Section 9.2.5.2 and is now addressed in Section 9.2.3.3.**

Section 9.3

23. Each laboratory needs to titer to determine a screening level. The suggested level is appropriate only as a guideline, as a place to start.

- **See Section 8 (reference interval determination) and Section 9.3.1.**

24. The statement, "In contrast, a 1:20 titer of a significant pattern of autoantibody(s) in a young person may suggest that overt disease may occur later" is misleading. It might encourage the screening of samples at a lower dilution when titers of 1:40 or 1:80 will detect clinically relevant autoantibody(s) levels.

- **This is an excellent suggestion. The statement was rewritten to clarify this point (see Section 9.3.2).**

Section 10.1

25. The last sentence should read, "(The undiluted 66/233 contains 100 IU/mL.)" instead of 64/233.

- **This sentence was corrected in Section 10.1.**

Section 10.2

26. In addition to the reference sera listed, we also have available reference sera for antibodies to Scl-70 and Jo-1, as well as for the homogeneous and centromere patterns.

- **This information was corrected in Section 10.2.**

27. Reference sera are obtained from the AF/CDC ANA Reference Laboratory at the CDC and not from the Arthritis Foundation.

- **This information was corrected in Section 10.2.**

Section 10.2.1

28. I suggest the following rewording of the second paragraph: "The vial contents are sterile, as determined by United States Pharmacopeia (USP)-approved methods of sterility testing; negative for hepatitis-B surface antigen by radioimmunoassay; negative for antibody to HIV by Western blot; and free of rheumatoid factors, as determined by latex agglutination and radioimmunoassay. Mean dry weights, water content as determined by the Karl Fischer residual moisture test, results of formal thermal stability studies, and data related to their practical stability for routine laboratory use are provided with the ampules."

- **The wording in Section 10.2.1 is consistent with the labeling information for the AF/CDC ANA sera. The committee agrees that the wording suggested in the comment is appropriate for any new materials produced.**

29. New lots should also be assayed for hepatitis C virus.

- **Although the committee agrees, the current wording reflects the labeling of the AF/CDC reference sera.**

Section 10.2.2

30. This section should include discussion on the EIA method.

- **The AF/CDC ANA reference sera has not been validated with the various EIA methods; thus, no change has been made to Section 10.2.2.**

Section 13.0

31. I question the appropriateness of reference values by age.

- **The committee believes that the recommendations in Section 13 apply to the results for any patient population.**

32. A PBS or buffer control should be included.

- **A buffer control may be used to evaluate the contribution of fluorescein to assay background. A negative serum control would be more useful if incorporated into a standard assay run.**

Section 14.0

33. With the increase of ELISA test kits on the market, standardization of Sm, RNP, SSA, and SSB units is needed. Because these tests are more sensitive, is there any work in progress to develop international standards with specific cut-off points for disease states?

- **The committee is aware of efforts by the Association of Medical Laboratory Immunologists (USA) to develop standards that will be useful with ELISA test kits. Other European groups are developing similar materials.**

34. The second paragraph should include information on EIA.

- **Section 14 includes information on EIA methods.**

Related NCCLS Publications*

C28-A **How to Define, Determine, and Utilize Reference Intervals in the Clinical Laboratory; Approved Guideline (1995).** C28-A contains protocol for the determination of reference ranges for defined populations as an aid to the interpretation of laboratory data.

DI1-A2 **Glossary and Guidelines for Immunodiagnostic Procedures, Reagents, and Reference Materials—Second Edition; Approved Guideline (1992).** DI1-A2 provides common terminology and basic methodology for users and manufacturers of immunodiagnostic systems.

I/LA18-A **Specifications for Immunological Testing for Infectious Diseases; Approved Guideline (1994).** I/LA18-A outlines specimen requirements, performance criteria, algorithms for the potential use of sequential or duplicate testing, recommendations for intermethod comparisons of immunological test kits for detecting infectious diseases, and specifications for the development of reference materials.

M29-T2 **Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue—Second Edition; Tentative Guideline (1991).** M29-T2 offers guidance on the risk of transmission of hepatitis B virus and the human immunodeficiency virus in the laboratory. It gives specific precautions for preventing the transmission of bloodborne infection during clinical and anatomical laboratory procedures.

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* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.