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Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition

This document addresses specimen collection, handling, and storage, as well as performance criteria for the comparison of immunological test kits and specifications for reference materials.

A guideline for global application developed through the NCCLS consensus process.

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Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition

Abstract

Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition (NCCLS document I/LA18-A2) is intended for use by laboratorians who perform immunodiagnostic testing within clinical and reference laboratories. The document addresses the generic problems of preparation and characterization of antigens and antibodies; testing using these reagents; and understanding the results. *Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition* offers recommendations on specimen collection, handling, and storage, and performance criteria for the comparison of immunological test kits, as well as specifications for reference materials.

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Contents

Abstract i

Committee Membership v

Active Membership ix

Foreword xvii

1 Introduction 1

2 Scope 1

2.1 Standard Precautions 2

3 Definitions 2

4 Preliminary Information 7

4.1 Clinical Questions and Traditional Laboratory Methods 7
4.2 Immunoassays in Detection of Antigens from Infectious Agents 7
4.3 Immunoassays in Detection of Patient-Generated Antibodies 8

5 Immunochemical Methods 9

5.1 Tests for Antigen or Antibody in Liquid or Surface Specimens 9
5.2 Testing of Tissue Specimens (Primarily for Antigen) 10
5.3 Classes of Clinical Testing for Antigens and Antibodies 10

6 Qualification of Antigens 11

6.1 Types of Antigens 11
6.2 Ascertainment of Purity 12
6.3 Reporting by Manufacturers 12

7 Qualification of Antibodies; Immunochemical Specificity 12

7.1 Testing for Specificity 12
7.2 Causes of Nonspecificity 13
7.3 Monoclonal Antibodies 13
7.4 Reactivity with Immunoglobulin Classes and Subclasses 13
7.5 Methods 14
7.6 Antiserum Production 16
7.7 Nonspecificity, Undesirable Specificity, or Cross-Reactivity 17

8 Quantification of Antibodies (Titer) 18

9 Reference Materials, Proficiency Testing, and Challenge Panels 19

9.1 Calibrators, Controls, and Verifiers 19
9.2 Reference Materials 19
9.3 Proficiency Testing 19
9.4 Challenge Panels 21

10 Specimen Collection, Handling, and Storage 22

10.1 Specimen Collection 22
10.2 Specimen Handling 23
10.3 Specimen Storage 24

Contents (Continued)

11 Laboratory Interpretation of Tests 24
11.1 General Considerations 24
11.2 Criteria for Selection of Cut-Off Values 25
11.3 Clinical Predictive Value 25
11.4 Borderline Results 26
11.5 Evaluation of a Test 26
11.6 Reporting of Results 26
12 Evaluation of Test Results 27
13 Test Confirmation and Optimization 27
13.1 Precision vs. Accuracy 27
13.2 Physiologic Dynamic Range 28
13.3 Comparison of Quantitative Tests 28
13.4 Comparison of Qualitative Tests 28
13.5 Equivocal Results in Test Comparisons 28
14 Product Clearance; Recommendations for Package Inserts 29
14.1 Clearance by the Appropriate Regulatory Organization 29
14.2 Recommendations for Manufacturers of Kits and Reagents 29
14.3 Recommendations for the Clinical Laboratory 30
14.4 Recommendations for the Selection of Referral (Reference) Laboratories 31
References 33
Appendix. Interpretation of Laboratory Tests—Mathematical Concepts 36
Summary of Comments and Committee Responses 39
Related NCCLS Publications 41

Foreword

The intended audience for I/LA18-A2 — *Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition*, is clinical and reference laboratories that perform immunodiagnostic testing for infectious diseases, as well as the manufacturers of commercial test kits. To improve their positive and negative predictive values in diagnosis of disease, and to enhance interlaboratory comparability and performance, I/LA18-A2 promotes a better understanding of the requirements, capabilities, and limitations of these diagnostic tests.

The use of immunochemical methods for detection and quantification of agents of infectious disease, and of related antibodies, is increasing rapidly. Many of the assays in use, however, have significant problems with sensitivity and specificity. There are two basic types of immunodiagnostic tests for infectious disease: those that test for the presence of antigen(s) produced by the infectious agent(s), and those that measure antibody response to such antigens. Although the immunochemical methods are similar for both, potential problems with the two types differ with regard to sensitivity, specificity, correlation with the clinical stage of infection, and the like. Tests that are currently available vary greatly in reliability and accuracy.

Few, if any, guidelines or standards exist that address serological or immunological tests for infectious diseases. This document sets forth guidelines for the development and performance of immunodiagnostic tests for the detection of antigens from, and of antibody responses to, infectious agents. Also, the most commonly used immunological tests share basic specifications that apply to testing for many infectious agents and related antibodies; these specifications are the primary focus of this document. At the same time, it is recognized that there are exceptions, a few of which are addressed. Infections with the human immunodeficiency virus (HIV) and hepatitis viruses A-E, among others, have special requirements for testing and are not addressed in this document.

Key Words

Antibody, antigen, cross-reactivity, immunoassay, immunogen, sensitivity, specificity

Specifications for Immunological Testing for Infectious Diseases; Approved Guideline — Second Edition

1 Introduction

Despite the great strides made in prevention and treatment, infectious diseases continue to exact a heavy toll from humankind.[1] While traditional pathogen-detection methods, such as culture, have established their credibility over time, they are often slow and relatively insensitive. More recently developed rapid immunoassay methods show great promise as adjuncts to the traditional methods used in clinical diagnosis. Immunoassay methods have been used for many years in the detection of antigen from infectious agents and immune response; newer methods have increased the speed (and often the specificity) of this type of testing as well. However, there are many problems—recognized and potential—that should be considered in the development and use of immunoassays for the detection of infectious disease.

2 Scope

The number of specific immunochemical tests, and modifications thereof, is already large and is increasing rapidly. Therefore, this document addresses testing only in a general manner; examples are given as appropriate. It is anticipated that many current and future tests will have characteristics and problems not addressed in this document. The basic concepts of sensitivity and specificity, from both a laboratory and clinical standpoint, will remain important in regard to future tests.

This document addresses several issues to which particular attention should be paid, including the following:

- Determination of purity and structure of antigens used as immunogens or as detection molecules in assays;
- Antibody specificity and quantification (titer or other measurement);
- The use of enhancing and amplifying agents;
- Interfering substances;
- Sensitivity and specificity criteria for test kits and procedures for assessing sensitivity and specificity;
- Patient preparation and sample collection and handling;
- Laboratory and clinical evaluation of test results;
- Specifications for reference materials;
- Storage conditions for samples and reference materials;
- Performance criteria for ensuring comparability of results among methods and laboratories; and
- Recommendations for product labeling and product literature, including limitations of the procedure(s).

2.1 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

3 Definitions [a]

Definitions of the following terms are specifically directed toward the subject matter of I/LA18. Definitions of relevant terms other than those in the following list, or other uses of these terms, appear in NCCLS document NRSCL8 — *Terminology and Definitions for Use in NCCLS Documents.*

Absorption, *n*- The removal or neutralization of antigen and/or antibody from a solution by the addition of the other soluble reactant, i.e. the antibody and/or antigen; **NOTE:** See also **Adsorption**.

Accuracy, *n* - Closeness of the agreement between the result of a measurement and a true value of the measurand.

Adsorption, *n* - A process by which a substance is bound at the surface of a material (adsorbent); **NOTE:** See also **Absorption.**

Affinity, *n* - A measure of the attraction, or force of association, between a single antigenic site and a single antibody to that site; **NOTES:** a) The affinity constant is usually expressed as the equilibrium constant for the receptor + ligand reaction; b) Because of their heterogeneity, average or mean affinity constants are usually described for polyclonal antisera; c) See also **Avidity**.

Amplification, *n* - The use of substances that directly increase signal in proportion to quantity of analyte; examples include avidin-biotin labels and substrates which, following hydrolysis by an enzyme label, produce fluorescent components.

Analyte, *n* - A substance or constituent for which the laboratory conducts testing.

Antibody, *n* **- 1)** The functional component of antiserum, composed of a population of Y-shaped protein molecules, each member of which is capable of reacting with (binding to) a specific antigenic determinant. **NOTE:** These antibodies are produced by B-lymphocytes as a primary immune defense.

Antigen, *n* - Any substance that can stimulate the production of antibodies by an organism and combine specifically with them.

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

Antigenic determinant//Epitope//(determinant), *n* **- 1)** The minimum molecular structure of the antigenic site that will react with a monoclonal antibody; **2)** Any site on an antigen molecule at which an antibody can bind; the chemical structure of the site determining the specific combining antibody.

Antigen excess, *n* - The presence of an amount of antigen, in relationship to antibody concentration, that results in increased solubility of immune complexes, decreased apparent reactivity, and in underestimation of antigen quantity.

Antiserum, *n* - A serum, produced in animals or human beings, containing antibodies to one or more antigens of interest.

Ascitic fluid, *n* - Serous fluid from the peritoneal cavity. Monoclonal antibodies are commonly raised *in vivo* by implantation of hybridomas in the peritoneal cavity of mice, followed by purification of the antibodies from the resulting ascitic fluid.

Avidity, *n* - The net affinity of all binding sites of all antibodies in the antiserum, under specified physicochemical reaction conditions; **NOTE:** It is a function of the affinities of the antibody-combining sites on all antibodies present in an antiserum and all of the antigenic determinants of available macromolecules.

Bias, *n* - The systematic, {signed} deviation of the test results from the accepted reference value.

Blocking, *n* - The reaction of uncomplexed binding sites or of coupling agents to prevent nonspecific binding of test reactants.

Borderline positive, *n* – A test result that is neither positive nor negative, and thus the test has to be repeated or the results verified or extended by a confirmatory assay.

Calibration material//Calibrator, *n* - A material or device of known, or assigned quantitative characteristics (e.g., concentration, activity, intensity, reactivity, responsiveness) used to adjust the output of a measurement procedure or to compare the response obtained with the response of a test specimen and/or sample. **NOTES:** a) The guideline document for the HCFA regulations in the U.S. ***(Appendix C, Survey procedures, PC122)*** defines a calibration material as "a solution which has a known amount of analyte weighed in or has a value determined by repetitive testing using a reference or definitive test method"; b) The quantities of the analytes of interest in the calibration material are known within limits ascertained during its preparation and may be used to establish the relationship of an analytical method's response to the characteristic measured for all methods or restricted to some; c) Calibration materials with different amounts of analytes may be used to establish a calibration or response "curve" over a range of interest; d) The term "standard," commonly used in clinical laboratory protocols and having there a meaning as described here, has a specific and different meaning in the ***US CFR493 February 28, 1992***; e) The terms "primary" and "secondary standard" are used by WHO and ISO to refer to calibration materials.

Conjugate, *n* **-** A material produced by attaching two or more substances together; **NOTE:** Conjugates of antibody with fluorochromes, radioactive isotopes, or enzymes are often used in immunoassays.

Control//Control material, *n* - A device, solution, or lyophilized preparation intended for use in the quality control process; **NOTES:** a) The expected reaction or concentration of analytes of interest are known within limits ascertained during preparation and confirmed in use; b) Control materials are generally not used for calibration in the same process in which they are used as controls. See also **Standard** and **Reference material.**

Cross-reactivity, *n* - The reaction of an antibody with an antigen other than that which elicited its formation as a result of shared, similar, or identical antigenic determinants.

Denaturation, *n* - Loss of native structure or configuration of a macromolecule, usually with resulting loss of biological or immunological reactivity or solubility.

Dynamic range, *n* - **1)** Analytically, the functional range of an assay over which concentrations of an analyte can be measured with accuracy and precision; **2)** Physiologically, the full range of analyte levels to be expected in patient samples.

Efficiency, *n* - The percentage (number fraction multiplied by 100) of results that are true results, whether positive or negative.

Enhancement, *n* - The use of a reagent that nonspecifically increases signal in an assay (e.g., the use of polyethylene glycol to increase the rate of formation of antigen-antibody complexes).

Enzyme conjugate, *n* - One of the reagents of an immunoassay that has an antigen, analyte, or antibody complexed to an enzyme by a covalent linkage.

Epitope, *n* - See **Antigenic determinant.**

F(ab) fragment, *n* - The fragment consisting of a single antibody-combining site, embodied in an intact light chain and the F(d) fragment of one heavy chain, held together by means of a disulfide bond.

F(ab′)$_2$ fragment, *n* - The fragment obtained after papain treatment without subsequent reduction and consisting of a dimer of two F(ab′) fragments held together by two disulfide bonds.

False-negative result//False negative, FN, *n* - A negative test result for a patient or specimen that is positive for the condition or constituent in question.

False-positive result//False positive, FP, *n* - A positive test result for a patient or specimen that is negative for the condition or constituent in question.

F(c) fragment, *n* - The crystallizable fragment containing the complement and rheumatoid factor-binding regions and consisting of two heavy chain fragments joined by two disulfide bonds.

Fluorescence, *n* - Brief electromagnetic radiation emitted as a result of absorption of radiation (photons) by an atom, molecule, or ion; **NOTE:** Generally, fluorescent radiation has a longer wavelength than the absorbed radiation.

Heterophilic antibody//Heteroantibody, *n* - **1)** An antibody that has an affinity for an antigen other than its specific antigen; **2)** Antibodies in a patient's sample that can bind to immunoglobulins from other species.

Homogeneous immunoassay, *n* - An immunoassay in which no separation step is performed.

Hybridoma, *n* - A cell made in the laboratory by fusing a normal cell with a cancer cell to combine certain features of each; **NOTE:** Most specifically**,** a cell line derived by the fusion of a B cell and a plasmacytoma cell, usually for the production of monoclonal antibodies.

Immune complex, *n* – An aggregate formed of antigen and specific antibody molecules; **NOTE:** It can also include complement components and other molecules.

Immune threshold, *n* - The minimal level of specific antibody necessary for protection of a person against an infectious agent.

Immunoassay, *n* - **1)** Any laboratory method for detecting a substance by using an antibody reactive with it**; 2)** A ligand-binding assay that uses a specific antigen or antibody capable of binding to the analyte.

Immunogen, *n* - Any substance that elicits a cellular and/or humoral immune response and the production of antibody in a biological system; **NOTE:** See also **Antigen**.

Interference, *n* - Artifactual increase or decrease in apparent concentration of an analyte due to the presence of a substance that reacts nonspecifically with either the detecting reagent or the signal itself.

Label, *n* - A substance that is linked to a reagent (e.g., antigen or antibody) to facilitate detection in an immunoassay system using either a measurable property of the label or an entity produced by the label; **NOTE:** In EIA the label is the enzyme; in FIA the label is a fluorescer; in RIA the label is the radionuclide.

Ligand, *n* - An entity that binds to a receptor; **NOTES:** a) For example, an atom, ion, molecule, antibody, hormone, or drug; b) In immunological testing, the terms "ligand" and "analyte" are frequently used synonymously.

Matrix, *n* - All components of a material system, except the analyte.

Monoclonal, *adj* - Arising from a single clone of B lymphocytes or plasma cells; **NOTE:** All molecules of a monoclonal antibody have a single class and subclass of both heavy and light immunoglobulin chains, and a single antigenic-determinant specificity.

Monospecificity, *n* - The immunoreactivity of an antibody and/or antiserum with only its designated antigen or antigenic determinant.

Neutralization, *n* - See **Blocking**. **NOTE**: Can also be used to refer to inhibition of growth in culture (e.g., of viruses) or of the effect of toxins.

Nonspecificity, *n* - The reactivity of an agent in a test system to substances other than the analyte of interest.

Peritoneal fluid, *n* - An ultrafiltrate of plasma in the peritoneal cavity.

Polyclonal, *adj* - Pertaining to cells or cell products derived from different clones and having affinity for different epitopes on the same antigen and/or different antigens.

Polymerase chain reaction, PCR, *n* - A technology used to synthesize multiple copies of a defined region of target DNA; **NOTE:** A common method of DNA amplification, utilizing pairs of oligonucleotide primers as start sites for repetitive rounds of DNA polymerase-catalyzed replication alternating with denaturation in successive heating-cooling cycles.

Potency, *n* - **1)** The characteristic of an antibody that represents the concentration of antibody and the avidity for a given substrate antigen in a defined method; **2)** The characteristic of an antigen solution that represents the concentration of the antigen in a defined method.

Precision, *n* - The closeness of agreement between independent test results obtained under prescribed{/stipulated} conditions; **NOTE:** Precision is not typically represented as a numerical value but is

expressed quantitatively in terms of imprecision–the SD or the CV of the results in a set of replicate measurements.

Predictive value of a negative test result//Predictive value negative, *n* - The probability that a subject with a negative test result actually does not have the disease; **NOTE:** This is *a posteriori* (or post-test) probability.

Predictive value of a positive test result//Predictive value positive, *n* - The probability that a subject with a positive test result actually has the disease; **NOTE:** This is *a posteriori* (or post-test) probability.

Prozone effect, *n* - The result of a suboptimal antigen-antibody reaction in which either the antibody or antigen is in excess, incomplete, or blocks an optimal reaction.

Qualitative assay, *adj* - See Section 5.1.1.1.

Quantitative assay, *adj* - See Section 5.1.1.3.

Reactivity, *n* - The qualitative assessment of binding of an antigen or antibody with another substance; **NOTE:** "Reactive" is sometimes used as a synonym for "positive" when reporting test results.

Recombinant-derived antigens, *n* **-** Peptides or proteins produced by the introduction of genetic material into cells of another genus, species, or class of organism; **NOTE:** See also **Immunogen.**

Recovery, *n* - The measurable increase in analyte concentration or activity in a sample after adding a known amount of that analyte to the sample.

Reference material/Reference preparation, **RM,** *n* **-** A material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Reference range//Reference interval//(Normal range), *n* - The range of test values expected for a designated population of individuals; **NOTE:** For example, 95 percent of individuals that are presumed to be healthy (or normal).

Rheumatoid factor, *n* - Antibodies in serum samples that react with one or more classes of immunoglobulins of human (and sometimes other species) origin and that may be class- or subclass-specific.

Semiquantitative assay*:* See Section 5.1.1.2.

Sensitivity, *n* – ***In Qualitative Testing***, the test method's ability to obtain positive results in concordance with positive results obtained by the reference method; **NOTE:** If the true sensitivity of a device is better than the reference method, its apparent specificity will be less and the level of apparent false-positive results will be greater.

Seroconversion, *n* - The conversion of a patient's serum from negative to positive in a given test for antibodies to the infectious agent in question.

Signal//Measurement signal, *n* - A quantity that represents the measurand and which is functionally related to it.

Specificity//Analytical specificity, *n* – ***In Quantitative Testing***, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample.

Standard, *n* - **1)** An authoritative "document" setting forth criteria for performance and characteristics; **2) Primary standard,** *n* - A standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity; **3) Secondary standard,** *n* - A standard whose value is assigned by comparison with a primary standard of the same quantity **4) Reference standard,** *n* - A standard, generally having the highest metrological quality available at a given location or in a given organization from which measurements made there are derived; ***5*) Working standard,** *n* - A standard that is used routinely to calibrate or check material measures, measuring instruments, or reference materials; **NOTES:** a) A working standard is usually compared against a reference standard; b) A working standard that is used routinely to ensure that measurements are being carried out correctly is called a "check standard." See also **Reference material, Calibration material.**

Titer, *n* - **1)** The reciprocal of the dilution factor required to produce a defined outcome in a defined system; **NOTE:** The titer is usually proportional to the analyte concentration; **2)** ***In Radioimmunoassay***, the dilution of the antibody at which a specified percentage of the radiolabeled analyte is bound under defined conditions.

Verifier, *n* - The equivalent of a calibrator but used to verify calibration rather than for calibration *per se*; **NOTE:** Unlike a control, a verifier should have an absolute level designated (i.e., not a range of acceptable values).

4 Preliminary Information

4.1 Clinical Questions and Traditional Laboratory Methods

When evaluating a patient who has a possibly infectious disease, it is necessary for the clinician to decide upon the necessity for, and the type of, therapy as rapidly as possible. The history, physical examination, and other ancillary tests, such as radiography, can suggest the presence of one or more pathogens, but the final answer should be based on laboratory confirmation of the infectious agent.[1-5] For some fastidious pathogens, traditional pathogen-detection methods, such as culture, can require days or even weeks; in addition, such methods are not widely available for detecting some pathogens, especially viruses and parasites.[1,2,6] A few methods, such as the gram stain for bacteria and immunofluorescence for viruses, permit rapid presumptive diagnosis, but these are useful only when the pathogen can literally be seen in the microscopic examination of a sample from the area of infection.[1,2,7,8] As a result, clinicians often begin therapy before laboratory information is available.[1-5]

4.2 Immunoassays in Detection of Antigens from Infectious Agents

Immunoassays for antigens are useful in several contexts in relation to infectious disease. The rapid, specific diagnosis of infection with viruses, bacteria, fungi, and other agents is important to the management of the individual patient and the patient's contacts, for prevention of the spread of disease, and for an increased understanding of the epidemiology of the disease. For some infectious agents, immunoassays are the only practical means of diagnosis. For other agents, these assays permit specific diagnosis in minutes to hours.[9-11] Many assays permit quantification of the amount of pathogen present, at times providing helpful clinical information. To identify pathogens, such as *Mycobacterium tuberculosis,* immunochemical tests for antigens may be used after doing a culture.[12]

The detection of different antigenic epitopes, and the presence of related antibodies, can aid in the determination of the stage of infection, as, for example, in a syphilis or adenoviral infection.[1-4,9-10,13-15] Specific epitope studies can also be used, in some instances, to determine whether a pathogen represents a new strain in the community or whether diverse patient isolates represent a single outbreak.

Immunochemical tests for pathogens or their antigens should address several potential problems.

4.2.1 Epitopes

Antibodies used to detect antigens should be directed toward a consistently present, but pathogen-specific, epitope(s). The lack of knowledge about such epitopes is the single most inhibiting factor in the development of reliable immunoassays for infectious pathogens.[1-3,9,10,16]

4.2.2 Antigen or Antibody Excess

Pathogen (and antigen) concentrations vary widely from patient to patient and from sample to sample from the same patient, sometimes covering several orders of magnitude.[13] As a result, antigen or antibody excess is a distinct possibility with some assays (e.g., latex tests for *Cryptococcus* spp.).

4.2.3 Detectability

Due to the early stage of infection, clearance by host defenses, inadequacy of the specimen, nature of the pathogen itself, or the type of infection (e.g., a well-encapsulated abscess), antigen concentrations can be below the limit of detectability.[9,10,17] Complex antigen structure can also limit the accessibility of epitopes for reaction.

4.2.4 Sample Sources

Patient samples for pathogen detection may be taken from many sources (e.g., blood, urine, cerebrospinal fluid, feces, and pus). The samples supplied vary from patient to patient and from pathogen to pathogen. Sample matrix differences can markedly affect test performance.[1,2,4]

4.2.5 Effects on Immunoassays of Inappropriate Handling of Specimens

Many pathogens and antigens are labile, i.e., sensitive to such factors as pH, temperature, atmosphere, and the presence of leukocytes or bacterial contaminants. Specific recommendations for specimen handling and transport should be based on the nature of the assay and specimen lability. Such recommendations should be defined and followed carefully.

4.3 Immunoassays in Detection of Patient-Generated Antibodies

Immunoassays for antibodies to infectious agents are also important clinically for (1) determining whether a patient has been exposed to an agent and has developed antibodies to it; (2) evaluating a person's immunoresponsiveness to infection or immunization; (3) determining whether the disease is under control; and (4) studying the extent of exposure and the spread of infection within populations.

In many respects, assays for antibodies are associated with fewer problems than are those for antigens. First, the only specimens commonly used are serum, plasma, or cerebrospinal fluid, so matrix problems are less likely. Second, there is usually less variability in concentration, so assays can be developed with less likelihood of antigen or antibody excess.

Potential problems associated with antibody assays include the following:

4.3.1 IgG Antibodies

The presence of immunoglobulin G (IgG) antibodies can indicate previous exposure to the pathogen rather than current, active infection.[1-3] As a result, the detection of either IgM antibodies or a rising level (titer or other measurement) of IgG over a period of several weeks can be necessary for diagnosis.[3,4] In the latter case, the diagnosis often becomes of historical interest only. (Because of between-run differences in assay response, paired samples should be run at the same time; if a sample is run initially, freeze a portion of the untreated sample and rerun it when the second sample is obtained.)

4.3.2 Lysates as Antigens

The use of crude lysates of a pathogen (and often cells or medium) as antigen in tests for the detection of antibodies can render an assay unacceptably nonspecific. Purification of the desired antigens, or the use of recombinantly generated antigens, can greatly improve specificity without compromising sensitivity.[1-4] Otherwise labor-intensive confirmatory tests, such as western immunoblotting, can be necessary on a large number of samples.

4.3.3 Confirmatory Tests

Because of a lack of adequate characterization and knowledge about proper interpretation, confirmatory tests can be unreliable. Also, many confirmatory tests are available only for investigational or research purposes.

4.3.4 Suppression of Antibody Levels

Conditions, such as severe infection and immunosuppressive drug therapy, can result in the suppression of antibody levels below the detection threshold for the assay.[3,4] As a result, false-negative test results can be seen in patients who are, or have been, infected with the agent in question.

4.3.5 Correlation of Antibody Levels

Antibody levels might not correlate with the severity of infection, the degree of immunity, or the ability of the patient's immune system to mount an immunologic response.[1-4]

4.3.6 Detection of IgM Antibodies

Many assays designed to detect immunoglobulin M (IgM) antibodies, either alone or in combination with IgG, are unreliable. Particularly with combined tests (IgG plus IgM), the manufacturer and the laboratory should document adequate performance with samples that contain only IgM antibodies to the agent or antigen of interest before such tests are used to assay clinical samples.

5 Immunochemical Methods

5.1 Tests for Antigen or Antibody in Liquid or Surface Specimens

5.1.1 Qualitative vs. Quantitative Assays

Immunochemical tests for infectious disease can be qualitative, semiquantitative, or quantitative. Each type of assay has special performance criteria insofar as sensitivity, specificity, and controls are concerned. In each case, specify the precise analyte being assayed (e.g., true antigen vs. "process product").

5.1.1.1 Qualitative Assays

Qualitative assays report only the presence or absence of the analyte, without quantification. A positive test result implies only that the assay signal exceeds the analytical threshold (detection limit), or a cut-off point set to give an arbitrary combination of sensitivity and specificity. In simplistic and idealistic terms, detection of the analyte should correlate with the presence (and nondetection with the absence) of the infectious agent or of related antibodies, resulting from either natural exposure or immunization.

5.1.1.2 Semiquantitative Assays

Semiquantitative assays are essentially qualitative assays with an additional option for response range (degree of positivity, dilution to which positive results are obtained, or comparison to a color chart).

5.1.1.3 Quantitative Assays

Quantitative assays generate a spectrum of signal responses that correlate with the concentration of the analyte of interest. If analyte preparations with known concentrations are available for calibration, the actual concentration of the analyte can be determined.

5.2 Testing of Tissue Specimens (Primarily for Antigen)

Sections or smears of patient tissues, obtained by biopsy, aspiration, or swab, are often tested for the presence of infectious agents and/or related antigens by immunohistochemical methods. The most commonly used methods are fluorescent and enzyme immunoassays. Immunohistochemical methods permit visualization of the adequacy of the specimen and of the specific morphology of the antigen (often the intact organism). Disadvantages include the subjective nature of interpretation, the need for special equipment, and labor intensity (partly due to the need for multiple controls). The same methods can be used for detection and identification of organisms, particularly viruses and fastidious bacteria, after growth in tissue culture.

Immunofluorescent assays are used most frequently for tissue testing in clinical laboratories and they can be direct or indirect. In the former, a fluorescence-labeled antibody specific for the antigen (organism) to be detected is applied to the specimen (tissue section or fixed smear); this is sufficient for the detection of relatively abundant antigens, such as cell surface components of target organisms. In indirect immunofluorescence, an unlabeled antibody specific for the antigen (e.g., mouse monoclonal IgG anti-*Chlamydia trachomatis*) is applied first; for detection, a labeled antibody directed toward the first antibody (e.g., fluorescein-labeled goat antimouse IgG) is used.

Discussion of optimization and of potential problems of these assays is beyond the scope of this document. Because proper collection and processing of tissues is necessary for the assay to be valid, the clinician should always consult with the laboratory before samples are obtained.

5.3 Classes of Clinical Testing for Antigens and Antibodies

The recommended levels of sensitivity and specificity for tests in the following classifications will vary, depending on the consequences of disease; the failure to detect disease if it is in fact present; the availability of adequate treatment; the negative effects (costs, psychological trauma, etc.) of false-positive test results; and the prevalence of the disease in the population being tested. Suggested levels of sensitivity and specificity are, therefore, only general guidelines. These classes of clinical tests are not mutually exclusive.

5.3.1 Screening Tests

Screening tests are used for testing entire populations or subsets of such populations for the presence of a characteristic (such as the presence of an infectious agent or of related antibodies. In general, they should have high clinical sensitivity (i.e., clinical detection rates over 95%). The recommended degree of specificity (and positive predictive value) depends on the factors mentioned above plus the difficulty and cost of confirmation of positive screening results. In most cases, however, the recommended specificity and positive predictive value are somewhat lower than those recommended for the following tests. In other words, a negative screening result should infer that the person has a high probability of being free of the characteristic, whereas a positive test result might reflect only the need for further, confirmatory testing.

5.3.2 Diagnostic Tests

Diagnostic tests are used for the evaluation of persons suspected of having a given characteristic (e.g., a particular type of infection). If the characteristic is important, either for treatment or for prognostic considerations, sensitivity should be as high as possible. However, if an accurate confirmatory test is readily available, a high degree of specificity might not be necessary. The majority of clinical tests for infectious disease are for diagnostic use.

5.3.3 Confirmatory Tests

After positive screening or diagnostic test results, supplemental tests may be used to document (in an attempt to confirm) the positivity of the persons' previous results. In this case, specificity and positive predictive value, rather than sensitivity and negative predictive value, are usually the primary considerations; specificity should be over 98 to 99%. Confirmatory tests may be nonimmunochemical (e.g., culture or deoxyribonucleic acid studies) or immunochemical. Examples of the latter include western immunoblotting and antigen- or antibody-blocking assays. In most cases, an assay method other than that used for screening or diagnosis should be used for confirmation. Confirmatory tests might not be necessary if the screening or diagnostic test has high specificity or positive predictive value.

6 Qualification of Antigens

Antigens from infectious agents are used as:

- immunogens to produce antibodies (usually in nonhuman species) for the detection of similar antigens in patient specimens, and

- reagents to detect the presence of antibodies in such specimens.

As a result, the antigens used affect all immunochemical tests for infectious disease, and the criteria and methods for preparation, purification, and testing of these antigens are crucial.

6.1 Types of Antigens

Antigens can be native (i.e., naturally occurring, unmodified); denatured (or otherwise altered); or synthetic. Particularly for the last of these, fragments and even single epitopes may be used. In some cases, whole organisms (usually killed or lysed) are used as antigens for testing. Because of the possibility of cross-reactivity with related organisms, preparations made from whole organisms should be tested extensively with all noted cross-reactivities specified.

Were it not for the variability of antigens among strains of the same organism (either from normally occurring strain differences or mutation) and, in some cases, for the sharing of similar antigens among different classes of organisms, the use of highly purified antigens (or even single epitopes) would be preferred for all testing. It is therefore necessary to document not only the purity of antigens used but also their within- and among-strain/class/species reactivity. The lack of cross-reactivity with antigens from human tissues (normal or diseased) should be documented as well, especially for antigens prepared from human cells or cell cultures.

Spontaneous mutations in an organism can result in antigenic changes without associated changes in the virulence of the organism. When this is known to occur (e.g., Sabin D antigen), assays should be designed to detect the mutant antigens, and/or the manufacturer should state the possibility of such an occurrence.

6.2 Ascertainment of Purity

Methods used to ascertain purity should be as rigorous as possible. Testing should be performed with sufficient antigen to permit the detection of minor components. Sensitive detection methods for contaminants are essential.

6.3 Reporting by Manufacturers

Antigens used for immunization or as assay reagents should be derived from generally accepted strains of the infectious agent (i.e., not from atypical or mutant strains). If the information (which may be considered proprietary) is not given in the package insert, the laboratorian should request it from the manufacturer before using the reagents or kit. If the information is proprietary, the manufacturer should be willing to certify to the user that it is a generally accepted strain and not atypical or mutant. If there are any subsequent changes in antigen source, the manufacturer should notify users.

If cell cultures are used for the preparation of antigens, the type of cells and the methods used in purification of the antigen should be specified. If cellular antigens [e.g., human leukocyte antigens (HLA) from human cells], which can react with patient materials, remain in the antigen preparation, this should be stated, along with recommended methods of testing for such reactions and of confirmation of test results.

7 Qualification of Antibodies; Immunochemical Specificity

Antibodies used for the detection of antigen or of other antibodies may be monoclonal, mixed monoclonal, polyclonal, or combined monoclonal-polyclonal. High affinity and avidity are usually associated with higher sensitivity of the tests derived from such antibodies; however, in some cases, high affinity can be associated with reduced specificity. The antibodies used should therefore be matched to the criteria necessary for the performance of the particular method.

7.1 Testing for Specificity

Testing for the specificity of antibodies should be performed using multiple strains of the organism of interest, other biologically related organisms (e.g., other spirochetes in the case of antibodies to *Borrelia burgdorferi*), and normal specimens (i.e., from unaffected persons). Any identified cross-reactivity should be specified in the package insert. Likewise, the potential absence of reactivity with some strains of the organism of interest should be specified.

7.2 Causes of Nonspecificity

Nonspecific reactivity can result from "abnormal" human immunoglobulins, including rheumatoid factor and heterophilic antibodies[18,19]; such reactivities are usually anti-ruminant, anti-avian, or anti-murine in their origin. All reagents should be tested against sera containing IgG and IgM rheumatoid factor at several levels, with the results specified in the package insert. If heterophilic antibodies are likely to be a problem with the population to be tested (e.g., farmers, veterinarians, persons receiving monoclonal antibodies in therapy), this should be stated. In some cases, the use of $F(ab')_2$ IgG fragments in the assay can reduce nonspecific reactivity.[20]

It is important to distinguish between antibodies intended to detect purified or partially purified antigens and those intended to detect the infectious agents themselves. The latter can react with multiple antigens from the agent. Immunochemical specificity in this case refers to the lack of reactivity with other agents (especially closely related ones) or with normal tissues. In either case, specificity can be increased by using two different antibody species for capture and detection.[19]

7.3 Monoclonal Antibodies

Monoclonal antibodies can be powerful immunochemical tools because of the three following attributes: single epitope-binding specificity, homogeneity, and potentially unlimited supply. Due to their high degree of specificity, monoclonal antibodies can be used successfully, under some conditions, when polyclonal antibodies cannot. For example, screening and cloning techniques can permit the production of specific monoclonal antibodies when sufficiently pure antigen is not available for *in vivo* production of specific polyclonal antibodies. However, production of a "good" monoclonal antibody is often a difficult, laborious, and costly task.

Depending on the application, the use of monoclonal antibodies is not always advantageous, especially if affinity-purified polyclonal antibodies are available. Monoclonal antibodies often show low affinity and avidity. Recognition of a single epitope can limit the usefulness of a monoclonal antibody for species- or genus-wide detection of infectious agents and for test methods dependent on immunoprecipitation if epitope density on the antigen is low. In addition, the antigen epitope might be shared by other infectious agents, thus making the monoclonal antibody nonspecific.

The pooling of two or more monoclonal antibodies having different epitope specificities can overcome these problems. Also, screening procedures can select for higher affinity monoclonal antibodies. Because these antibodies can behave differently from one assay protocol to another, screening of monoclonal antibodies should employ a method as close as possible to the proposed application. The immunologic assay design should include careful evaluation of the many factors that influence the choice of an antibody reagent.

The best current method for testing the specificity of monoclonal antibodies is western immunoblotting, using one- or two-dimensional electrophoresis of crude antigen preparations as the first step.[21]

Clinical laboratories should not be expected to test for antibody characteristics. However, the laboratorian should be aware that test performance is not always improved by the use of monoclonal antibodies.

7.4 Reactivity with Immunoglobulin Classes and Subclasses

Manufacturers of antibodies (and kits that utilize them) designed to detect a single class or subclass of human antibodies should indicate the type of specificity (e.g., anti-μ or anti-γ chain or anti-F(c) fragment). The lack of reactivity with light chains must be documented. It is especially important for the

detect all antibody classes, or IgM plus IgG, in reality react poorly with IgM. The detection of this problem should not be the responsibility of the user. In addition, antibodies to IgG should be documented to react with all subclasses unless one or more subclasses are not important in detection of disease or immune response.

7.5 Methods

7.5.1 Detection of Unwanted Antibody Specificities

"Contaminant" antibodies (or unwanted specificities) in antisera often cannot be detected by the method used for the final assay. In such cases, use alternative methods to detect these specificities. To avoid antigen or antibody excess, several concentrations of each antigen preparation should be tested against serial dilutions of relevant antisera. Sensitive methods, such as two-dimensional immunoelectrophoresis (crossed antigen-antibody electrophoresis)[22,23] or sieving electrophoresis (e.g., polyacrylamide gel) followed by immunoblotting,[24] should be used whenever possible. Less sensitive methods, such as immunoelectrophoresis and Ouchterlony immunodiffusion, should only be used when more sensitive methods are not available.

Although thorough washing of gels or blots is essential, excessive washing and/or the use of acidic washing solutions (including unbuffered saline) can dissociate immune complexes. Sensitive fixation and staining methods are also essential.

Boiling of samples and the addition of detergents or other chemicals to samples can expose additional antigenic epitopes, or alter those already exposed. Such treatments can be helpful in testing for unwanted antibody specificity. If treatments are to be used in the final, clinical assay, the manufacturer should document that unwanted reactivities do not result and that the desired epitopes will react as expected.

Monoclonal antibodies should be tested by competitive inhibition techniques, using radioimmunoassay, enzyme immunoassay, or immunoblotting.

7.5.2 Detection of Unwanted Materials in Antigen Preparations

Unwanted or nonspecific reactivity can result from the presence of contaminant materials in antigen preparations. Antigens should be tested for reactivity with antisera to possible cross-reacting agents (e.g., related strains of bacteria). For direct detection of contaminants in an antigen preparation, run several concentrations of the preparation using sieving gel electrophoresis, isoelectric focusing, two-dimensional electrophoresis, or similar methods. Also, a sensitive detection method, such as direct silver staining or immunoblotting followed by a sensitive staining method, should be used.

7.5.3 Reporting of Cross-Reactivity and Interference

The manufacturer should indicate known assay cross-reactivities in the package insert. Measures to counteract interference should be provided when known (e.g., addition of nonreacting immunoglobulins to inactivate heterophilic antibodies). If these measures are used in the assay, the manufacturer should state this fact.

7.5.4 Permissible Reactions

The following do not constitute nonspecificity in an antiserum and are considered permissible in most cases:

7.5.4.1 Cross-Species Reactivity

The reactivity of an antiserum to human immunoglobulins with antibodies from other species is anticipated and is not a deterrent, unless antibodies from the other species are used in the assay.

7.5.4.2 Precipitation of Plasma

The formation of a precipitate or the clotting of plasma samples by an antiserum does not indicate nonspecificity. However, the manufacturer should indicate whether the possibility of such precipitation might interfere with the use of the antiserum in the assay of plasma samples.

7.5.4.3 Removal of Contaminating Antibodies by Absorption or Adsorption

Many polyclonal antisera are absorbed or adsorbed with materials to remove unwanted antibodies. "Absorption" uses soluble antigen. As a result, free antigen or antigen-antibody complexes can remain in solution and potentially interfere with certain assays. It is recommended that solid-phase "adsorption" be used to minimize this problem. If liquid absorption is used, the manufacturer should indicate this and warn against potential problems with assay performance.

7.5.4.4 Use of Enhancing or Amplifying Agents

The use of agents such as polyethylene glycol (PEG), used to enhance the formation of antigen-antibody complexes, can result in nonimmunological precipitation, particularly of high-molecular weight proteins or lipoproteins. Amplifying agents, such as fluorescent substrates for enzyme immunoassays or avidin-biotin complexes, can increase nonspecific binding and reactivity. Either type of agent usually increases test sensitivity but can increase or decrease specificity. If such agents are used, the manufacturer should address and document any possible interference with the assay.

7.5.4.5 Unusual Assay Conditions

Certain forms of support media might not be conducive to antigen-antibody complex formation in the presence of unusual ionic strength or pH. Consequently, the user or manufacturer should pretest recommended or commonly used support media and recommended buffers with the reagents in a given assay.

7.5.5 Species of Antiserum Production

The manufacturer should indicate the species in which antisera were raised.

7.5.6 Use of Binding or Adhesive Agents

Using agents to bind antigen preparations such as cell cultures, patient specimens, microscope slides, or other solid-phase substances can result in the generation of a nonspecific signal, often because of altered surface charge. When used in a kit or when recommended for use with reagents, agents should be tested for nonspecific reactivity, and specify methods to minimize this problem.

7.5.7 Blocking of Unbound Reactive Sites

The use of blocking agents to bind unreacted sites, such as on solid-phase supports, is a common procedure (e.g., skim milk for immunoblotting membranes). In many cases, this can reduce assay sensitivity and/or specificity.[25] In addition, some blocking materials can cause nonspecific reactivity. The effect of such agents should be extensively tested in an assay and any undesirable effects specified.

7.5.8 Blocking for Confirmation

"Blocking" antibodies or antigens are sometimes used in enzyme immunoassays for the confirmation of positive test results,[26,27] particularly in situations where other organisms have epitopes in common with the organism(s) to be detected.

A blocking antibody is generally a mouse monoclonal antibody specific for an epitope(s) unique to the organism of interest. This antibody interferes with binding of the regular test antibody (e.g., rabbit anti-organism antibody), resulting in a reduced signal. The manufacturer should set guidelines for confirmation; one format is that a true positive test result will be positive on retesting and demonstrate at least a 50% reduction (blocking) in reactivity with the addition of the blocking antibody.[26,27]

Highly purified antigens, peptides, or known positive specimens may also be added to an assay in order to complex with specific antibodies and therefore inhibit reactivity with the patient sample. Such materials should be highly specific and bind with high affinity to the antibodies in use.

7.5.9 Dissociation of Immune Complexes

In some cases, a patient specimen might contain immune complexes that include the antigen or antibody to be assayed. Dissociation of the complexes can aid in the detection of the antigen or antibody, although there are few documented circumstances in which this is the case. One suggested method is the precipitation of the complexes with PEG, followed by their dissociation through incubation in alkaline borate buffer.[28] Because of the potential interference afforded by PEG or borate, this procedure should not be used with commercial kits or reagents unless it is certified by the manufacturer.

7.6 Antiserum Production

7.6.1 Fibrinogen Removal

Antiserum that contains clottable fibrinogen can produce precipitates upon mixing with serum or plasma samples. Antiserum should not contain thrombin-clottable fibrinogen.

7.6.2 Removal of Antigen-Antibody Complexes

Liquid absorption of antigen or antibody reagents usually results in the presence of antigen-antibody complexes, factors which should be removed to prevent nonspecific reactions. Relevant methods for removal of such complexes include water dialysis, precipitation with 4 to 6% PEG 8000, immunoglobulin or antigen purification, or a combination of these methods.

7.6.3 Atypical Reactions Due to the Presence of Anti-Antibodies or Heterophilic Antibodies

"Reverse" immunological reactions can occur when patient samples contain antibodies to antigens (including antibodies) present in the antibody preparation in use. Relatively common examples include heterophilic antibodies and rheumatoid factor (see Section 7.2).[18-19] In particular, false-positive test results for IgM antibodies can be observed in the presence of some rheumatoid factors. Each of these possibilities should be assessed and documented. In some instances, $F(ab')_2$ fragments of purified animal antibodies can circumvent these problems.[20] Alternatively, the patient samples can be adsorbed to remove the interfering antibodies.[19]

7.6.4 Interfering Substances

Hemoglobin, bilirubin, bile pigments, and other substances can interfere with some assays, especially homogeneous assays using colorimetric detection. In addition, manufacturers sometimes add pigments

for reagent identification. The potential interferences afforded by such substances should be specified in the package inserts.

7.6.5 Processing of Antibody Reagents

Monoclonal immunoglobulins from ascitic fluids should be purified to remove all irrelevant proteins. Several methods for purification have been used, including ammonium or sodium sulfate precipitation, ion-exchange liquid chromatography, and high-performance chromatography. Of these, the last is the preferred method.[29]

The same methods may also be used to purify polyclonal immunoglobulins. Antisera of animal origin are often clarified by removal of lipids and lipoproteins (i.e., immunoglobulins are not purified). Reagents used for clarification, such as silicon, can react with antigens in patient specimens if the reagents are not completely removed. Hence the manufacturer should test for the presence of such materials in the final reagent and/or document the lack of interference in the assay of clinical samples.

7.7 Nonspecificity, Undesirable Specificity, or Cross-Reactivity

For tests in which antigen or antibody is to be identified or measured in a patient specimen or extract of a patient specimen, specificity is defined as the ability of an assay to correctly identify as negative those samples that do not contain the antigen or antibody associated with the etiologic agent or the specific immune response to an infection by the etiologic agent.

The extent to which an assay yields positive results with samples not containing antigen or antibody associated with the etiologic agent is determined by the specificity of the detection molecule for the ligand, the prevalence of the ligand in heterologous agents, and contributions from other components in the assay. The latter components can be related to the signal-producing system of the assay or to nonspecific interactions with the medium or the sample. Thus, from the viewpoint of the laboratorian, specificity has two elements, one related to the detection molecule for the ligand and a second related to interactions between the test sample and assay components other than the detection molecule.

7.7.1 Identification of Nonspecific Reactions or Cross-Reactions

True cross-reactions (i.e., reactions of the antigen or antibody used in the detection system with heterologous ligands) can often be recognized because of prior experience with the assay or information provided by the manufacturer. This information can include listings of organisms tested and shown to be reactive or nonreactive with the assay. Because the impurities themselves might not be fully identified and quantified, cross-reactions caused by impurities in the antigen or antibody mixture can be more difficult to discern.

Nonspecific reactions are nonimmunochemical reactions that result in a false-positive test result. They are often due to the reactivity of specimens with components of the assay. Typically, assays use a solid phase to which is attached an antigen or antibody that is similar in structure and origin to the specific detecting antigen or antibody but lacking the same specificity for the analyte. The solid phase or the reagents used in the assay can cause nonspecific reactions. Sample pretreatment methods compatible with a particular assay including boiling, filtering, and chemical extraction, can reduce nonspecific reactivity. For example, for assays to detect polysaccharide, carbohydrate, or lipopolysaccharide (LPS) antigens, boiling or chemical treatment can remove interfering proteins, including immunoglobulins. However, it is the committee's position to avoid using such methods unless the manufacturer has certified their performance in the assay.

Incompatibility of an assay with some methods for sample collection, transportation, or storage can also cause nonspecific reactivity. Information pertaining to the compatibility of an assay with various

collection devices (e.g., tubes and swabs), transport media, specimen storage conditions (including heating and freezing), pH, temperature, lipemic or hemolyzed specimens, and anticoagulants should be indicated by the manufacturer whenever relevant (see Section 10). To avoid such incompatibilities, the user should adhere strictly to the procedure provided by the manufacturer.

Blocking assays, in which specimens are preincubated with specific antibody or antigen, can be useful in differentiating true positive results from those caused by either nonspecific reactions or cross-reactions with other epitopes (see Section 7.5.8).

7.7.2 Undesirable Specificity vs. Inconsequential Specificity

The adverse effect of a nonspecific reaction can be limited if the cause of the reaction is unlikely to be encountered in clinical specimens. For example, while an antibody preparation can be shown to cross-react with heterologous agents, the clinical specificity of the assay will not be affected if these agents are rarely, if ever, present in the body fluid or location sampled. Nonspecificity can also be inconsequential if the user routinely screens specimens for cross-reacting heterologous organisms before or during testing. This is often accomplished with assays using culture, with growth characteristics on culture media (e.g., hemolysis) and simple biochemical procedures (e.g., lactose fermentation) serving as common tools for screening for heterologous organisms that can share antigenic determinants with the infectious agent.

Testing of patient samples with a low probability of having the disease in question will increase the proportion of samples that yield a low predictive value. As an example, persons with rhinitis without pharyngitis (and therefore suspected to have uncomplicated viral upper respiratory infections) should not be tested for Group A streptococci because many, if not most, positive test results will reflect a carrier state rather than disease. Factors to be considered in testing for a specific agent include the prevalence of the disease, local endemics or epidemics, season of the year, age of the patient, and clinical data (e.g., history, physical examination) as well as results of other laboratory tests.

7.7.3 Tests for Broad Versus Specific Categories of Antigens

Low specificity in some test systems can offer advantages over more specific tests. If the test antigen is a virulence factor, specificity for a disease state will be more important than the ability to differentiate taxonomically distinct agents. For example, serological assays for antistreptolysin O are unable to differentiate between infections with Group A, C, and G streptococci, but they are useful in monitoring sequelae of infection with Group A, such as rheumatic fever. Specific antibodies to the capsular polysaccharide of *Neisseria meningitidis* Group B will also react with *Escherichia coli* K1, because both agents share common determinants. However, detection of this antigen from either organism in body fluids can be important diagnostically.

The desired specificity of an assay can also depend on the level of reporting to which the laboratory is committed. Assays for *Chlamydia* spp. that use antibody to identify antigens to the genus level, such as LPS, can be either advantageous or undesirable, depending on whether species-level identifications are necessary. The lower specificity of anti-LPS antibodies offers the opportunity to identify species for which specific reagents might not be available (e.g., *C. pneumoniae*). This situation is also true for several other infectious agents with genus- and species-specific antigens.

8 Quantification of Antibodies (Titer)

The manufacturer or distributor of antibodies intended for use as reagents (i.e., not in kits) in testing for infectious diseases should list recommended methods and approximate working antiserum dilutions for these methods, based on actual testing. However, the recommended dilutions should be accepted as only a starting point; the individual laboratory should assume responsibility for optimizing sample and reagent dilutions in this case.

9 Reference Materials, Proficiency Testing, and Challenge Panels

All materials discussed in this section should mimic the reactivity of patient specimens to be tested (i.e., matrix effects of all materials should be similar). The manufacturer should specify the expected results for each of these materials; for example, the expected range of signal levels or enzyme immunoassay (EIA) indices should be given for calibrators.

9.1 Calibrators, Controls, and Verifiers

9.1.1 Calibrators

Calibrators are materials used to standardize the instrument or other assay method each time testing is performed. In most assays, several calibrators (or dilutions) covering the entire spectrum of levels expected in patient samples should be included in each run. These calibrator values should be within the designated absorbance range for the run to be counted. Extrapolation of values beyond the range of calibrator concentrations is usually not acceptable. If dilutions of a single calibrator are used, the diluent should not result in a change in reactivity or in altered matrix effects.

9.1.2 Controls

Controls are materials intermixed with patient samples and assayed in order to ascertain the reliability of the assay. Controls usually have a range of acceptable values; the range should be consistent with acceptable variability in the testing of patient samples. If the control values are outside this range, the assay is invalid and should be repeated. Controls should contain levels of analyte at or near the decision cut-off points.

9.1.3 Verifiers

Verifiers are similar to controls, except that single, exact values are assigned. They are also used to ascertain reliability but are not generally necessary in clinical testing.

9.2 Reference Materials

Reference materials are used to standardize an assay initially and periodically, in contrast to calibrators and controls, which are used daily. It is recommended that reference antisera be uniform in their ability to react with a specific antigen, and they should yield reproducible results when reacted with a reference antigen. A reference antigen may consist of whole organisms, or of partially or highly purified components of the organism. Reference antigens should provide reproducible results when reacted with a reference antiserum to the antigen. It is recommended that either type of reference material behave like patient samples in the test system (e.g., have no unusual matrix effects).

National and international reference materials are also used to permit standardization among laboratories. When such materials are available, each manufacturer and laboratory should ascertain that secondary or tertiary reference materials have a known reactivity in relation to the relevant national or international standards.

9.3 Proficiency Testing

Proficiency tests are tests run by a clinical or reference laboratory on materials known to contain, or not to contain, the analyte of interest. These tests are used to confirm the reliability of clinical tests and to challenge a laboratory's skills in the performance of such tests, rather than to aid directly in clinical diagnosis. Such tests should be designed to test sensitivity and specificity, and they should include

samples at all decision cut-off points (e.g., the values used to separate "positive" from "negative" results). Proficiency testing was once voluntary; regulations now require it of every laboratory that assays clinical samples, and it will henceforth be used to determine which laboratories qualify to perform specific clinical tests. Such testing should be a regular practice of every manufacturer as well.

A laboratory's performance on proficiency testing provides feedback as to the laboratory's ability to attain results that agree with other laboratories using the same method and shows how results from one method agree with results found using other methods. Results from proficiency testing do not necessarily mean that one method is better able to detect or measure a pathogen, or products related to the pathogen of interest in actual clinical circumstances, compared to other methods.

9.3.1 Specimens for Proficiency Testing vs. Reference Materials

Specimens for proficiency testing are normally obtained from an external source but should not be considered reference materials. Antigens in these specimens are often altered from their natural state and might or might not work in a particular assay.

9.3.2 Antigens Used in Proficiency Testing Specimens

In proficiency testing for infectious diseases, the nature of the antigens used is a vital determinant of the results obtained. Factors to be considered include the following:

9.3.2.1 Molecular Weight and Structure

Subtle differences in the molecular weight and in the primary and tertiary structure of an antigen can greatly affect reactions with antibodies used in a given test. For the development of meaningful proficiency specimens, the selection of widely occurring antigens with stable epitopes is necessary.

9.3.2.2 Antigenic Determinants

The antigenic determinants present in proficiency testing specimens should be adequate to ensure immunological specificity. The source of antigen, and its method of purification, have been shown to influence performance of tests for *H. pylori* and for antibodies to rubella.[30,31]

9.3.2.3 Structural Components

Antigens should include structural components of the agents of interest in most cases. Commonly available isolates, representative of the taxonomic group that includes the infectious agent (e.g., "type" cultures), may be used as long as the tests in use can detect the agent specifically.

9.3.2.4 Chemical Structure of Antigens

The complex molecular basis of infectious agents should be recognized. Tests often measure antigens that are not structural antigens or that are not easily accessible on intact organisms. The chemical structure of such antigens is often variable or poorly characterized. Often, such antigens result from complex interactions between the infectious agent and specific and nonspecific host responses. Factors such as specific immune responses, enzymatic activity, and chemotherapeutic agents can alter the molecular weight and conformation of the antigen. Examples of frequently altered antigens include the capsular polysaccharides of bacterial agents of meningitis and the circulating, soluble antigens of *Candida* spp.

9.3.2.5 Epitope-Specific Antibodies

Proficiency samples that contain high levels of antibody specific for a single epitope (or group of epitopes) will give more positive results in assays dependent upon the restricted epitope(s), compared to assays dependent upon multiple epitopes and specificities. This issue is particularly relevant in comparing assays using crude whole cell lysates as antigen sources versus tests based on highly purified material, including antigens produced by recombinant methods.

9.3.2.6 Matrix Consideration

Whenever possible, proficiency testing specimens should include samples in the same matrix (e.g., serum) on which patient testing is performed.

9.3.2.7 Liquid Standards and Reference Materials

With few exceptions (e.g., international units for rubella antibody), recognized liquid standards and reference materials (see Section 9.2) are not available for antibody or antigen testing for infectious diseases. When national or international standards are available, however, assays should be referenced to them to assure comparability among laboratories, as noted in Section 9.2.

9.3.3 Differences Among Test Methods

The proficiency test results are expected to vary with different assay methods. This does not imply superiority of one method over another. In general, first-generation tests are less well defined and less specific than later tests, even though the former may be considered to be the "gold standard." Acceptable performance characteristics and results should be specified for each acceptable test method.

9.4 Challenge Panels

Challenge panels are designed to test the reproducibility, sensitivity, and specificity of a new assay. Reproducibility and sensitivity can usually be determined quickly, using replicates, repeat assays, and dilutions. Specificity is often much more difficult to document. Often, years of use in clinical laboratories is necessary to determine specificity, especially if the infectious agent itself, or a potentially cross-reacting agent, is uncommon. Also, test sensitivity and/or specificity can change because of mutations in the infectious agent.

Challenge panels should contain specimens from persons both with and without the disease of interest, with varying levels of the analytes to be assayed. In addition, they should contain specimens from persons known to have diseases that might interfere with the assay, resulting in false-negative or false-positive results. Examples include multiple myeloma, rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases; infectious diseases related to the agent in question (e.g., panels for a Lyme disease test should incorporate syphilis and other spirochetal diseases); and heterophilic antibodies (human anti-rabbit, anti-mouse, or other appropriate specificities).

When challenge panels are used for premarket testing of reagents and kits, the manufacturer should specify the numbers and types of patient samples in the panels. They should also indicate how the patients were categorized and by whom.

There is a need for a generally available panel of specimens from patients with rare diseases. These would be useful for both manufacturers and reference laboratories.

10 Specimen Collection, Handling, and Storage

The methods used for collecting, transporting, and storing specimens can greatly influence the results of immunodiagnostic tests. Among the factors that can have a major effect on the outcome of tests are the type of swab, container, or medium; temperature of storage; and time before processing. With the exception of IgG, which is relatively resistant to degradation from environmental factors other than proteases, most antibodies and antigens are highly susceptible to alteration by temperature, oxidation, and proteolytic degradation. Samples should be handled as aseptically as possible and stored at recommended temperatures until assayed.

10.1 Specimen Collection

Selecting the proper sample for analysis and collecting it in an adequate manner are essential for optimal test performance. If specific instructions for specimen collection and preparation are not provided by a test manufacturer and are not available from a laboratory procedure manual, locate the proper instructions before specimens are collected. Factors to be considered when determining the adequacy of a specimen for immunodiagnosis of an infectious disease include the following:

10.1.1 Timing of Collection During the Infectious Process

Specimens collected too early or too late in the disease process can give negative results. Obtain paired specimens, such as acute and convalescent sera, far enough apart to permit appropriate comparison; specify the recommended timing of such specimens (see also Section 14.3). In some chronic infections, serological markers can show unique patterns that aid in diagnosis.

10.1.2 Patient Medications

Certain medications can cross-react with diagnostic test reagents, or inhibit or slow the growth of an organism in culture. Manufacturers should list medications known to cause interference, and laboratories should report possible interferences to the manufacturer.

10.1.3 Preparation of Collection Sites

The cleaning of a sample site to avoid microbial contamination of the specimen or to remove unwanted substances (e.g., mucus) is sometimes necessary. Conversely, excessive cleaning before collection of a surface specimen (e.g., by swab) can remove or destroy the microorganism or antigens of interest, thereby decreasing test sensitivity. It is strongly recommended that specimens be collected by trained personnel. (See NCCLS documents H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*, and GP16—*Routine Urinalysis and Collection, Transportation, and Preservation of Urine Specimens.*)

10.1.4 Collection Technique

The manner in which the specimen collection device is used can affect the outcome of the test. This is especially true for specimens obtained from tissue surfaces (e.g., swabs or scrapings), when it is possible that the infected site was inadequately sampled or inappropriate surfaces were contacted.

10.1.5 Sample Order

If the collection process itself releases the specimen (e.g., urination, swabbing), the order of specimen collection can influence test results. Midstream urine is preferred to "first-catch" urine for most purposes (exceptions include testing for *Chlamydia* spp. and *Neisseria gonorrheae*), and mucosal surfaces can yield more analyte with repeated swabbing.

10.1.6 Cross-Reactivity with Sampling Device

The device used to collect the specimen (e.g., swabs or a blood collection tube) can adsorb antigens or antibodies from the specimen, resulting in false-negativity. Less commonly, the device can alter antigens and their immunoreactivity or add interfering substances to the specimen.

10.1.7 Release of Analyte by Collection Device

Ideally, a collection device should be able to release all of the collected analyte into the diagnostic system if adsorption occurs.

10.1.8 Specimen Volume

Whenever possible, obtain an adequate volume of specimen to allow for repeat and/or confirmatory testing. The manufacturer and laboratory should indicate not only the recommended volume but also the minimum acceptable volume for a given assay.

10.1.9 Dilution of the Specimen

Some collection devices contain media to enhance storage, transport, or handling; unless sample volume is large in proportion to medium volume, dilution of the analyte can lower test sensitivity.

10.1.10 Sample Type

Some tests might perform well with serum but not with plasma or whole blood. In some cases, the type of anticoagulant used in plasma samples affects test results. Sample types (such as saliva or urine) other than those described as suitable in the test kit package insert might not be validated for use by the test kit manufacturer. Testing such specimens can be valueless and misleading.

10.1.11 Identification of the Specimen

Proper identification of the patient from whom the specimen is obtained, the type of specimen, and the time of collection are essential and should be required for all specimens accepted for testing.

10.2 Specimen Handling

The effects of transportation on sample integrity vary with the type of sample and the nature of the assay. In general, to minimize the growth of contaminants, the release of proteases from any cells that are present, and the like, transport samples to the laboratory as expeditiously as possible.

Within the laboratory, it should be recognized that certain treatments, such as heating, might be necessary for one methodology but preclude use of the specimen for further tests. If necessary, split samples before treatment.

It is the manufacturer's responsibility to specify the conditions needed to yield optimal test performance, but it is the laboratory's responsibility to inform its users about appropriate collection and transport of specimens. Specimens that arrive in a condition unsuitable for accurate testing should be rejected after notification of the physician responsible for care of the patient. In general, laboratories should not offer tests for which specimens cannot realistically be obtained and stored in the manner recommended for the test methods available.

10.3 Specimen Storage

Aberrant results obtained after the retesting of stored materials might be secondary to changes in the assay being used or in the materials themselves. The latter can alter reactivity in some assay methods but not in others. For example, aggregation of molecules can affect results in gel diffusion methods but not in assays in solution. Treatment of samples before storage (e.g., heating or sterilization procedures) can also influence storage effects. Although many diagnostic tests yield accurate results on specimens stored under suboptimal conditions for prolonged periods, the results should be interpreted with great caution. This is particularly important when using stored specimens to compare the performance of tests being considered for routine use with fresh clinical specimens. If a portion of a specimen is to be stored for use at a later date, follow the manufacturer's recommendations for storage.

Preferred conditions for storage of specimens vary; in general, however, the stability of most antibodies and many antigens is inversely proportional to temperature. Adsorption onto the surface of containers can be a problem for some antigens and for fluids with low protein content, such as urine and cerebrospinal fluid. In some cases, this can be minimized by selection of the storage container and/or by addition of other substances that inhibit binding to the surface, as long as the substances do not interact with the analyte of interest or with the detecting reagents.

Specimen containers should be tightly sealed before storage. For freezing, the use of self-sealing, air-tight tubes is recommended. Whenever possible, avoid the use of self-defrosting freezers because of the danger of desiccation of specimens. Avoid repeated freezing and thawing in all circumstances; if repeated analysis over time is to be done, split the specimens.

11 Laboratory Interpretation of Tests (Also refer to NCCLS document EP12—*User Protocol for Evaluation of Qualitative Test Performance*)

11.1 General Considerations

Several interrelated concepts are of vital importance in the interpretation of laboratory tests. These include detection limits, cut-off values, sensitivity and specificity (laboratory and clinical), positive predictive value, negative predictive value, false-positive results, false-negative results, prevalence, efficiency, and "gold standards." These terms are defined in Section 3, and the mathematical concepts are presented in the appendix. However, a brief discussion is warranted here.

The lower immunochemical detection limit for an assay represents the lowest level of an analyte that can be reproducibly detected. The laboratory or test sensitivity is identical to the detection limit. Detection limits are expected to vary with sample type; for example, an assay can show significantly different detection limits in testing saliva and serum.

In most immunoassays, there is overlap of test results between samples from infected and uninfected populations, and a cut-off point for assignment of positivity should be established. From a clinical standpoint, persons with the disease (or other trait) who test negative are falsely negative, while those without the disease (or other trait) who test positive are falsely positive. In many cases, to attain an acceptable positive predictive value, cut-off points for test positivity should be set substantially above the immunochemical detection limit. An example is testing for *Candida* spp. antigens, which are detectable in a high percentage of specimens from normal persons if highly sensitive assay methods are used.

The remaining terms can also be defined from both laboratory and clinical standpoints. From a clinical standpoint, the positive predictive value of a test is the percentage of patients who have a positive test result and are indeed positive for the trait or analyte. Conversely, the negative predictive value is the percentage of persons with a negative test result who are indeed negative. Efficiency (the percentage of

results that are true results, whether positive or negative) is of clinical importance if false-positive and false-negative results are equally undesirable. Sensitivity, in contrast, is the percentage of patients who are positive for a trait and who test positive, and specificity is the percentage who do not have the trait and test negative.

11.2 Criteria for Selection of Cut-Off Values

As implied by the overlap of values previously mentioned, it is generally not possible for a test to have perfect (100%) sensitivity, specificity, or predictive value. The selection of a cut-off value, and the reporting of results from a test, should take into consideration which of these is most important. The following criteria are suggested by Galen and Gambino[32]:

- The highest sensitivity is desired when the disease is serious, should not be missed, and is treatable; when false-positive results will not lead to serious psychic or economic trauma; and when inappropriate treatment has minimal consequences (e.g., in the absence of an allergy to penicillin, treatment of persons with positive rapid tests for Group A streptococci).

- The highest specificity is desired when the disease is serious but is not treatable; when the absence of disease has either psychological or public health value; or if false-positive results might cause serious psychic or economic trauma (e.g., confirmatory tests for antibodies to HIV).

- The highest predictive values are desired when inappropriate treatment has serious consequences (e.g., therapeutic abortion because of increasing levels of anti-rubella antibodies).

- The highest efficiency is desired when the disease is serious but treatable, and when false-positive or false-negative results are equally serious (e.g., antigen assays for bacterial causes of meningitis).

In determining the appropriate balance of these factors, the manufacturer or other test developer should evaluate the performance of the test in samples from three populations: persons infected with the disease, healthy persons without the disease, and ill people with diseases other than the one in question. Often the last group is not included in the evaluation; this could be the population for which a test is likely to be performed but show poor specificity, positive predictive value, efficiency, or all of these.

Remember that the "normal" reference interval varies with age and population, and the definition of multiple cut-off levels might be necessary. The manufacturer should designate how the recommended cut-off points were defined and in what population. Ideally, the laboratory should confirm the intervals and cut-off points in its own population(s). However, deviating from the package insert criteria should be done with caution.

11.3 Clinical Predictive Value

To the clinician, the predictive value of a test is often the most important parameter as long as the clinical relevance of the test is defined (e.g., "Does a positive test result predict disease, infection, or exposure?). It is therefore important to establish valid, accurate predictive values and to define what the test is predicting (e.g., disease vs. prior infection). The prevalence of a disease in a population profoundly affects the predictive value of a test result. (Prevalence represents the total number of cases per unit population; incidence is the number of new cases per year per unit population. These are often defined as number per 100,000 population.[32]) The effect of different prevalences is illustrated in Table 4 in the appendix. Predictive values should be based on a broad sample of the total population (all three groups of persons previously mentioned, in proportion to their representation in the total population).

11.4 Borderline Results

Values around the cut-off point chosen for reporting results as positive or negative are perhaps the most important range for which accurate predictive values should be established. It is important for the manufacturer and the clinical laboratory to establish criteria for further testing: repeat assay of the sample, assay of a second sample, assay of a sample obtained after several weeks or months, or testing by another method that is more sensitive and specific, if such a test is available. For example, borderline positive results in testing for antibodies to *Borrelia burgdorferi* might suggest obtaining a repeat sample in a few weeks or an alternative test, whereas a borderline positive result for antibodies to HIV might indicate western immunoblotting or another confirmatory test.

11.5 Evaluation of a Test

The common means of evaluating tests is by comparison with an alternative method for determining "disease present." This method may be clinical diagnostic criteria or another test, often a so-called "gold standard." In most cases, these standards are also imperfect, but the imperfection is neither obvious nor appreciated in the evaluation. As a result, sensitivity and specificity of a new test are usually underestimated or overestimated. Valenstein[33] and DeNeef [34] give examples of comparisons of tests to imperfect "gold standards"; Tables 2 and 3 in the Appendix demonstrate results when the tests are independent of each other and when the two misclassify the same patients, respectively. "Gold standards" should be selected carefully; for example, the use of culture plus bacitracin sensitivity to detect Group A beta-hemolytic streptococci is not as reliable as cultures plus actual typing of the organism. Therefore, the latter should be used to evaluate the sensitivity and specificity of immunochemical assays that are intended to detect the presence of this organism.[34]

In test evaluation, use specimens that are known to react like fresh samples in both (or all) methods. Ideally, evaluation should include prospective, fresh clinical samples tested in laboratories similar to those that will use the assay for clinical purposes.

Many rapid-detection tests are more sensitive than the traditional reference methods, such as viral or bacterial culture. Thus, the reference methods are more likely to give false-negative results, compared to the rapid tests, particularly in the presence of localized or loculated infections. The availability of other specific, but more sensitive, confirmatory tests can be crucial for adequate evaluation of new tests.

11.6 Reporting of Results

The manufacturer of test kits or reagents should give clearly stated guidelines for reporting of results. As mentioned previously, the recommended follow-up of borderline results should be clearly stated. The laboratorian and clinician should not accept tests that do not come with interpretive guidelines. Ultimately, however, the responsibility for reporting belongs to the clinical laboratory, which should interpret results in relation to the population being tested. The responsibility for clinical interpretation belongs to the clinician, who should interpret results in light of clinical information (see Section 12). A dialogue between the laboratory and the clinician is, obviously, crucial for the proper utilization of laboratory data.

Predictive values, if reported, should be based on the local prevalence of the disease in question. Reports should clearly indicate whether a test indicates a high likelihood of disease (or lack thereof) and/or the need for further testing. If further testing is already in progress, this should be indicated in the report.

The significance of the reported result should be clearly indicated. In most cases, the result should not be expressed simply as positive or negative, e.g., within the normal reference interval, borderline positive, or significantly elevated for the given population.

When relevant, the interpretation of results should include a brief presentation of the results of IgM and IgG antibody tests. One suggested scheme follows:

Table 1. Results of IgM and IgG Antibody Tests

IgM	IgG	Interpretation
Neg	Neg	Patient not exposed or too early
Pos	Neg or Pos	Acute or recent infection (< 7 days)
Neg	Pos	Immunity from past exposure, not acute disease

Adapted from Johnson RB, Nakamura RM. In Nakamura RM, Dito WR, and Tucker III, eds. *Immunoassays: Clinical Laboratory Techniques for the 1980's*. New York: Alan R. Liss: 1980:155. Copyright © John Wiley and Sons, Inc. Reprinted by permission of Wiley–Liss, Inc., a subsidiary of John Wiley and Sons, Inc.

12 Evaluation of Test Results (Also refer to NCCLS document EP12—*User Protocol for Evaluation of Qualitative Test Performance)*

In the interpretation of the results of immunologic tests for antigen or antibody, a positive result should not always be equated with clinical disease or the need for specific therapy. Positive results for some tests do strongly suggest infection; for example, detection of polyribose-ribitol phosphate in the serum of a pediatric patient virtually always reflects an active or partially treated *Haemophilus influenzae* infection, unless the patient has recently received the *H. influenzae* vaccine. In contrast, a positive assay for *Clostridium difficile* toxin does not always indicate that the patient has active antibiotic-associated colitis or that therapy is indicated to eradicate the organism, because asymptomatic colonization is common with this organism.[35-39] Similarly, detection of Group A streptococcal polysaccharide in a throat swab from a patient with no symptoms, or with a viral upper respiratory infection, does not always indicate acute streptococcal pharyngitis, but it can indicate a carrier state. Therefore, antigen-detection tests should usually be ordered only when clinical signs and symptoms are compatible with the specific infection under consideration, not as screening tests in patients with a low probability of disease. Even tests with high specificity will have a low positive predictive value if used indiscriminately.

In clinical interpretation, it is also important to consider what specific antigen (or related antibody) a test is detecting. In some cases, antigens have not been well characterized or do not reflect the presence of the virulence factor necessary for the organism to cause disease. For example, an immunodiagnostic test that did not distinguish between the invasive and the nonvirulent forms of *Entamoeba histolytica* would provide misleading clinical information and lead to unnecessary therapy. It is essential, in such cases, that the clinical picture be the final determination of whether therapy is needed.

13 Test Confirmation and Optimization

For a general discussion of immunoassay optimization and validation, the reader is referred to an article by JP Gosling (especially pages 1420 ff).[40]

13.1 Precision vs. Accuracy

The terms "precision" and "accuracy" are often confused, particularly by clinicians who are unfamiliar with laboratory terminology. Precision refers to the ability of a test to produce the same result on repeat testing of the same sample; it is usually expressed as the coefficient of variation (CV), which is a measure of imprecision—i.e., lower values reflect higher precision. In contrast, accuracy refers to the ability of a test to produce the correct result, as ascertained by a test with known accuracy. Bias refers to the degree of systematic inaccuracy of a test, whether positive or negative.

Obviously, a test can be either precise or accurate, both, or neither. The recommended accuracy and precision of a test for clinical decision making depend on the seriousness of the condition, the degree of separation of results obtained from affected and unaffected persons, and the like. The evaluation of a new test should include adequate documentation of both accuracy (and bias, if present) and precision at all expected levels of positivity, not just overall, pooled results.

13.2 Physiologic Dynamic Range

As a part of this testing, the physiologic, dynamic range (the range of values expected in the population of affected persons) should be established. Given the physiologic dynamic range, the manufacturer (and/or the clinical laboratory) should optimize the test to afford the desired levels of sensitivity and specificity (see Section 11). It is crucial that an informed decision be made about the number and types of samples, and the number of replicates of each sample, necessary to obtain this information.

13.3 Comparison of Quantitative Tests

The comparison of quantitative data from different test methods is commonly performed by simple linear regression, with a high correlation coefficient (*r*) taken to indicate comparability of results. This approach is fraught with difficulties, however. First, a non-zero intercept, which (if correct) suggests differences in behavior of the two assays, is usually ignored. Second, the *r* value is unduly influenced by the highest values; if testing includes a few very high results, a high *r* may be associated with poor comparison for the lower values more commonly seen in patient samples. Third, simple linear regression implies superiority of the method used as the independent variable. Deming regression can be used to overcome the last problem. However, more sophisticated approaches are necessary to overcome the others. The logarithmic transformation method of Finney,[41-43] which recommends similar behavior of samples at all levels (or of dilutions of samples) for colinearity, is one suggested approach. The correlation of results can be demonstrated visually in package inserts by scatter plots and/or residual plots covering the entire assay range, but without correlation coefficients.

For the comparison of similar reagents (e.g., different antigen preparations, calibrators, controls, etc.) within a test method, the functional linear relationship between the materials (rather than simple regression) is a simpler, but statistically better, approach. In this case, the plot of the test results for the two materials, assayed in multiple dilutions, should give a straight line with a zero intercept. The absence of linearity implies antigen or antibody excess at the extremes of the assay (i.e., the assay is not optimized for the full range of concentrations tested); the lack of a zero intercept, determined statistically, implies matrix or antigenic differences between the two materials.

13.4 Comparison of Qualitative Tests

In the comparison of qualitative tests, the previously mentioned methods are not useful. It is essential that identical, split samples be assayed by both tests, and that an adequate number of clinically positive, negative, and equivocal samples be included. The comparison of a new test with the “gold standard” is also essential; the practice of comparison only with tests previously shown to be "equivalent" can result in digression from accuracy. It is also essential that samples that might show cross-reactivity be tested (samples from patients with closely related diseases, with rheumatoid factor, with heterophilic antibodies, and the like).

13.5 Equivocal Results in Test Comparisons

The management of equivocal data from test comparisons is currently a matter of debate. Of particular concern is the question of whether the equivocal results should be included as false-positive results, false-negative results, or both in the data analysis. A decision about this point is beyond the purview of this

document. However, it is crucial that a manufacturer indicate the percentage of equivocal results to be expected in actual patient testing and how such results were interpreted in the data analysis used for product licensure (e.g., inclusion or exclusion in calculations of positive and negative predictive values, sensitivity and specificity, efficiency, etc.). If this information is not included in the package insert, the laboratorian should request it from the manufacturer. Obviously, a test that has a high percentage of equivocal results should be viewed with caution.

14 Product Clearance; Recommendations for Package Inserts

14.1 Clearance by the Appropriate Regulatory Organization

Product clearance by the appropriate regulatory organization does not guarantee test performance. (For example, in the U.S. the FDA does not approve tests[44]; it merely approves their sale, in most cases either after demonstration of efficacy in premarket approval [PMA] or license approval, or after demonstration of equivalence to a previously licensed test [510(k)]. In many cases, "grandfathered" assays have not been reviewed by the FDA, and lot-to-lot variability is not tested routinely. Thus, the individual test facility should validate test kits and new lots of previously used kits, using samples from appropriate patient populations [affected and unaffected], and accept liability for results reported.)

In general, reports in refereed scientific journals are preferred to manufacturers' literature and citations as informational sources about test performance. Even these should be read critically, however.

14.2 Recommendations for Manufacturers of Kits and Reagents

Manufacturers should adhere to the appropriate regulatory organization's (in the U.S., the FDA[45]) *in vitro* diagnostic labeling requirements, both for labels and for package inserts. Additional information should include the following:

- The intended use and limitations of a kit should be specified.

- Warnings and precautions should be given in detail in the required section of the package insert. If these considerations are significant, they should be emphasized by use of bold print, inclusion in a box, or the like.

- The specific analyte should be defined, as well as its specificity. The means used to determine this specificity should also be stated. In the case of assays for specific classes of immunoglobulins (e.g., IgG, IgM, or both), the manufacturer should indicate the specificity of the detecting antibodies (e.g., anti-μ chain, anti-F(c), etc.). The manufacturer should document adequate reactivity with all indicated classes (and subclasses for IgG) to be included; this is particularly important for IgM in combined-class assays. For assays of antigen, the exact antigen(s) recognized by the kit reagents should be listed when these are known.

- Assay procedures should be spelled out in detail, especially for critical factors, but they should be expressed in language that is easy to understand.

- Most clinical laboratories test specimens once. If, for the purposes of product clearance, the evaluation of an assay was performed in replicate, this should be so stated. The manufacturer should state when/if duplicate or repeat specimens should be assayed.

- If samples require (or can require) dilution, the material for dilution should be specified. In many cases, the dilution of a sample with buffers will change the matrix effects in an assay. If nonreactive

("negative") serum is to be used for this purpose, the manufacturer should either include such serum in the kit or make available serum that is certified as being nonreactive in the assay.

- Any known or reasonably expected interfering agents or conditions should be listed, with possible methods for detection or elimination of these when relevant.

- Precision (imprecision) should be specified individually for all levels of the assay range, especially at decision cut-off points. For a qualitative procedure, a CV of 50% at high levels of analyte can be acceptable, but this should be stated.

- Recommendations for interpretative reports should be given, along with recommendations for further testing or follow-up for samples at or near decision points (i.e., borderline results).

- The types of samples (and documentation of their validity) used for confirmation of the test should be stated in detail.

- Comparisons with previous assays should be presented as completely but clearly as possible (see Section 13). Assays should be compared with a widely accepted reference method. For quantitative tests, scatter plots and/or residual plots should be shown, with specimens covering the entire functional assay range.

- For qualitative tests, the method of dealing with equivocal results in test comparison, and the percentage of such results to be expected in clinical testing, should be specified.

- The sites chosen for clinical trials should include the entire span of proposed users–clinical and reference laboratories, plus private physicians' offices, if relevant. The manufacturer should conduct adequate, well-controlled studies to ensure that the assay will perform satisfactorily in all such sites. Each participating site should test the assay in a real patient setting with real patient specimens, including, if possible, both stored and fresh (prospective) samples. Specimens should include all proposed types to be used (e.g., urine, cerebrospinal fluid, serum, plasma, swabs, and the like).

- There should be an appropriate means for notification of any changes that might affect reagent or kit performance. Examples include changes in antigen or antibody source, in the detection system or the level of signal to be expected, in quality control parameters, and the like. Whenever such changes are made, a letter should be sent to the users and a brightly colored label (e.g., fluorescent pink) should be placed on the front of the package insert indicating where such changes have been made.

14.3 Recommendations for the Clinical Laboratory

- Whenever possible, data published in a refereed journal should be used, rather than the manufacturer's marketing data, in the evaluation of test kits and reagents. Even published data should be read critically.

- The use of monoclonal antibodies is not always advantageous (see Section 7.3) and should not be a major criterion for test acceptance. The type of antibody used is less critical than the overall performance of the assay.

- Kits should not be used to assay specimens other than those for which they are designed. Differences in matrix effects and expected levels of analyte can significantly affect the performance of the assay.

- The laboratorian should request information about the number of equivocal results to be expected in a patient population and whether the manufacturer included such results in the company's statistical analysis (see Sections 11.4 and 13.5).

- Whenever possible, the laboratory is encouraged to confirm the validity of an assay in its own patient population before accepting the assay for testing of clinical samples. If the manufacturer's claims cannot be duplicated, the reasons for this should be determined and corrections made before the assay is used for testing patient samples.

- If the manufacturer does not give data comparing the assay with a widely accepted reference method, the laboratory should request such data before using the assay.

- Laboratory manuals should be updated whenever the package insert is changed, whether for procedural reasons or for evaluation of results.

- Whenever the laboratory's procedure deviates from that recommended by the manufacturer, certification of acceptability of that deviation should be requested from the manufacturer. If this information is not available, the laboratory assumes full responsibility for the performance of the assay.

- The laboratory director should encourage communication between the laboratory personnel and clinicians about sample collection and handling, meaning of test results, and clinical outcome. In particular, equivocal results should be discussed with the clinician.

- The clinician should be encouraged to base therapeutic decisions on the patient's clinical status in addition to the laboratory test results. Discrepancies between the two can indicate a need for further confirmation of the laboratory results.

- The laboratory should inform the clinician of possible cross-reactivities and possible clinical or therapeutic interferences with the assay method. This information can usually be communicated via laboratory manuals for the clinician or the laboratory requisition, but in many cases it should be reported with the results of testing.

- Samples should be split before using harsh treatments, such as boiling; the remaining untreated sample should be stored for further studies as indicated.

- To minimize assay variation, paired serum samples for antibody titer (or other measurements of specific antibody concentration) should always be run at the same time. If the first ("acute") sample is to be run at the time of collection, a portion of the sample should be stored and rerun when the second ("convalescent") sample is assayed.

14.4 Recommendations for the Selection of Referral (Reference) Laboratories

Few clinical laboratories have the capability of performing even a small fraction of the rapidly increasing number of immunological tests for infectious diseases. Therefore, it is often necessary to send out specimens to referral (reference or commercial) laboratories. The principles outlined in this document should help clinical laboratory personnel evaluate the performance of tests performed by others. In general, it is appropriate to ask referral (reference or commercial) laboratories to answer the following questions about the tests they perform:

- Does the laboratory perform the test itself, or send it elsewhere? If it sends it out, to whom is it sent?

- Does the laboratory use a commercial product?

  * If so, which specific product is used? When this is determined, review the package insert.

  * If not, what method is used? By whom was it developed, and are references available for review? What is the quality of the reagents (see Section 7)?

- Is the test intended for screening, diagnosis, or confirmation (see Section 5.3)?

- What are the sensitivity, specificity, precision, and accuracy of the test (see Sections 11.2 and 13.1)? What is the cut-off value for a positive test result and how was it established? How are equivocal results handled?

- In which patient populations has the test been evaluated, and are these patients representative of the population served by the clinical laboratory referring the specimen?

- What are the known cross-reactions for the test?

- What are the quality control procedures for the test? Are positive and negative controls included in test runs? How often? What is the range of acceptable control values?

- If instrumentation is used, how often is it calibrated?

- What are the qualifications of the personnel performing the test and of the laboratory manager and director? Does the laboratory participate in a proficiency testing program? Is this specific test included? Is the laboratory accredited? By which agency?

- What specimen types are acceptable? Specifically, how should specimens be collected, stored, and transported?

- Are residual specimens saved in case retesting is necessary? For how long?

- What is the turnaround time?

- What is the fee?

(For more information, refer to NCCLS document GP9—*Selecting and Evaluating a Referral Laboratory*.)

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Appendix. Interpretation of Laboratory Tests—Mathematical Concepts

Table A1. Calculation of Sensitivity, Specificity, and Predictive Values for an Assay

Test Being Evaluated	Disease Status		
	Number with Disease	**Number without Disease**	**Totals**
Number with (+) test Results	True (+) (TP)	False (+) (FP)	TP + FP
Number with (-) test Result	False (-) (FN)	True (-) (TN)	FN + TN
Totals	TP + FN	FP + TN	TP + FP + FN + TN

TP = The number of persons with the disease correctly identified by the test.

FP = The number of persons without the disease incorrectly classified by the test.

TN = The number of persons without the disease correctly classified by the test.

FN = The number of persons with the disease incorrectly classified by the test.

Sensitivity (the percentage of positivity in patients with disease)

$$\frac{TP}{TP + FN} \cdot 100$$

Specificity (the percentage of negativity in patients without disease)

$$\frac{TN}{TN + FP} \cdot 100$$

Predictive value of a positive test result

$$\frac{TP}{TP + FP} \cdot 100$$

Predictive value of a negative test result

$$\frac{TN}{TN + FN} \cdot 100$$

Efficiency (represents the percentage of all results that are true results)

$$\frac{TP + TN}{TP + FP + TN + FN} \cdot 100$$

Appendix (Continued)

Table A2. Effect of the Use of an Imperfect Standard Test for Comparison to a New Test

	Disease Status Imperfect Standard Test		*Disease Status* New Test	
Test Results	**Present (+)**	**Absent (-)**	**Present (+)**	**Absent (-)**
(+)	94	22	98	18
(-)	46	838	2	882

	Perceived Values for New Test	**True Values for New Test**
Sensitivity (TP/[TP+FN])	67.1%	98.0%
Specificity (TN/[TN+FP])	97.4%	98.0%
Predictive value – Positive	81.0%	84.5%
Predictive value – Negative	94.8%	99.8%

Table A3. Test Evaluation When the Test and the Standard Make Similar Errors

	Disease Status Imperfect Standard Test		*Disease Status* New Test	
Test Results	**Present (+)**	**Absent (-)**	**Present (+)**	**Absent (-)**
(+)	175	45	85	135
(-)	5	775	15	765

	Perceived Values for New Test	**True Value for New Tests**
Sensitivity (TP/[TP+FN])	97.2%	85.0%
Specificity (TN/[TN+FP])	94.5%	85.0%
Predictive value – Positive	79.5%	38.6%
Predictive value – Negative	99.3%	98.1%

Appendix (Continued)

Table A4. Effect of Prevalence on a Test with 95% Sensitivity and Specificity

Test Result	*Disease Status (10%)* **Imperfect Standard Test**		*Disease Status (1%)* **New Test**	
	Present (+)	Absent (-)	Regent (+)	Absent (-)
(+)	9,500	500	950	50
(-)	4,500	85,500	4,950	94,050

Sensitivity (TP/[TP+FN])	95%	95%
Specificity (TN/[TN+FP])	95%	95%
Predictive value – Positive	67.9%	16%
Predictive value – Negative	99.4%	99.9%

NCCLS consensus procedures include an appeals process that is described in detail in Section 9.0 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Committee Responses

I/LA18-A: *Specifications for Immunological Testing for Infectious Diseases; Approved Guideline*

General

Section 3 and Section 13.1

1. There are some minor inconsistencies in the use of certain terms in this document from those defined in other NCCLS documents: 1) accuracy…NCCLS, inaccuracy; 2) precision…NCCLS, imprecision.

- **The area committee reviewed and revised the terminology in Section 13.1 to be consistent with NCCLS document NRSCL8 – *Terminology and Definitions for Use in NCCLS Documents.***

Section 4.3.6

2. Are the authors implying that all samples be adsorbed or have IgG removed before IgM testing? If so, it is not clear.

- **It is not being implied that all samples be adsorbed.**

Section 7.5.1

3. References 23, 24, and 25 seem very impractical.

- **The area committee believes that these references do not detract from the document, but provide additional resources to the user.**

Section 7.6.3

4. $F(ab')_2$ reference 20 seems very impractical.

- **See response to Comment 3.**

Sections 11.4, 11.6, and 14.2

5. It is wise to define 'borderline positive.' In our understanding, "borderline" means not positive and not negative – and thus the test has to be repeated or the result verified or extended by a confirmatory assay. The same question arises on page 28 in the first paragraph for the interpretation of a borderline positive result.

- **The area committee agrees and has added the definition for "borderline positive" to Section 3. The committee also deleted the last sentence in Section 11.4.**

Section 13

6. Test Confirmation & Optimization, the sentence "Remember that increasing numbers of anomalous results…." is a perhaps excessively elaborate way of stating the fact that with time and experience the true utility and limitations of any assay become apparent.

- **The area committee agrees and has deleted the last two sentences in Section 13.**

Section 14.4

7. I believe the name for the type of laboratory being talked about is a REFERRAL laboratory (refer to NCCLS document GP9) and NOT reference.

- **The commenter is correct that NCCLS document GP9 – *Selecting and Evaluating a Referral Laboratory* uses the term "referral." The subcommittee that developed GP9 believes that "reference" laboratory can be perceived as a laboratory that performs high– powered esoteric tests normally not performed by the referring laboratory. The subcommittee wanted to use a term that would be more inclusive, also accommodating the provision of routine testing and backup services. The subcommittee decided to use the term "referral laboratory," believing it to be more inclusive. For consistency in NCCLS documents, "referral" was incorporated and "reference" was placed in parentheses in Section 14.4.**

Related NCCLS Publications*

DI2-A2 **Immunoprecipitin Analyses: Procedures for Evaluating the Performance of Materials—Second Edition; Approved Guideline (1993) (Reaffirmed 1999)**. This document gives a description of, and procedures for, evaluating the performance of materials used in immunoprecipitin analysis, including a discussion of specificity.

DI3-A **Agglutination Analyses: Antibody Characteristics, Methodology, Limitations, and Clinical Validation; Approved Guideline (1993) (Reaffirmed 1999).** This document offers guidelines that describe specificities of antibodies and their required potency, labeling information, and characteristics and limitations of agglutination methods.

EP12-P **User Protocol for Evaluation of Qualitative Test Performance; Proposed Guideline (2000).** This document contains a protocol that optimizes the experimental design for the evaluation of qualitative tests, to better measure performance and provide a structured data analysis.

GP9-A **Selecting and Evaluating a Referral Laboratory; Approved Guideline (1998).** This guideline provides an outline of reasons and criteria for choosing a referral laboratory. A checklist for evaluating potential referral laboratories is included to assist in the decision process.

GP16-A **Routine Urinalysis and Collection, Transportation, and Preservation of Urine Specimens; Approved Guideline (1995).** This guideline describes routine urinalysis test procedures that address materials and equipment, macroscopic examinations, clinical analyses, and microscopic evaluation.

H3-A4 **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard —Fourth Edition (1998).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. It also includes recommendations on order of draw.

I/LA2-A **Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline (1996).** This document offers guidelines for the development of reference sera of defined antibody specificity to ANA and standardization of the immunofluorescent test for ANA.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** A consolidation of M29-T2 and I17-P, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

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

Related NCCLS Publications (Continued)

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Guideline (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory.

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