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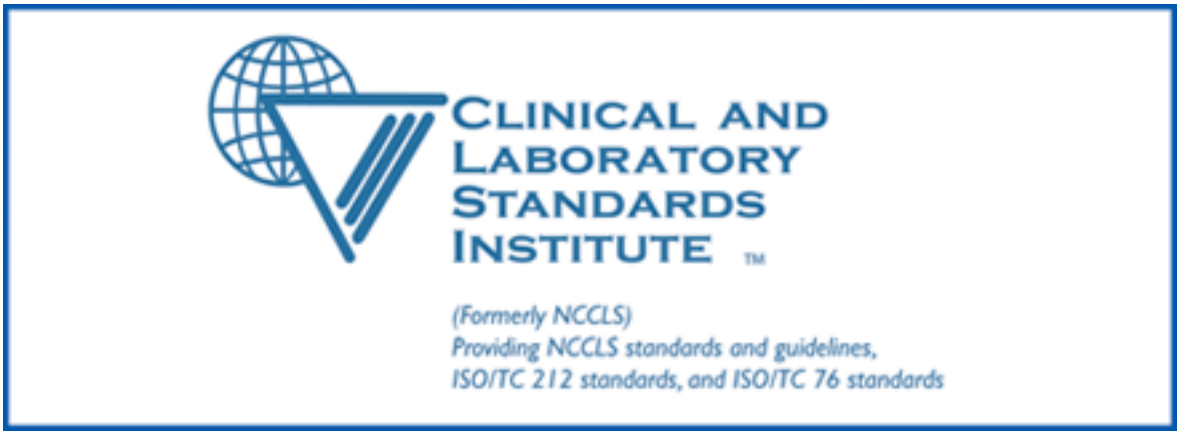
Assessing the Quality of Immunoassay Systems: Radioimmunoassays and Enzyme, Fluorescence, and Luminescence Immunoassays; Approved Guideline



This guideline addresses components for harmonizing and assessing the quality of immunoassay systems for several commonly used dose-response indicator categories, e.g., radioisotopes, enzymes, fluorescence, luminescence, reagents, and experimental components criteria essential to characterizing an immunoassay.

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

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Assessing the Quality of Immunoassay Systems: Radioimmunoassays and Enzyme, Fluorescence, and Luminescence Immunoassays; Approved Guideline

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Abstract

NCCLS document I/LA23-A—*Assessing the Quality of Immunoassay Systems: Radioimmunoassays and Enzyme, Fluorescence, and Luminescence Immunoassays; Approved Guideline* addresses components for harmonizing and assessing the quality of immunoassay systems for several commonly used dose-response indicator categories, (e.g., radioisotopes, enzymes, fluorescence, luminescence, reagents, and experimental components criteria) essential to characterizing an immunoassay.

The Area Committee on Immunology and Ligand Assays merged NCCLS documents LA1-A2—*Assessing the Quality of Radioimmunoassay Systems; Approved Guideline—Second Edition* and DI4-T—*Enzyme and Fluorescence Immunoassays; Tentative Guideline* into one document assimilating the residual segments of LA1-A2, and updating information in DI4-T into a more generic model, along with the addition of new information for each topic. I/LA23-A has broader utility and applicability while providing resource information previously available in the other two documents.

This new guideline describes the iterations in the development, performance characterization, and certification from sample collection to method transferability. Specific nuances of each of the different dose-response systems for immunoassays are addressed while placing emphasis on mechanisms to assess the quality of the different immunoassay systems—factors that contribute to reliable and reproducible results. This guideline is particularly useful for specific details on optimization and harmonization of immunoassays, especially for those measurands (analytes) that are measured only by quantitation of antigen-antibody reactions (e.g., protein hormones, IgG, serum specific proteins).

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Committee Membership

Area Committee on Immunology and Ligand Assay

Acknowledgement

The Area Committee on Immunology and Ligand Assays would like to extend its appreciation to Jeffrey Warren, M.D. (University of Michigan) and Marvin Goldsmith (Goldsmith Graphics), past committee members, for their contributions. We also recognize the contributions of Peter E. Maxim, Ph.D. (deceased), a past committee member representing the Food and Drug Administration. These individuals contributed substantially to the development of this important NCCLS protocol.

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Foreword

The intended audience for I/LA23-A—*Assessing the Quality of Immunoassay Systems: Radioimmunoassays and Enzyme, Fluorescence, and Luminescence Immunoassays; Approved Guideline*, is manufacturers of assay reagents and kits, regulatory and accrediting bodies, and scientists and healthcare professionals that develop and apply immunoassays for a variety of analytical purposes. The purpose of this guideline is to improve the quality and performance of immunoassays and to enhance laboratory and product comparability by promoting a better understanding of the requirements, capabilities, and limitations of these tests. Immunoassays are unique tests using antibodies of defined specificity to measure analytes. Each assay configuration and detection system has advantages and disadvantages. An understanding of the specific application is essential to assay production and use. The range of applications for immunoassays is extensive. The degree of variations in configurations is large and may involve a hierarchy of antibodies used with different specificities for capture, separation, measurement, and dose amplifications.

A comprehensive coverage of the field of immunoassays is too large for the scope of this document. The area committee, during development of this guideline, focused on the core quality management issues. For detailed information, other publications cited in the general references should be consulted. I/LA23-A replaces NCCLS documents LA1-A2—*Assessing the Quality of Radioimmunoassay Systems; Approved Guideline—Second Edition* and D14-T—*Enzyme and Fluorescence Immunoassays; Tentative Guideline*.

A Note on Terminology

NCCLS, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. Despite these obstacles, NCCLS recognizes that harmonization of terms facilitates the global application of standards and is an area that needs immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In keeping with NCCLS's commitment to align terminology with that of ISO, the following describes the metrological terms and their uses in I/LA23-A:

The term a*ccuracy* refers to the "closeness of the agreement between the result of a (single) measurement and a true value of a measurand" and comprises both random and systematic effects. *Trueness* is used in this document when referring to the "closeness of the agreement between the average value from a large series of measurements and to a true value of a measurand"; the measurement of trueness is usually expressed in terms of *bias. Precision* is defined as the "closeness of agreement between independent test/measurement results obtained under stipulated conditions." As such, it cannot have a numerical value, but may be determined qualitatively as high, medium, or low. For its numerical expression, the term *imprecision* is used, which is the "dispersion of results of measurements obtained under specified conditions." In addition, different components of precision are defined in ILA23-A, primarily *repeatability,* i.e., "the closeness of the agreement between results of successive measurements of the same measurand carried out under the same conditions of measurement"; while *reproducibility* describes the "closeness of agreement of results of measurements under changed conditions."

The NCCLS Harmonization Policy recognizes ISO terms as the preferred terms. When appropriate, alternative terms are included parenthetically to help avoid confusion.

The term *measurand* (a particular quantity subject to measurement) is used in combination with the term *analyte* (component represented in the name of a measurable quantity) when its use relates to a biological fluid/matrix; and the term *measuring range* in combination with *reportable range* when referring to "a set of values of measurands for which the error of a measuring instrument (test) is intended to lie within specified limits."

The term *diagnostic sensitivity* is combined with the term *clinical sensitivity*, and correspondingly the term *diagnostic specificity* is combined with the term *clinical specificity*, because in Europe, the term "clinical" often refers to clinical studies of drugs under stringent conditions.

Users of I/LA23-A should understand, however, that the fundamental meanings of the terms are identical in many cases, and to facilitate understanding, terms are defined in the Definitions section of this guideline.

All terms and definitions will be reviewed again for consistency with international use, and revised appropriately during the next scheduled revision of this document.

Key Words

Antibody, assessment, enzyme immunoassay, fluorescence immunoassay, fluorescence system, heterogeneous immunoassay, homogeneous immunoassay, labeling, performance evaluation, quality control, radioimmunoassays, reference materials, separation systems

Assessing the Quality of Immunoassay Systems: Radioimmunoassays and Enzyme, Fluorescence, and Luminescence Immunoassays; Approved Guideline

1 Scope

This document presents guidelines for immunoassays of macromolecular analytes. The factors likely to be important in achieving reliable and reproducible results are emphasized. Use of this document should promote greater reliability and comparability in immunoassay results.

The definitions, information, and procedures necessary to properly assess the quality of immunoassay systems are described. Awareness of the evaluation process allows laboratory personnel to better assess systems that meet the specific needs of the patient population.

Immunoassays are widely used to quantitate specific measurands (analytes) in complex mixtures such as clinical samples. Immunoassays using enzymes or fluorescers as labels are recent developments. Enzyme immunoassays (EIA), fluorescence immunoassays (FIA), and luminescence immunoassays (LIA) were developed to provide a simple, sensitive immunoassay technique that does not use unstable and potentially dangerous radioisotopes. At present, enzyme, fluorescence, and luminescence immunoassays are typically less sensitive than radioimmunoassays (RIA). However, high sensitivity is not necessary in many applications, and there are reasons to expect that sensitivity comparable to radioimmunoassay can and will be achieved by EIA and FIA in the near future. There are no criteria on whether RIA, EIA, FIA, or LIA is the best method for a particular analyte measurement. When radioisotopes cannot be used or when radioisotope decay counters are not available, techniques such as EIA, FIA, or LIA are obligatory. In practice, EIA, FIA, and LIA systems have exhibited other advantages, including high specific activity, reagent stability, and applicability to simple instrumentation. Immunoassays using luminescent technologies are now among the most sensitive, with analytical detection limits as low as one zeptomole (10^{-21} moles).

2 Introduction

Immunoassays have become essential tools for the analytic operation of clinical diagnostic and research laboratories. Numerous advances in immunoassay techniques continue to drive new technologies, especially for application to research in proteomics and the human genome: highly sensitive dose-response indicators, methods for reduction in nonspecific binding and background signal, simultaneous analyte measurements, improved automation, and miniaturized analytic systems.

At the scheduled review of several immunoassay documents, the Area Committee on Immunology and Ligand Assay decided to develop one new document rather than expand the older ones. The area committee combined the most relevant parts of these existing documents on radioimmunoassay and enzyme and fluorescence immunoassays. A new section for luminescence was added to reflect its popularity and wide use by manufacturers of automated instruments. The sections on antigen-antibody components, sample requirements, quality assurance, and assay performance were enhanced for improved utility of the guideline for developers and users of immunoassays. Also, the sections on antibody components were provided in greater detail, because the antibody is probably the most important element in the development and performance of a high-quality and low-bias immunoassay. This guideline will provide information critical to the understanding of immunoassays to the manufacturer, the researcher, and the healthcare professional.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are 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 the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

4 Terminology

4.1 Definitions

Accuracy – Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93)[1]; **NOTES:** a) "Accepted reference value" may be used in place of "true value"; See **Trueness**, below.

Activity (of a radioactive material) – The number of radioactive transitions taking place in a sample per unit time; **NOTE:** See **Specific activity.**

Adjuvant – A substance admixed with an immunogen to elicit a more marked immune response (RHUD1.7CD).[2]

Affinity – **1)** The force by which atoms, ions, molecules, prosthetic groups, and particles are attracted or held together in chemical compounds; **2)** ***In Immunology***, a measure of the attraction or force of association between a single antigenic site and a single antibody to that site.

Analyte – Component represented in the name of a measurable quantity; **NOTES:** a) In the type of quantity "mass of protein in 24-hour urine," "protein" is the analyte. In "amount of substance of glucose in plasma," "glucose" is the analyte. In both cases, the long phrase represents the **Measurand** (ISO 17511)[3]; b) In the type of quantity "catalytic concentration of lactate dehydrogenase isoenzyme 1 in plasma," "lactate dehydrogenase isoenzyme 1" is the analyte. **(**ISO 18153)[4]; c) In this document, the term **Analyte** is combined with the term **Measurand** when its use relates to a biological fluid/matrix.

Analytical method – Set of operations, described specifically, used in the performance of particular measurements according to a given method (VIM93)[1]; **NOTE:** The term **Analytical method** (U.S.) is equivalent to **Measurement procedure** (Europe).

Analytical specificity – Ability of a measurement procedure to determine solely the measurable quantity it purports to measure; **NOTES:** a) *In quantitative testing*, the ability of a measurement procedure 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; b) *For qualitative or semiquantitative tests,* the method's ability to obtain negative results in concordance with negative results obtained by the reference method; c) *In Immunology*, specificity is an antiserum quality defining its reactivity with defined antigens and lack of specificity is the inaccuracy introduced by cross-reacting and/or interfering substances, because cross-reacting substances compete with the analyte for antibody-binding sites.

Antibody – Specific immunoglobulin formed by B lymphocytes in response to exposure to an immunogenic substance and able to bind to this; **NOTE:** The molecule of an immunogenic substance contains one or more parts with a characteristic chemical composition, an epitope.

Antigen – Any substance that can stimulate the production of antibodies by an organism and combine specifically with them.

Antiglobulin – An antibody produced by an animal in reaction to the introduction of globulin from an animal (RHUD1.7CD).[2]

Assay – **1)** A quantitative determination or measurement of the amount, activity, or potency of a constituent or characteristic; **2) Qualitative assay** - Reports only the presence or absence of the analyte, without quantification; **3) Quantitative assay** - Generates a spectrum of signal responses that correlate with the concentration of the analyte of interest; **NOTE:** If the analyte preparations with known concentrations are available for calibration, the actual concentration of the analyte can be determined; **4) Semiquantitative assay** - Essentially a qualitative assay with an additional option for the response range (degree of positivity, dilution to which positive results are obtained, or comparison to a color chart).

Avidity – The net affinity of all binding sites of all antibodies in the antiserum, under specified physicochemical reaction conditions. **NOTES:** a) 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; b) See **Affinity.**

Bias – Difference between the expectation of the test results and an accepted reference value; **NOTES:** a) Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value; b) The measure of **Trueness** is usually expressed in terms of bias. (ISO 3534-1)[5]

Calibrator – Reference material whose value is used for the independent variable in a calibration function.

Clinical sensitivity – The proportion of patients with a well-defined clinical disorder whose test values are positive or exceed a defined decision limit (i.e., a positive result and identification of the patients who have a disease); **NOTES:** a) It is the fraction of clinically true positive classification divided by the sum of clinically true positive and clinically false negative; b) The clinical disorder must be defined by criteria independent of the test under consideration; c) The term **Clinical sensitivity** (U.S.) is equivalent to **Diagnostic sensitivity** (Europe).

Clinical specificity – The proportion of subjects who do not have a specified clinical disorder and whose test results are negative or within the defined decision limit; **NOTES:** a) It is the fraction of clinically true negative classifications divided by the sum of clinically true negative plus clinically false positive classifications; b) The term **Clinical Specificity** (U.S.) is equivalent to **Diagnostic specificity** (Europe).

Competitive assay – An assay based on the competition of labeled and unlabeled analytes for a receptor.

Cross-reactivity – ***In Immunology***, 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.

Cut-off values – The quantitative value of a measured analyte that is used to decide whether the result is considered above or below a clinical or analytical decision point (usually positive or negative).

Detection limit//Limit of detection – The lowest amount of analyte in a sample which can be detected but not quantified as an exact value (WHO-BS/95.1793).[6]

Dynamic range – The total span over which an analysis can provide results; **NOTES:** a) Analytically, the functional range of an assay over which concentrations of an analyte can be measured with accuracy and precision; b) Physiologically, the full range of analyte levels to be expected in patient samples.

Efficiency – ***In Immunoassay,*** the percentage (number fraction multiplied by 100) of results that are true results, whether positive or negative.

Epitope – Any site on an antigen molecule at which an antibody can bind; the chemical structure of the site determining the specific combining antibody.

False-negative result – A negative test result for a patient or specimen that is positive for the condition or constituent in question.

False-positive result – A positive test result for a patient or specimen that is negative for the condition or constituent in question.

Fluorescer//fluophor – A substance that fluoresces when excited by electromagnetic radiation.

Gold standard – A nonspecific term that indicates that a process or material(s) is the best available approximation of the truth.

Heterogeneous immunoassay – An immunoassay that requires the physical separation of free labeled antigen (or antibody) from the labeled antigen (or antibody) bound in an immune complex, prior to measurement of the quantity of label.

Heterophilic antibodies – See **Antibody**.

Heterospecificity – Describes an antiserum with reactivity against a variety of antigens.

Homogeneous immunoassay – An immunoassay that requires only the mixing of a sample (analyte) and immunochemical reagents (antibodies or antibody conjugates) with no wash step(s) to disturb the binding equilibrium before the bound fraction is measured; **NOTE:** The measurand (analyte) must produce a detectable dose-response signal upon binding that distinguishes it from unbound analyte.

Idiospecificity – A characteristic that describes the reactivity of an antiserum with a unique subset of a broad antigen class.

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

Imprecision – Dispersion of independent results of measurements obtained under specified conditions; **NOTE:** It is expressed numerically as standard deviation or coefficient of variation.

Isotype – An immunoglobulin heavy or light chain class or subclass characterized by antigenic determinants in the constant region.

Lumophore – A molecule capable of emitting a luminescent response, or light measured as photons, upon appropriate excitation.

Measurand – Particular quantity subject to measurement (VIM93)[1]; **NOTES:** a) This term and definition encompass all quantities, while the commonly used term "analyte" refers to a tangible entity subject to measurement. For example, "substance" concentration is a quantity that may be related to a

particular analyte; b) In this document, the term **Analyte** is combined with the term **Measurand** when its use relates to a biological fluid/matrix.

Negative predictive value (NPV) – The likelihood that an individual with a negative test result does not have the disease, or other characteristic which the test is designed to detect; **NOTE:** This varies with prevalence of the disease unless the test is 100% sensitive.

Noncompetitive assay – An immunoassay in which the analyte (antigen) is captured by the receptor (or first antibody), and a second labeled antibody is used to measure the amount of analyte bound or captured by the binding site.

Positive predictive value (PPV) – The likelihood that an individual with a positive test result has a particular disease, or characteristic, which the test is designed to detect; **NOTE:** This varies with prevalence of the disease unless the test is 100% specific.

Postzone effect – The increased solubility of immune complexes resulting from the presence of a marked excess of antigen in relationship to antibody concentration; **NOTE:** See also **Prozone effect.**

Potency – ***In Immunologic Testing,*** **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 – Closeness of agreement between independent test results obtained under stipulated conditions; **NOTES:** a) Precision depends only on the distribution of random errors and does not relate to the true value or the specified value; b) The measure of precision usually is expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation. (ISO 3534-1).[5]

Prevalence – The extent of occurrence expressed as a fraction of the numbers affected by the disease or condition compared to the total number of members in the specified group.

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

Quantity (measurable) – Attribute of a phenomenon, body, or substance that may be distinguished qualitatively and determined quantitatively); **NOTE:** The term "quantity" may refer to a quantity in a general sense (length, time, mass, etc.) or to a particular quantity (volume of a given beaker, amount of substance concentration of glucose in a given sample of blood plasma) (VIM93)[1]; See **Analyte, Measurand.**

Quencher – Any molecular species that reduces the radiance measured from an emitting molecular species (generally applied to fluorescence emission).

Reference value (accepted) – A value that serves as an agreed-upon reference for comparison and which is derived as: a theoretical or established value based on scientific principles; an assigned value based on experimental work of some national or international organization; or a consensus value based on collaborative experimental work under the auspices of a scientific or engineering group (ISO 5725-1).[7]

Repeatability (of results of measurements) – Closeness of the agreement between results of successive measurements of the same measurand carried out under the same conditions of measurement (VIM93).[1]

Reportable range – A set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits; **NOTE:** In this document, the term **Reportable range** (U.S.) is equivalent to **Measuring range** (Europe).

Reproducibility (of results of measurements) – Closeness of the agreement between the results of measurements of the same measurand carried out under changed conditions of measurement (VIM93).[1]

Sensitivity – In quantitative testing, the change in response of a measuring {system or} instrument divided by the corresponding change in the stimulus (modified from VIM93)[1]; **NOTE:** In the context of QC, the power of error detection of a QC system.

Specific activity – A measure event per unit mass per unit time; **NOTE:** See **Activity.**

Specificity – The ability of a measurement procedure to measure solely the measurand; **NOTES:** a) Specificity has no numerical value in this context; b) See **Measurand**.

Titer – **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.

Tracer – A radiolabeled analyte for use in dose-response for radioimmunoassay; **NOTE:** The production of the tracer may use the replacement of one or more atoms in the analyte with a radioisotope or the covalent labeling of the analyte with an isotope tag, most commonly ^{125}I.

Trueness – The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1)[5]; **NOTE:** See also **Accuracy, Bias**.

Turnover number – The number of substrate molecules converted by an enzyme to product molecules per unit time when the enzyme is saturated with substrate (under optimum conditions).

4.2 Acronyms and Abbreviations

ABTS	diammonium salt of 2, 2′ -azino-di(3-ethlybenzthiazoleine-6-sulfonate)
AMPPD	adamantly 1,2-dioxetane phenyl phosphate
CPM	counts per minute
CV	coefficient of variation
EMIT	Enzyme multiplied/mediated immunoassay technique
EIA	Enzyme immunoassays [also known as ELISA (Enzyme-linked immunosorbent assay)]
EU	European Union
FDA	Food and Drug Administration
FIA	fluorescent immunoassay
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IMA	immunometric assay
IRP	international reference preparation
LIA	ligand immunoassay
MOPC	mineral oil induced plasmacytoma
NADH	nicotinamide adenine dinucleotide with hydrogen
NIST	National Institute of Standards and Technology
OPD	o-phenylenediamine
QC	quality control

RIA	radioimmunoassay
SRM	standard reference material
TMB	3, 3′, 5, 5′ - tetramethylbenzidine
^{3}H	tritium
WHO	World Health Organization

5 Primary Components for Immunoassays

5.1 Antibody

The principle function of the antibody is to specify and aid in the quantification of the analyte being measured. 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 usually are associated with higher sensitivity of the tests derived from such antibodies; however, in some cases, high affinity can be associated with reduced specificity. Therefore, the antibodies used in a particular immunoassay should be matched to the criteria necessary for the performance of the particular method.

5.1.1 Monoclonal and Polyclonal Antibodies

Two general methods exist for production of antibodies:

- *in vitro* hybridization of antibody-secreting B-cells obtained from the spleen of an antigen-immunized mouse with myeloma cells (e.g., mineral oil induced plasmacytoma (MOPC)—an immortalized tissue-culture cell line) followed by cloning and continuous cell culture (i.e., monoclonal antibodies);

- immunization of an animal with an antigen followed by the subsequent collection and purification of serum (i.e., polyclonal antibodies).

Monoclonal antibodies can be powerful immunochemical tools due to the attributes of 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.

5.1.2 Immunogen

By its definition, an immunogen is any substance that can elicit an immune response. For the purpose of designing an immunoassay, the immunogen will generally be the chosen analyte itself or a substance sharing critical structural features with the analyte. For optimal reagent development, the immunogen will preferably be pure and well characterized. Methods to demonstrate purity and characterization include:

- application of a high-resolution analytical technique such as polyacrylamide gel electrophoresis
- measurement of the specific activity (e.g., enzyme-specific activity)
- comparison of assay results using an assay specific for the immunogen (e.g., an immunoassay) and an assay for total mass (e.g., total protein assay)
- immunochemical characterization using techniques such as immunoprecipitation, immunodiffusion, or immunoelectrophoresis. Several types of antisera are useful in this context, including:
  - a known, specific antibody directed against the immunogen
  - antibody(s) specific for likely contaminant(s)
  - antibody raised against a pool of samples of the same character as the designated assay samples
  - antibody raised against the source material from which the immunogen was purified.

5.1.3 Production

The following immunization and collection parameters are important in antibody production:

- form, nature, and storage for the immunogen
- use of adjuvant
- quantity of immunogen and adjuvant
- immunization schedule
- species of immunized animal
- location of injection sites
- bleeding schedule and technique for withdrawal (e.g., blood removal, plasmapheresis).

For the production of monoclonal antibodies, the following methods are also important:

- immunization
- hybridization
- cloning
- antibody production.

5.1.4 Purification and Processing

Serum is the preferred choice for the antibody preparation and may be obtained from plasma by standard procedures. Some minimal processing is required and includes:

- filtration or centrifugation to remove cells, particulate matter, and lipids;

- preservation against:
  — bacterial growth through addition of antibacterial agent
  — thermal denaturation by storing at low temperature or freezing
  — proteolysis through the addition of agents that inactivate proteases.

It may also be necessary to process the antibody to remove specific interferences or an undesired antibody fraction. This processing may involve:

- isolation of the gamma-globulin fraction or an IgG fraction by removal of other proteins

- removal of immune complexes present naturally, or remaining after absorption of antibodies of undesirable specificity.

In other situations, it may be necessary to alter the specificity of the antibody preparation. Examples of such alterations include:

- blending of antibody preparations to change their epitopic specificity

- removal of antibodies of undesirable specificity by their absorption with soluble antigen or by absorption to a solid phase antigen. The latter method is preferable, since it does not add free antigen to the preparation and leaves no soluble immune complexes that can subsequently interfere with measurement of analyte.

5.1.5 Characterization

5.1.5.1 Species of Antiserum Production

Indicate the animal species in which antisera were raised.

5.1.5.2 Titer

In general, the titer of an antibody is less important than its specificity, provided that the titer lies within the range of values that permit the preparation of useful reagents. In certain situations, it may be necessary to raise or lower the titer of an antibody preparation through concentration or with the addition of nonimmune material.

5.1.5.3 Immunoglobulin Classes and Subclasses

Manufacturers of antibodies (and kits that utilize them) should indicate the form(s) of antibody supplied with immunoassay (e.g., IgM, IgG). The manufacturer should also document reactivity with the isotype and subclass of immunoglobulins (e.g., anti-μ or anti-ν chain or anti-F(c) fragment) for the reagent, for kits designed to identify immunoglobulins (either a single class or subclass of antibodies). The lack of reactivity with light chains must be documented. In addition, document that antibodies to IgG react with all subclasses unless one or more subclasses are not important in detection of disease or immune response.

5.1.5.4 Reporting of Cross-Reactivity and Interference

Indicate known assay cross-reactivities in the package insert. Provide measures to counteract interference 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.

5.1.5.5 Specificity

Conduct specificity studies in the presence of the measurand (analyte) in biological samples. A test for undesired functions, such as cross-reactivity, should be at least as sensitive as the actual immunoassay. Ideally, determine the level of cross-reactant needed to change the assay response by a stated amount throughout the normal analyte range. Many interfering substances do not give response curves parallel to that of the analyte.

Antibody specificity tests commonly include investigations for the following:

- undesirable cross-reaction with endogenous substances likely to be present in the sample
- interference by exogenous factors (e.g., drugs) likely to be present in the sample
- impact of common pathological states, physiological states, and sample mishandling that might alter the specificity
- interference by related but inactive metabolites.

5.2 Antigen

An important difference exists in operational definitions between an immunogen (see Section 5.1.2) and an antigen, with the latter being a substance capable of binding to a specific antibody. Whereas all immunogens are antigens, not all antigens are immunogenic. In the design of an immunoassay, antigens are used as reagents in the assay system and as compounds to develop the antibodies that serve as the source for immunodetection. Depending on the purpose of the assay, the antigen reagent may be composed of discrete molecules (e.g., purified proteins) or a defined complex of molecules (e.g., virus).

5.2.1 Source and Purification

Antigens may be obtained from a natural source or prepared from an *in vitro* system. To obtain antigen that is representative of all variants, a pool of materials may be required.

Unwanted or nonspecific reactivity in immunoassays can result from the presence of contaminant materials in antigen preparations. In general, the more highly purified the antigen, the smaller potential for contaminant reactivity, and the better the performance in the assay. For direct detection of contaminants in an antigen preparation, analyze several concentrations of the preparation using sieving-gel electrophoresis, isoelectric focusing, two-dimensional electrophoresis, or similar methods. Also, use a sensitive staining method, such as direct silver staining or immunoblotting, followed by a sensitive detection method.

Choose the purification techniques to prevent antigenic changes caused by the removal or destruction of some particular antigen or changes in the physical form of the antigen (e.g., proteolytic digestion, degree of glycosylation, dissociation, aggregation, denaturation, small molecule binding) that may be relevant to assay performance.

Purification of antigens from a microbiological system (e.g., viruses grown in tissue culture) requires the following additional considerations:

- Contaminating microorganisms must be excluded from the tissue culture so that their presence does not complicate purification.

- Harvest the antigens using a technique that minimizes contaminants from the culture and culture medium.

- If a crude or partially purified antigen (e.g., virus preparation) is used, reactivity caused by contaminants can be partially addressed by using a control antigen. A control antigen is prepared from a culture (as well as the control sample) not infected with antigen microorganism but one that is otherwise identical in composition and purification protocol. Immunological reactivity with contaminants can be accounted for by subtracting the control antigen reactivity. However, this process will not compensate for new host antigens arising from the infection.

5.2.2 Characterization

Test the antigenic preparation by physical, chemical, and immunological methods. Analyze the preparation to ensure that each of the required antigens is present in the sample.

The following information pertaining to the antigen may be useful:

- chemical nature of the antigen

- source of the agent

- method of preparation and/or purification

- criteria for satisfactory purity and function

- purity of the antigen and method of assay

- known contaminants and likely cross-reactants

- storage conditions and methods of handling

- precautions when pathological materials are being used.

5.3 Separation Procedures

The ideal separation technique will completely separate free analyte from immunochemically bound analyte. The separation technique should not significantly distort the equilibrium that exists between free and bound entities. Good separation techniques are rapid, easy to perform, not strongly subject to minor variations in process, and not affected by the sample matrix.

5.3.1 Solid-Phase Separation

In this technique, one of the reagents is linked to a solid support that can be readily manipulated during the assay to cause separation. The types of solid supports can differ considerably in design and composition. The solid phase can be some device (e.g., dipstick or bead) that can be moved from reagent to reagent; or it can be the reaction vessel itself, so that various assay reagents are added to the vessel. General considerations on the solid support include:

- The composition of the solid-phase surface should be controlled, so that binding of assay reagent is reproducible and stable.

- Conditions for binding of assay reagents (e.g., pH, temperature) may need to be controlled to ensure reproducibility and stability.

- Because the final assay system is not homogeneous, a bulk, which is representative of the whole lot of reagents, cannot be sampled. Consequently, the final quality control of the product should be carefully designed to provide sufficient statistical sampling.

- The method of binding should be designed so that reagent loss from the solid support during the assay is minimal, because reagent loss can affect reproducibility and sensitivity.

- Binding may alter the immunochemical reactivity of the antibody or antigen reagent.

- Care should be taken to ensure that all antigens of interest bind to the solid phase in the proper proportions.

- Where light absorption or fluorescence measurements are made in the presence of the solid support, special precautions are recommended to assure sufficient optical uniformity.

The reagent is typically bound to the solid support either through adsorption or covalent binding. Adsorption is easier to accomplish but is less reproducible and allows for loss of adsorbed reagent during the immunoassay.

Covalent linkage is easier to monitor and control than adsorption. In addition, there is less potential for loss of reagent during the assay. There are numerous reagents available for covalently linking a compound to a solid support. A linking reagent should be selected which minimizes any damage to the immunochemical properties of the assay reagent. After linking the desired reagent, remaining functional linking groups are usually rendered nonreactive.

5.3.2 Double (Second) Antibody

A second antibody directed against one of the primary assay reactants can be used to precipitate the complex. Alternatively, the second antibody can be bound to a solid support. These techniques have the potential advantage of increasing specificity of the assay by the additional immunochemical binding. There is, however, the disadvantage that an additional biological reagent must be carefully controlled.

Antibody used to achieve separation should be of sufficient specificity to bind mainly the protein to be separated. It should be free of interfering impurities and of adequate titer. When used for immunoprecipitation, a broad antibody concentration range is preferable, whereby all immune complexes are precipitated between the prozone and postzone regions.

Other binding proteins (e.g., Staph protein-A, biotin-avidin, and biotin-streptavidin) may be used to bind the primary antibody.

5.3.3 Other Techniques

There are various other techniques for separation which should be chosen on the basis of individual circumstances, including salt (e.g., ammonium sulfate) or solvent (e.g., ethanol, polyethylene glycol) precipitation. These techniques rely on the differential solubility of proteins in the presence of these compounds. Because the efficiency of precipitation depends on the amount of total protein present, protein concentration must be controlled. In addition, the precipitations are often strongly temperature

dependent. These techniques also have the potential for disrupting complexes and shifting the immunochemical equilibrium.

6 Sample 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 test outcome are the type of swab, container, or medium; storage temperature; and time elapsed 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. Handle samples as aseptically as possible and store at recommended temperatures until assayed. Standard and universal precautions should be followed (see Section 3, Standard Precautions).

6.1 Sample Collection

Proper sample selection and collection techniques are essential for optimal test performance. Before specimens are collected, specific instructions should be available either from a test manufacturer or provided in a laboratory procedure manual.

6.2 Sample Requirements

Immunoassays are applicable to a variety of matrices. The user should identify the appropriate matrices, (e.g., blood spots, serum, plasma, urine) with the necessary patient preparation, collection techniques, and devices that are known to work for the assay. Any anticoagulants that may be used should be indicated. (Refer to NCCLS documents H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*, H4—*Procedures and Devices for the Collection of Diagnostic Blood Specimens by Skin Puncture*, H11—*Procedures for the Collection of Arterial Blood Specimens*, GP16—*Routine Urinalysis and Collection, Transportation, and Preservation of Urine Specimens* and LA4—*Blood Collection on Filter Paper for Newborn Screening Programs.*) Provide the storage conditions and stability for the samples. Note any container restrictions.

Assay performance must be proven on all sample types for which it is intended. Minimum sample types include both fresh and frozen sera which have been centrifuged to remove particulates. Additional sample types may include whole blood, cerebrospinal fluid, amniotic fluid, urine, and saliva.

6.3 Sample Handling

Samples should be transported to the laboratory as expeditiously as possible to minimize the growth of contaminants and the release of proteases from any cells that are present. The effects of transportation on sample integrity vary with the type of sample and the nature of the assay.

Within the laboratory, certain treatments, (e.g., heating) might be necessary for one methodology, but preclude use of the sample for further tests. If necessary, samples should be split before treatment.

The manufacturer has the responsibility for specifying required conditions for optimal test performance, but it is the laboratory's responsibility to inform its users about appropriate collection and transport of samples. Manufacturers are responsible to notify users of the effect of high lipid or hemolyzed samples on the test performance. Samples that arrive in unsuitable condition for accurate testing should be rejected after notifying the healthcare professionals (e.g., physicians) responsible for care of the patients. In general, laboratories should not offer tests for which samples cannot realistically be obtained and stored in the manner recommended for the test methods.

6.4 Sample Storage

Appropriate sample storage can be critical in obtaining accurate test results. If the assay cannot be run immediately, processed samples (e.g., serum/plasma separated and remove from cells) should be stored at 4 °C or per assay manufacturer's instructions. If a longer delay is expected, freezing of samples may be necessary. The manufacturer should recommend the preferred temperature for freezing. However, the excessive freeze/thaw cycles should be avoided, and the user may consider the use of stored control samples with the test samples. Storage conditions can also alter reactivity in some assay methods but not in others.

7 Radioimmunoassays

7.1 Radioisotopic Systems

The most commonly used radioisotope for radioimmunoassays (RIA) is iodine (^{125}I). Tritium (^{3}H) is also used for some applications. These assays are referred to as "gamma" or "beta" immunoassays, respectively. Competitive assays involve the use of radiolabeled analyte as a tracer to estimate the bound antibody and the free fractions, whereas immunometric assays use tracer-labeled antibody for measuring the amount of bound analyte. The level of radioactivity (ionizing radiation) is measured (disintegrations per minute) using a scintillation counter. The higher the specific activity of the tracer, the more sensitive the assay. However, with protein analytes, radiolysis can damage the proteins. Although radiolabeled immunoassays have high sensitivity and the tracer can be similar to the analyte, the assay kits have a short shelf life, and safety regulations and radioactive waste disposal create additional concerns relative to other immunoassays.

Specific information about the intended use and a summary explanation of the principle of the test for the user should be provided. The method of radiolabeling should be specified, and any chemical modifications to the analyte in order to make radiolabeling possible should be described. The information should be sufficient to ensure the authorized user has a generic appreciation of the test applications and an adequate understanding of the chemistry involved.

7.2 Reagents

The quantity of reagent supplied (mass, volume, or number of units, as appropriate) should be indicated. The user should also note if the separation reagent was specifically selected or pretreated, because similar reagents from different manufacturers, or different lots from the same manufacturer should not be interchanged.

The biological source (e.g., animal species) in which a second antibody was produced, as well as the general characteristics of immunogen (e.g., purified rabbit gamma globulin) in immunoprecipitant separation (see Sections 5.1.1 through 5.1.4), should be identified. The buffer used, its concentration, and pH should be indicated, and all other reagents identified. Stability, under carefully defined conditions of storage, should be stated. Preservatives used in reagents (e.g., sodium azide) should be identified, together with any necessary precautions. All shelf-life and storage conditions for reconstituted or freshly prepared reagents should be noted.

7.2.1 Separation Procedures

A variety of techniques and reagents may be used to separate the bound antibody from free fractions. All reagents used to separate bound fraction from the free fraction should be identified. Reagents should be identified by chemical name, if no proprietary information is involved (e.g., charcoal, polyethylene glycol, or ammonium sulfate), as well as by general chemical composition (e.g., anion-exchange resin, silicate, immunoprecipitant in the solution or attached to a solid phase [inside of a tube, polymer beads,

etc.]) and by physical separation techniques (e.g., ultrafiltration, dialysis, gel filtration, or electrophoresis). Any effect of interfering substances on the separation process should be described, and pertinent references listed. No additional information other than that required by regulatory bodies is recommended to assess the quality of separation reagents for use in RIAs.

7.2.2 Conjugates

The chemical process of the radiolabeling techniques for preparation of the tracer should be described. The specific sites of the isotope label and the number of labels for the tracer should be identified, if known. For labeled proteins, the specific sites may not be known. The specific activity should be listed, and the conjugation process used for production of the specific antibodies should be described (see Section 5.1).

7.2.3 Calibrators

Calibrators should show traceability to certified reference materials, if available. If certified reference materials are not available, the source of relevant information and physical properties for the substance used should be specified. Calibrators prepared from substances of attested chemical and physical purity should be identified with their sources.

For substances with unknown chemical and physical purity (or which are unstable in highly purified form), other types of calibrators are necessary. The source, and any physical properties and stability concerns, should be identified. Many substances exist in numerous forms, and suitable reference materials may be difficult to select. For biological reference material, dose-response relationships parallel to the substance to be quantified in the assay should be given. The assay should measure the reference material in the same biological matrix as the unknown samples (see Section 11). Although this procedure may not give absolute concentrations, the relative concentrations so determined may be clinically useful. Potency, in terms of the appropriate reference material (including confidence limits) and the number and type of independent assays performed to determine this potency, should be provided. A sufficient supply of calibrators should be maintained for repeated use over extended periods of time. Recalibration of new calibrator preparations at frequent time intervals should be avoided.

The book, *A National Understanding for the Development of Reference Materials and Methods for Clinical Chemistry*,[8] discusses many generalities, as well as specific aspects of calibration and reference materials. Additional literature about calibration and reference materials should be reviewed. (See NCCLS document NRSCL13—*The Reference System for the Clinical Laboratory: Criteria for Development and Credentialing of Methods and Materials for Harmonization of Results*.)

7.3 Assay Description

The classic RIA is a competitive binding (displacement) immunoassay in which the analyte from the sample displaces the radiolabeled species of the analyte or its close analog. A dose-response curve for measurements is produced by measuring the ratio of radioactivity bound in the absence of analyte (B_0) versus the bound radioactivity (B) in the presence of known quantities of analyte. Sandwich immunoassay configurations for RIA are used also where one antibody binds the analyte to a solid phase and a second radiolabeled antibody (dose-response indicator) is added.

For both assay types, the separation of bound and free radiolabeled materials is critical for measurements. A total count and blank sample are important components of the RIA (see Section 7.4). Separation may be accomplished by precipitation of immune complexes, adsorption of the free label, or by washing the solid phase (see Section 7.2.1). For some beta isotope assays, the RIA may use proximity to the scintillation chemicals embedded in the solid phase to produce a dose-response curve. Radioactivity is generally measured as counts-per-minute (CPM), and at least 100 total counts for a measurement should

be accumulated to avoid imprecision due to stochastic counting errors. Radiolabeled reagents should be stored as specified by the manufacturer, and they must be handled and disposed of according to national and regional regulatory guidelines for radioisotope use (see Sections 7.5 and 7.6). Since denaturation may alter the immunoreactivity of analytes, especially for proteins, the radiolabeled reagents should not be used beyond their expiration dates, even if they are still providing adequate CPM rates.

7.4 Detection and Quantitation

The radioactivity should be measured using a gamma or beta scintillation counter, and the level of bound and free fractions relative to each level of calibrator used should be determined. Background, nonspecific binding, and total count tubes should be measured in the process and used to determine quenching and other assay performance characteristics.

As an example, a tabular worksheet showing the sample number, other identification, count rate, dose-response variable (*y*-axis), and analyte concentration (*x*-axis) should be prepared. The method of calculation should be noted, including the computer algorithm, if appropriate. Written and graphic examples of typical calculations should be given, e.g., documentation of numerical steps, count-rate conversions, percent-binding calculations, or a computer-generated curve. A typical calibration curve (dose-response curve), including the effective assay range, should be shown.

Data on standard deviation or coefficients of variation over the clinically significant parts of the calibration curve should be included. The method of calculation should be stated and references cited.[9]

7.5 Limitations and Precautions

The RIA kit has a short and defined shelf life. The activity of the radioactive isotope gradually decreases as it decays. The rate of decay is specific to the isotope. Iodine^{125}, commonly used for assays, has a half-life of 60 days. One or more atoms are replaced in the analyte to produce the tracer.

Sources of error, interferences, and inherent limitations of the assay should be identified. A comprehensive listing of the interfering substances (drugs that test patients might be taking and structural analogs of the analyte) and the relative percent cross-reactivity to the specific analyte measured should be provided. The appropriate literature reference should be included.

RIAs can be used only in approved institutions or by individuals operating under a general license for radioisotope use.[10] Radioactive materials may be received, acquired, and used only by authorized laboratories and then only for *in vitro* clinical or laboratory tests not involving internal or external administration of radioisotopes to humans. Appropriate national and local regulations must be adhered to with the use of radioisotopes. Storage of isotopes must be limited to specifically designated areas. Access to radioactive materials must be limited to authorized personnel only.

Containers must be labeled with the type and actual amount of radioactivity contained at the calibration date of the isotope; and the package insert must indicate the theoretical radioactivity in the kit. All known items that might influence the validity of the assay results should be delineated.

Good laboratory practices imply safe laboratory operations. Diligence must always be exercised to ensure that one is working in a safe environment and not subject to unsafe conditions. RIAs include many steps that require caution by the analyst to avoid personal injury. Personal hazards are commonplace in the laboratory and should not be taken lightly (see the current version of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections).*

7.6 Radiolabeled Waste Products

The disposal of radioactive materials is subject to the regulation and conditions of the user license and in accordance with the recommendations of any local regulatory authority. (See NCCLS document GP5—*Clinical Laboratory Waste Management.*) Approved users must ensure complete internal controls for receipt, storage, use, transfer, and ultimate disposal of all radioactive materials. Regardless of the method of disposal, records of radioactive waste should be maintained, even if two or three methods of disposal exist for the laboratory. Complete records of the receipt, use, and disposal of all radioactive materials should be maintained, even if disposal is handled by a radioactive waste broker. Small-quantity generators using RIA kits only may be exempt.[10]

8 Enzyme Immunoassays

8.1 Enzyme Systems

Enzyme immunoassays (EIA) use an enzyme to label an antigen or antibody.[11,12] The high turnover number of enzymes provides a significant amplification, and this catalytic property makes enzymes an obvious choice as labels. Immunoassays that use enzyme labels have several advantages, including:

- they have strong signal strength, high sensitivity, and wide applicability
- enzymes are less hazardous and have a longer shelf-life than most radioactive tracers
- quantitative and qualitative assays may be rapid and can be automated
- a separation step is not required in all assays
- qualitative assays can be performed with minimal equipment and at low cost
- assays can be used by untrained personnel in point-of-care and home testing applications.

There are some disadvantages. Measurement of enzyme activity usually requires an extra incubation step in the assay procedure. In addition, enzymes are susceptible to interferences and/or changes in assay conditions (e.g., time, temperature, pH, inhibitory substances). Enzymes also have practical limitations related to their large size, and they tend to bind nonspecifically to reaction vessels.

The two most commonly used enzyme labels are horseradish peroxidase and alkaline phosphatase. Horseradish peroxidase is popular due to its high turnover number, its small molecular size (44 kDa compared to 140 kDa for alkaline phosphatase), and large number of hydrogen donors that can reduce hydrogen peroxide to generate colored, fluorescent, or luminescent products. Alkaline phosphatase is often favored because of its simple reaction kinetics and the variety of assay systems available. Glucose-6-phosphate dehydrogenase is a very common label for homogenous EMIT assays.

Increasingly, enzymes are being used as secondary labels. For example, antigen (or antibody) labeled with the low molecular weight cofactor biotin may be used as a primary label, and the biotin binding protein streptavidin labeled with enzyme may be used as a secondary label. Enzymes are also being used in assays with labels that contain enzyme substrates, enzyme inhibitors, coenzymes, or enzyme cofactors.

8.2 Reagents

8.2.1 Separation Procedures

Enzyme immunoassays, other than homogenous immunoassays,[13] require a procedure for physically separating the antibody-antigen complex from other reactants. Several methods are available, but solid phases with antigen or antibody attached have become dominant. Solid phases are usually plastics (e.g., polystyrene) that adsorb enzyme proteins noncovalently.

8.2.2 Conjugates

Enzymes are covalently linked to the antigen or antibody to form a conjugate. The optimal ratio of enzyme to antigen or antibody varies and is determined empirically. The bifunctional reagent glutaraldehyde is often used for alkaline phosphatase and horseradish peroxidase. Conjugation methods using the periodate method may be used with glycoproteins such as horseradish peroxidase. The most common methods for coupling haptens and enzymes are the mixed anhydride and active-ester procedures. Molecular biological techniques are also available for sensitive proteins that may be damaged during conjugation reactions.

8.3 Assay Description

Enzyme-labeled antibodies or antigens first react with the ligand of interest, and enzyme substrate is subsequently added. Measurement of the resultant decrease in substrate concentration or increase in product concentration is then used either to detect or quantitate the antigen-antibody reaction. Essentially all enzyme immunoassays can be classified as competitive (reagent-limited) or noncompetitive (reagent-excess). In a typical competitive immunoassay, enzyme-labeled analyte competes with unlabeled analyte in the sample for a limited number of antibody binding sites. After incubation, the unbound material is removed, leaving the bound enzyme attached to the solid phase. Substrate is added, and at the end of an incubation period, the enzyme reaction is stopped by addition of an inhibitor or by changing the pH. The strength of the signal depends on the amount of enzyme present, which in a competitive assay is inversely proportional to the amount of measurand (analyte) in the original sample.

Most enzyme immunoassays are heterogeneous and require a physical separation system. Homogeneous (separation-free) assays use kinetic measurements of the enzyme activity. When antibody reacts with the enzyme-labeled antigen, the activity of the enzyme is diminished. Enzyme activity is directly related to the concentration of the measurand (analyte) in the sample.

8.4 Detection and Quantitation

Enzymes are easily detected using convenient substrates that give rise to colored, fluorescent, or luminescent compounds. Colored products can be monitored visually with the naked eye or measured accurately in a photometer. The most common colorimetric substrate for alkaline phosphatase is p-nitrophenyl phosphate. ABTS, OPD, and TMB are common peroxidase substrates. TMB is often chosen, since it gives the highest absorbance values, low backgrounds, and is not mutagenic. Colorimetric signals generated with horseradish peroxidase are an order of magnitude greater than alkaline phosphatase.[14]

Fluorescent-labeled substrates or products are inherently more sensitive than colorimetric measurements, since fluorescent compounds can be repeatedly excited by incident radiation. The most common substrate is 4-methylumbelliferyl phosphate with alkaline phosphatase as the enzyme label. Luminescent enzyme immunoassays can achieve levels of sensitivity that are several orders of magnitude better than colorimetric or fluorometric assays. Substrates that yield luminescent end points are available for the common enzyme labels. A very sensitive assay for alkaline phosphatase, for example, uses a

chemiluminescent adamantyl 1,2-dioxetane arylphosphate substrate. Upon dephosphorylation, this substrate decomposes with a protracted glow of light (> 1 hour) and a low detection limit (1 zeptomole).

8.5 Limitations and Precautions

Colorimetric enzyme assays, particularly noncompetitive immunometric assays, are limited by the working range of the spectrophotometer (about 0.1 to 1.5 absorbance units). Extension of this dynamic range is possible by monitoring the absorbance at a wavelength that is offset from the peak maximum, or by monitoring color development kinetically.

Temperature, polarity, pH, and dissolved oxygen content affect fluorescent enzyme assays. Interferences may also arise from light scattering (particulate matter), natural background fluorescence (proteins, bilirubin, and NADH), and quenching effects from molecules in the sample.

Some luminescent enzyme immunoassays, particularly those using horseradish peroxidase, have low quantum yields and produce a weak light emission that rapidly decays. Addition of certain enhancer compounds can substantially increase the light output, reduce the background light emission from the substrates, and improve the signal-to-noise ratio.

The primary limitation of homogenous (separation-free) enzyme immunoassays is the difficulty in designing assays for large protein molecules, since antibody binding may need to occur near the active site of the enzyme in order to have a strong effect on enzyme activity. At low enzyme concentrations, only small amounts of product are generated, resulting in a weak signal that is difficult to measure because of background noise.

9 Fluorescent Immunoassays

9.1 Fluorescent Systems

There are several types of immunoassay systems based on fluorescence technology. Fluorescent methods use a fluorescent molecule to produce an emission as it passes from an excited state to the ground state. Emissions are captured and quantified with a fluorescence spectrophotometer. Fluorescein, green fluorescent protein, methylumbelliferone, and phycoerythrin are examples of fluorescers. Flow cytometry is an application of fluorescence technology to read streams of cells or particles labeled with multiple, unique fluorochromes. Fluorescence polarization methods use polarized light to distinguish bound and unbound antibody-analyte complexes. Time-resolved fluorescent methods use fluorescers with long decay times (typically lanthanide ions, such as europium) and instruments that excite with microsecond-pulsed light, and record fluorescence emission only during the dark phase of each pulse.

There are many factors to consider when developing a fluorescence immunoassay:

- **Fluorescence intensity.** A fluorescer with high quantum yield will maximize the sensitivity of detection.

- **Emission spectrum.** To minimize the interference caused by background fluorescence, use a fluorescer that emits at a wavelength different from that of the background fluorescence.

- **Fluorescer attachment.** The fluorescer will usually have a functional group that will form a covalent bond with the compound of interest. The fluorescer conjugate must be soluble.

- **Sample characteristics.** Nonspecific quenching by substances in the sample should be minimized.

- **Stability.** The fluorescer system must be stable during the assay and when stored.

- **Solvent pH, ionic strength, and viscosity.**

- **Linearity and dynamic range.** Fluorescence intensity readings should be linear with respect to the concentration of the fluorescer unless confounding factors interfere.

9.2 Reagents

9.2.1 Fluorescer/Quencher or Fluorescer Conjugate

The chemical and physical characteristics of fluorescer and/or quencher must be determined and should include purity, molecular weight, excitation, and emission spectra. If the fluorescer is conjugated to protein, the purity and stoichiometry of the conjugate should be determined. When conjugating fluorescers and proteins, the following should be considered:

- The fluorescence of the conjugate peaks at a defined ratio of label to protein, beyond which the fluorescence may decrease.

- Conjugation may change the solubility, stability, size, shape, and net charge of the protein.

- Conjugation may alter the immunological activity of the protein.

- The excitation and emission spectra of the conjugate may differ from that of the free fluorescer.

- Quenching effects cause nonlinear responses at higher fluorescer concentrations, while noise and photobleaching effects contribute to nonlinearity at lower concentrations.

9.2.2 Calibrators

Reference materials may be patient samples of known composition or a sample matrix to which a quantitative amount of reference material has been added. The reference material may be:

- a pure, well-defined material, such as the fluorescein reference material (Fluorescein SRM)[15,a] ;

- a material of known and reproducible purity and composition; or

- an impure material of unknown composition but defined origin, obtained by a reproducible procedure.

Certain analytes have differing and complex characteristics in different samples (e.g., rheumatoid factor may be IgG, IgA, IgM, or a mixture). In this case, the reference material is defined by its immunochemical and functional property, rather than by a unique feature. Such a biological reference material must give a dose-response relationship that correlates to the substance to be quantified in the assay, and it should be stable in the biological matrix to be studied.

9.3 Assay Description

Immunoassay formats are characterized as either competitive or noncompetitive. In a competitive assay, a labeled and an unlabeled analyte (antigen) compete for a limited amount of specific antibody. Non-competitive assays may be direct or indirect. In a direct, noncompetitive assay, analyte is detected by a specific antibody with a fluorescent label. An indirect format utilizes both a primary and a secondary antibody to detect analyte and enhance signal emission. There are many variations on these basic formats,

[a] Available from NIST.

but the result is a fluorescent emission that is quantitatively proportional to the amount of analyte in the test sample.

9.4 Detection and Quantitation

Fluorescent signal detection may be accomplished by several instrumental techniques: filter fluorometers, spectrofluorometers, fluorescence polarization analyzers, and time-resolve fluorometers.[14] Appropriate excitation and emission wavelengths are characteristic of the assay system under development and are optimized for the detection system chosen.

Quantitation of signal and correlation to measurand (analyte) concentration requires the development of a dose-response curve. To develop a dose-response curve, two fundamental factors are required:

- the linearity of the detector for the fluorescer must be demonstrated.
- the linearity of the assay for the analyte must be determined.

The dynamic range of the assay must be within the capability of the detector. A series of equal-volume serial dilutions of the calibrator material across a defined range will demonstrate the limits of assay linearity. Linear regression of the log dilution factor (X) against log instrument reading (Y) should give a best-fit line with residuals at each point no greater than 10% of the expected dilution value. Multiple runs will allow an analysis of the accuracy and precision of the assay. The three biggest contributions to non-linearity are quenching, photodegradation, and noise. Most systems are generally capable of linear responses across at least two decades of fluorescer concentration (a hundredfold range). Quantitative measurements are less reliable outside the linear range, and assays should be optimized to use the linear response range for the most clinically relevant measurements. Because of reduced noise and resistance to quenching, time-resolved fluorescence has the largest linear dynamic range (a thousandfold) among the various platforms in common use.

9.5 Limitations and Precautions

There are several limitations that are unique to fluorescent systems. Interferences can produce either positive or negative bias: substances in the sample that fluoresce at the same wavelengths as the analyte-fluor will result in a falsely elevated signal. The presence of interfering substances may quench the fluorescence (e.g., by competing for antibody), resulting in a reduced signal. Fluorescence emission is sensitive to pH, temperature, ionic strength, and solvent viscosity. All of these parameters must be characterized, optimized, and controlled.

10 Luminescent Immunoassays

10.1 Luminescent Systems

Chemiluminescence, electroluminescence, and bioluminescence are all biochemical systems that produce light.[16] In a chemiluminescent system, a chemical reaction (usually oxidation) generates an organic molecule in an electronically excited state, which emits photons upon return to the ground state. The chemical reaction is initiated by the addition of an oxidizing agent. Light emission by electroluminescence is accomplished by generating an electronically excited state of an appropriate molecule through the application of an electric potential. A bioluminescent reaction utilizes a biological system (e.g., luciferin/luciferase) to catalyze a series of reactions, which results in light emission. Each of these systems offers the following advantages:

- Because sample radiation is not required, the high backgrounds due to light scattering and nonspecific excitation are eliminated, allowing greater sensitivity and dynamic range.

- Improved signal detection coupled with the power of immunoassay technology provides better specificity.

- Luminescent assays often occur on solid phases which capture the analyte and wash away interfering substances, enhancing sensitivity and specificity.

- Luminescent reagents and conjugates are generally stable and nontoxic.

10.2 Reagents

10.2.1 Lumophores

A luminescent molecule from one of the following classes typically provides the signal in chemiluminescent assays: acylhydrazides (luminol), 1,2-dioxetanes (AMPPD), acridinium esters and related analogs, or pyridopyridazines; the luciferin/luciferase pair is used in bioluminescent systems.[17] One application of electroluminescence employs an electronically excited state of a ruthenium complex.[18]

10.2.2 Conjugates

The lumophore may be attached directly to an analyte or antigen, a substrate, or an antibody. Characterization of the conjugate should include: optimization/quantitation of the conjugation reaction, characterization of the emission spectrum of the conjugate, and stability studies.

10.3 Assay Description

Assay formats are typically monoclonal/polyclonal sandwich assays on solid phases. Both direct and indirect competitive immunoassay protocols predominate in chemiluminescent systems. Quantitative light emission may result from direct chemical cleavage of an acridinium ester for example; or from the action of an enzyme or enzymatic system on a luminescent substrate (e.g., luciferin/luciferase or a luminol/enzyme pair); or from an electronically excited state of an appropriate molecule, resulting from the application of an electrical potential difference to the reaction.

10.3.1 Calibrators

There is no reference material for luminescence. The detector in use can be calibrated with a defined set of dilutions of an appropriate material of proven stability. For example, a quantitative set of dilutions of an acridinium conjugate would provide an assessment of the dynamic range of the system for an acridinium-based assay. Manufacturers of luminometers also use isotopes (^{14}C and ^{3}H) to standardize an instrument population. Assay calibration may be accomplished by strategies previously discussed for fluorescence assays.[b]

10.4 Detection and Quantification

The signal generated by a luminescent reaction is detected as photons by a photomultiplier tube for a defined period, and the count data are transferred to a central processing unit to yield a relative light unit measurement. A background measurement is subtracted from the reaction measurement to yield the final measurement. Immunoassays require nonlinear algorithms to fit curves and establish performance criteria.

[b] Calibrator materials should be NIST or WHO reference materials when available.

10.5 Limitations and Precautions

Because photon detection is a very sensitive method, contaminating signals may result from a variety of sources. Biological organisms produce luminescent materials; cleaning solutions and laboratory dust may contain substances that produce light; etc. The validity criteria for background measurements and assay measurements are safeguards against many interfering substances. The maintenance procedures provided by the manufacturer address system decontamination and cleanliness, but the importance of Good Laboratory Practices cannot be overstated when working with this technology.

11 Assay Performance Characteristics

11.1 Accuracy, Trueness, and Precision

Even those familiar with laboratory terminology are often confused by the international community's use of metrological terms. (See the *Note on Terminology* beneath the Foreword) To narrow the communication gap, it is important to have a general understanding that accuracy, trueness, and precision are typically qualitative terms, and therefore, have no numerical value. To express these terms quantitatively, the terms *error*, *bias*, and *imprecision* are used.

Accuracy (of measurement) refers to how close a *single* measurement result (test method result) comes to a true, expected, or accepted reference value. But it is usually expressed in terms of error (of measurement), or how far away one's result is from one's expectations (i.e., mathematically, the result of a *single* measurement minus a true, expected, or accepted reference value).

Trueness refers to the degree of agreement between an *average value* of a large series of measurements and a true, expected, or accepted reference value. *Bias* is the quantitative expression of trueness and refers to the degree of systematic difference from the accepted reference or true value, whether positive or negative. A larger systematic difference will mean a larger bias.

Precision refers to the degree of agreement between independent test/measurement results obtained under stipulated conditions. It depends only on the distribution of random errors, and it does *not* relate to a reference or true value. Determined qualitatively as "high," "medium," or "low," it is expressed mathematically in terms of *imprecision* (i.e., the dispersion of results of measurements obtained under specified conditions) and is measured as the standard deviation (SD) or coefficient of variation (CV). A lower CV value reflects higher precision.

The "stipulated conditions" are further broken down into "repeatability (within-run) conditions" (i.e., independent test results obtained with the same method, on identical test material, in the same laboratory, by the same operator, using the same equipment, within a short interval of time) and "reproducibility (total precision) conditions" (i.e., where test results are obtained with the same method, on identical test items, under different settings (e.g. in different laboratories), with different operators using different equipment.[5]

Traceability to an accepted reference value for an immunoassay should take into account that different reagents may react to different extents to the various epitopes of the reference material and yield different although related quantities.[3]

The recommended trueness and precision of an assay for clinical decision may depend on the seriousness of the condition, the degree of separation of results obtained from affected and unaffected persons, and the assay methods available. (See NCCLS document I/LA21—*Clinical Evaluation of Immunoassays.*) The analytical evaluation of a new immunoassay or comparison to existing immunoassays should include adequate documentation of trueness and precision (including repeatability and reproducibility) at all levels of diagnostic (clinical) performance.

11.2 Sensitivity and Specificity

Several interrelated concepts are of vital importance in the interpretation of laboratory tests. These include detection limits, cut-off values, sensitivity, and specificity (analytical and diagnostic [clinical]), positive predictive value, negative predictive value, false-positive results, false-negative results, prevalence, efficiency, and "gold standards." These terms are defined in Section 4.

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. Even laboratory sensitivity can be defined two ways: the detection limit (the lowest value that can be measured different from zero), and the smallest difference that can be measured reliably between any two values. Detection limits are expected to vary with sample type, e.g., an assay can show significantly different detection limits in the testing of saliva and serum.

Other 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. Diagnostic (clinical) sensitivity, in contrast, is the percentage of patients who are positive for a trait and test positive; and diagnostic (clinical) specificity is the percentage that do not have the trait and test negative.

11.3 Comparison of Quantitative Tests

The comparison of quantitative data from different immunoassays is commonly performed by simple linear regression, with a high correlation coefficient (r) taken to indicate comparability of results. This approach is often misused. First, a nonzero intercept suggests differences in behavior of the two assays, but is usually ignored. Secondly, 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. Finally, simple linear regression implies superiority of the method used as the independent variable. Deming regression can be used to overcome the last problem.[19] However, more sophisticated approaches are necessary to overcome others. The logarithmic transformation method of Finney,[20,21] 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.

To compare similar reagents (e.g., different antigen preparations, calibrators, controls, etc.) within an immunoassay, establish the functional linear relationship between the materials (rather than simple regression). NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach* provides a method for evaluating linearity. A plot of test results for 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 assay limits; a nonzero intercept implies matrix or antigenic differences between the two materials. An assessment of the linearity of a method allows the user to establish the dynamic range or range of clinical utility for that method.

NCCLS document EP9—*Method Comparison and Bias Estimation Using Patient Samples* provides guidelines for designing experiments to evaluate the bias between two methods that measure the same measurand (analyte). Ideally, a test method should be compared with a reference method. For the clinical laboratory, the comparative method is often the current method. The evaluation should determine whether the two methods yield equivalent results within the statistical limits of the experiment. NCCLS document EP9 allows the estimation of the bias (expected difference) between two methods at various

concentrations of analyte. If the comparative method is the same one used by the manufacturer in the statement of claims, it is possible to statistically compare the experimental results to the manufacturer's claims in order to verify acceptable performance.

11.4 Reference Intervals

Reference interval, also known as "reference range" (historically as "normal range"), is the span of test values expected for a designated (reference) population of individuals (i.e., the range of values representing 95% of the individuals presumed to be healthy [or normal]). A variety of factors determine the reference interval for the measurand (analyte). The size and characteristics should be provided for the selected reference population contributing to the reported reference interval. Diagnostic laboratories, test manufacturers, research test developers, and users of clinical immunoassays need to establish their own appropriate reference intervals to assess and understand the clinical applications of the immunoassay. Intervals provided by manufacturers may be useful as a performance example and guide. Reference intervals are necessary to meet requirements of reliability and usefulness for the quantitative test. (See NCCLS document C28—*How to Define and Determine Reference Intervals in the Clinical Laboratory* for detailed methodological approaches and recommended procedures.) Reference interval should not be confused with dynamic range, diagnostic cutoff, and measuring range (reportable range). Immunoassays may involve the use of medical decision limits, such as cutoff values, especially for screening applications. (See the current edition of NCCLS document I/LA21—*Clinical Evaluation of Immunoassays*.) A diagnostic cutoff is often based upon diagnostic (clinical) sensitivity and specificity for a specific medical disorder.

11.5 Reference Values (Expected Values)

A reference value provides a means of comparing or relating an observed data element to a reference database from a characterized population of subjects. Reference values may be from correlation data that have been determined by assessment of the immunoassay against a more specific measurement procedure, or against a reference method or a reference material. Expected values are produced by traceability and verification to a certified reference material to support the performance of the immunoassay (e.g., the WHO International Reference Preparation (IRP) for human Thyroid-Stimulating Hormone - IRP hTSH 80/558) and to establish traceable values for the calibrators. Reference materials prepared in a human serum matrix are most useful for immunoassay calibration and harmonization. Most international reference preparations are supplied as purified protein extracts, but a few are provided in a human serum matrix. An example of such a human serum reference preparation for immunoassays is the serum protein standard certified for 14 analytes.[22]

Definitive and reference methods are well-established, certified methods that are used for comparison and calibration of other methods for reference value assessments. For analytes measured by immunoassays, reference methods are considered the highest category achievable. However, the only proposed immunoassay for this category is for digoxin. NCCLS document I/LA9—*A Candidate Reference Method for Serum Digoxin: A Model for Radioimmunoassay Reference Methods* describes a working model that delineates and exemplifies the extent of documentation required for the development, evaluation, validation, and transferability of an immunoassay reference method. A few independent chemical reference methods (e.g., isotope-dilution gas chromatography-mass spectrometry method for cortisol) are available for some analytes that are measurable by immunoassays. These reference methods are very useful to evaluate method bias and matrix influences among immunoassays and to guide method improvements. When reference methods are not available, especially for proteins, pure analytes or certified reference preparations are used for calibration.

11.6 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. (See NCCLS document I/LA21—*Clinical Evaluation of Immunoassays*) 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, clearance, or approval (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.

12 Quality Assurance and Control

12.1 General

All quality control (QC) parameters must be determined within the laboratory in which they are to be used. Carry out at least ten laboratory runs to establish temporary confidence limits for the QC samples. Establish working limits after 20 runs and re-establish them after each additional 20 runs or a defined interval. Include at least two QC samples at different levels that bracket the decision point in each analytical run, in a matrix simulating the unknown sample to the extent possible, without contributing to the variance of the assay (e.g., human serum matrix). (See NCCLS document C24—*Statistical Quality Control for Quantitative Measurements: Principles and Definitions.*) The impact of the matrix on the performance of the immunoassay should be determined. (See NCCLS document EP14—*Evaluation of Matrix Effects.*) The matrix of any provided QC samples should be identified in the product insert.

For the pathological situations that the immunoassay is designed to study, statistically adequate sampling is required in a specified and (preferably) matched reference sample group to permit reliable differentiation from normal. State the distribution of the assay results from normal and disease reference sample groups with a 95% confidence limit. Clearly state the overlap (if any) of disease and reference interval sample groups.

12.2 Quality Control Enhancement Parameters

In addition to the routine quality control sample system and plots, other parameters for immunoassays should be monitored. Some suggested QC parameters for immunoassays include:

- the slope from the linear regression equation for the calibration curve;
- the y-intercept from the linear regression equation for the calibration curve;
- the mean percentage of bound/total (%B/T) for two calibrators (RIA);
- the corrected CPM for the total count tubes used for calibrators (RIA); and
- the coefficient of determination from the linear regression equation of the calibration curve.

For acceptable performance of the immunoassay, the mean value for the suggested parameters must fall within designated and established limits. If the calculated value for any of these parameters falls outside the defined limits, take appropriate remedial action based on the particular parameter. The mean value and upper and lower 95% confidence limits are determined for each parameter during the process of

setting the QC sample limits. The use of additional parameters in quality control of immunoassays targets the source of the problem and helps focus the corrective action for rapid response.

12.3 Technical Considerations

Several technical problems warrant special consideration for quality assurance of immunoassays: heterophile antibody interference; high-dose hook effects; and the quality of calibration curve-fitting. In contrast to the predictable and controlled impact of most technical errors, heterophilic antibodies can cause significant interference problems in most immunoassays and are difficult to detect. Circulating human heterophile antibodies have the ability to bind to immunoglobulins of other species, including the animal species used to generate immunoassay reagent antibodies. Immunometric "sandwich" assays for antigen detection are particularly susceptible to this interference. Heterophilic antibodies may either bridge the capture and signal antibodies, thereby producing a false-positive signal and a falsely elevated result; or they may bind exclusively to the capture or detection antibody, resulting in loss of signal and a falsely low result. The inclusion of serum or immunoglobulins from the same species as the reagent antibody is often effective in minimizing interference of this type. Unless an immunoassay is confirmed to be free of this interference, heterophilic antibodies should be suspected if test results are not consistent with the clinical picture. The laboratory should rerun questionable specimens using a different assay, employ blocking steps or serial dilution, or separate immunoglobulins from analyte by chromatography before the immunoassay.

Interference with antibody binding can also occur when extremely high concentrations of analyte in the patient sample exceed the binding capacity of the capture and signal antibodies, making these antibodies unavailable to form antibody-antigen complexes. This parameter is known as the "high-dose hook effect," and the phenomenon results in a severe underestimation of the measurand (analyte) concentration. This problem mainly affects immunometric assays where the range of analyte concentrations is very high, such as in assays for choriogonadotropin and many tumor markers. It is common practice to analyze samples in such assays at several dilutions to check on the validity of the result.

Linear transforms of dose-response curves for immunoassays may indicate large dynamic ranges, but caution should be used without a thorough understanding of these transformed calibration curves. The reliability of immunoassay results depends on the accurate interpretation of assay data using an appropriate calibration curve. Curve-fitting—the process of matching calibration data to a defined mathematical function (e.g., a straight line)—has the potential to introduce significant bias or imprecision. For example, severe nonuniformity of variance is introduced by the logit-log transformation, unless a weighted regression technique is used to provide protection against poor replicates or outliers. Many different curve-fitting algorithms are available, and the quality of the fit should be checked whenever a new immunoassay is introduced. A simple procedure to check for curve-fitting bias is to back-calculate calibrator values from their signal levels (using the equation for the fitted curve) and compare with preassigned values. Stability and analyzer-to-analyzer variation are important problems associated with master calibration curves that have been established by manufacturers and adjusted by users. Appropriate QC parameters (e.g., goodness-of-fit residuals, small and random) should be monitored to ensure that calibration integrity is maintained.

13 Necessary Product Insert Information for Immunoassay Systems

The following information is pertinent to manufacturers of diagnostic immunoassay kits and bulk reagents that are recommended for use in immunoassays. For diagnostic kits, this information should be in a user-friendly format and adhere to all local and international regulations.[23,24] For bulk reagents, only specific items in the list relating to product description (as well as reagent handling and labeling) are applicable. Users should consult local regulatory bodies for requirements regarding other items in the list. Date labeling can cause confusion between day-month-year and month-day-year; therefore, the way it is written should be clearly explained.

- proprietary name and established name
- intended use of the product
- summary and explanation of the test
- principles of the procedure
- listing of all components: common name, quantity, concentration
- warnings and precautions
- Handling instructions, including:
  — reconstitution or mixing
  — storage condition
  — indications of instability
- instrumentation instructions, including:
  — installation procedure
  — principles of operation
  — performance characteristics
  — operating instructions
  — calibration procedures
  — operational precautions
  — hazards
  — service/maintenance information
- specimen collection and preparation
  — standard and universal precautions, and safety guidelines
- assay procedure
- identify reagents, controls, and calibrators by lot
- results: data handling and interpretation
- limitations of the procedure
- expected values
- specific performance characteristics
- bibliography
- name and place of business
- date of issuance of the last labeling revision.

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NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Delegate Comments and Committee Responses

I/LA23-P: *Assessing the Quality of Immunoassay Systems: Radioimmunoassays, and Enzyme, Fluorescence, and Luminescence Immunoassays; Proposed Guideline*

General

1. This document is not useful as written. The document provides a broad and incomplete overview that lacks clear, concise explanations and descriptions for a novice; plus, it is clearly inadequate for a knowledgeable user.

- **The committee, during development of this guideline, focused on the core quality management issues for immunoassays and does not agree with this general comment. The development of I/LA23-A was implemented to replace NCCLS documents LA1-A2 and DI4-T by incorporating their pertinent information and updating them to include new detection systems. I/LA23-A presents guidelines primarily for immunoassays of macromolecular analytes. The factors likely to be important in achieving reliable and reproducible results are emphasized. Use of this document should promote greater reliability and comparability in immunoassay results. The definitions, information, and procedures necessary to properly assess the quality of immunoassay systems are described. The range of applications for immunoassays is extensive. The degree of variations in configurations is large and may involve a hierarchy of antibodies used with different specificities for capture, separation, measurement, and dose amplifications. A comprehensive coverage of the field of immunoassays is too large for the scope of this document.**

2. All parts of the document dealing with calibrators tell about traceability of the products to standards (reference materials or methods); however, the document never mentions calculation of the components of uncertainty. For this reason, maybe the word "calibrator" should be changed to the word "adjustor."

- **The committee believes (based on the definition of "calibrator") that it is the correct term. The definition for "calibrator" was added to Section 4.1 (formerly Section 3.1).**

Section 4.1, Definitions (Formerly Section 3.1)

3. The term "assay" is also used for qualitative tests.

- **Definitions of "qualitative assay," "quantitative assay," and "semiquantitative assay" have been added to the definition of "assay" in Section 4.1.**

4. Add a note following the definition of "clinical sensitivity" stating that it is the fraction of clinically true positive classification divided by the sum of clinically true positive and clinically false negative.

- **The suggested change has been made in Section 4.1 for the definition of "clinical sensitivity."**

Section 5.1.2, Immunogen (Formerly Section 4.1.2)

5. Fourth bullet, second dashed sentence: What if there is more than one contaminant?

- **The second dashed statement has been revised to read: "- antibody(s) specific for likely contaminant(s)."**

Section 5.2.1, Source and Purification (Formerly Section 4.2.1)

6. Third bullet: A control antigen is prepared from a culture as well as the control specimen.

- **The second sentence in the third bullet in Section 5.2.1 has been revised to read: "A control antigen is prepared from a culture (as well as the control sample) not infected with antigen microorganism but one that is otherwise identical in composition and purification protocol. Immunological reactivity with contaminants can be accounted for by subtracting the control antigen reactivity**."

Section 6, Sample Collection, Handling, and Storage (Formerly Section 5)

7. There is no mention of safety issues or guidelines during collection of samples.

- **The following sentence has been added to Section 6: "Standard and universal precautions should be followed (see Section 3, Standard Precautions).**

Section 6.3, Sample Handling (Formerly Section 5.3)

8. Manufacturers are responsible to notify users of the effect of high lipid or hemolysed samples on the test performance.

- **The commenter's statement has been added to the third paragraph in Section 6.3.**

Section 6.4, Sample Storage (Formerly Section 5.4)

9. The manufacturer should recommend the preferred temperature for freezing.

- **The commenter's statement has been added to Section 6.4.**

Section 8.1, Enzyme Systems (Formerly Section 7.1)

10. How about the quantitative assays?

- **The third bullet in Section 8.1 has been revised to read: "quantitative and qualitative assays may be rapid and can be automated."**

Section 11.5, Reference Values (Expected Values) (Formerly Section 10.5)

11. The first paragraph of this section refers to reference materials, and the second part tells about reference methods. However, the last part of this paragraph refers, again, to reference materials. So, the sentence beginning, "Since reference methods are not available, especially for proteins…" up to the end should be moved to the end of the first paragraph.

- **Revisions have been made in Section 11.5 to make this clarification.**

Section 12, Quality Assurance and Control (Formerly Section 11, Quality Assurance)

12. The term "quality assurance" usually reflects how to guarantee that the product is satisfactory for the intended use, and involves all phases (processes) of the medical laboratory. This document seems entirely focused on detecting and minimizing analytical errors. Consequently, the title of this section should be "Quality Control."

- **For clarity, the title of Section 12 has been changed to "Quality Assurance and Control" and appropriate revisions have been made in Section 12.2.**

Section 13, Necessary Product Insert Information for Immunoassay Systems (Formerly Section 12)

13. Bullet "Specimen collection and preparation." Are safety guidelines and universal precautions to be included?

- **The following statement has been added to Section 13: "Standard and universal precautions, and safety guidelines."**

14. Reagents, calibrators, and controls should also be identified with master lot and batch lot for traceability from manufacturer through to end user. Reagents are used internationally and therefore a consensus date format would be an advantage as the labeling can cause confusion between day-month-year and month-day-year.

- **For clarity, Section 13 has been revised to include a sentence reading: "Date labeling can cause confusion between day-month-year and month-day-year; therefore, the way that it is written should be clearly explained." A bulleted statement reading, "Identify reagents, controls, and calibrators by lot" has also been added.**

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

I/LA23-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section at the end of the document.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X C24 C28 D12 D13 EP6 EP9 I/LA18					X	GP5 M29
I/LA21			I/LA21		I/LA21	I/LA21					

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytic, analytic, and postanalytic. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

I/LA23-A addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section at the end of the document.

Preanalytic					**Analytic**		**Postanalytic**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Post-test Specimen Management
		X H3	X H3	X	X	X		X

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

Related NCCLS Publications*

C24-A2 **Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline—Second Edition (1999).** This guideline provides definitions of analytical intervals, plans for quality control procedures, and guidance for quality control applications.

C28-A2 **How To Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline – Second Edition (2000).** This document provides guidance for determining reference values and reference intervals for quantitative clinical laboratory tests.

DI2-A2 **Immunoprecipitin Analyses: Procedures for Evaluating the Performance of Materials—Second Edition; Approved Guideline (1993) (Reaffirmed 1999).** This guideline provides a description of and procedures for evaluating the performance of materials used in immunoprecipitin analyses. It also includes a discussion on specificity.

DI3-A **Agglutination Analyses: Antibody Characteristics, Methodology, Limitations, and Clinical Validation; Approved Guideline (1993) (Reaffirmed 1999).** This guideline describes the specificity of antibodies and antigens for agglutination techniques, guidance labeling information, and characteristics and limitations of agglutination methods.

EP6-A **Evaluation of the Linearity of Quantitative Measurement Procedure: A Statistical Approach; Approved Guideline (2003).** This document provides guidelines for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

EP9-A2 **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Second Edition (2002).** This document addresses procedures for determining relative bias between two clinical methods or devices; and for the design of a method comparison experiment using split patient samples and data analysis.

GP5-A2 **Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002).** Based on U.S. regulations, this document provides guidance on safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory.

HS1-A **A Quality System Model for Health Care; Approved Guideline (2002).** This document provides a model for healthcare service providers that will assist with implementation and maintenance of effective quality systems.

H3-A5 **Procedure for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003).** 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/LA18-A2 **Specifications for Immunological Testing for Infectious Diseases; Approved Guideline – Second Edition (2001)**. This guideline outlines: specimen requirements; performance criteria; algorithms for the potential use of sequential or duplicate testing; recommendations for intermethod comparisons of immunological test kits for detecting infectious disease; and specifications for development of reference materials.

I/LA21-A **Clinical Evaluation of Immunoassays; Approved Guideline (2002).** This guideline provides recommendations on designing trials that are appropriate for evaluating both the safety and effectiveness of immunoassays. It is a valuable resource in determining the necessary steps in designing an evaluation for new methods, new applications for existing methods, or variations on existing methods.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** 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.

NRCSL13-A **The Reference System for the Clinical Laboratory: Criteria for Development and Credentialing of Methods and Materials for Harmonization of Results; Approved Guideline (2000).** This document provides procedures for developing and evaluating methods and materials to provide a harmonized clinical measurement system.

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

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